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UNIVERSITY OF ALBERTA

TROPHIC REGULATION OF ION CHANNELS IN SYMPATHETIC NEURONS

BY

SAOBO LEI 

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DEDICATED TO MY WIFE BINQI, AND MY DAUGHTER JESSICA

ABSTRACT

Voltage-dependent ion (Ca^{2+}, Na^{+}, K^{+}) channels are subject to regulation by neurotransmitters, neuromodulators and trophic factors. This thesis describes the regulation of ion channels in adult bullfrog sympathetic ganglion (BFSG) B-neurons by nerve growth factor (NGF).

Whilst NGF-induced differentiation of pheochromocytoma (PC12) cells into sympathetic-like neurons involves the appearance of ion channels, high affinity receptors for NGF (TrkA) continue to be expressed in adult sympathetic neurons. The central hypothesis of the thesis is that NGF continues to be involved in ion channel regulation in adult neurons. A defined, serum-free medium culture system was developed. Dissociated BFSG neurons survived for at least 15 days in this system. NGF exerted three independent effects on the Ca^{2+} channel currents (I_{Ca}) of cultured BFSG B-neurons; it increased ω-conotoxin-GVIA-sensitive N-type I_{Ca}; it enhanced nifedipine-sensitive L-type I_{Ca} and attenuated the inactivation of total I_{Ca}. The NGF-mediated increment of I_{Ca} is transcription-dependent. Total I_{Ca} was 55.8% larger in the B-cells acutely dissociated from the sympathetic ganglia of frogs that had been pretreated with subcutaneous injections of NGF *in vivo*. Injection of NGF antiserum decreased total I_{Ca} by 29.4%. Inactivation of Ca^{2+} channel conductance of the cells from the animals injected with NGF was significantly reduced, compared with that of the cells from the animals injected with NGF antiserum ($p<0.001$).

NGF-mediated enhancement of I_{Ca} was generated via the high-affinity Trk A receptor, not via the low-affinity p75 receptor; the effect of NGF on I_{Ca} is tyrosine kinase-dependent; treatment of the cells with anti-Trk Ig-G antibody mimicked the NGF response, but anti-p75 antibody had no effect on NGF-mediated increase in I_{Ca}. Further investigation on the

downstream targets of Trk A showed that Ras and MAPKK were required for NGF-mediated increase in I_{Ca} because the effects of NGF were completely blocked by inhibitors of Ras post-translational modification, α-hydroxyfarnesylphosphonic acid and perillic acid, and the MAPKK inhibitor, PD98059.

To determine whether the effects of NGF are simply restricted to I_{Ca}, I tested whether NGF regulates other ion channels in adult BFSG B-neurons. NGF increased the total, TTX-sensitive and TTX-resistant Na^+ channel currents (I_{Na}) without altering their activation or inactivation kinetics. Of all the K^+ channels studied, only the voltage-sensitive Ca^{2+}-activated K^+ currents (I_C) were regulated by NGF, but this effect is possibly a consequence of the interaction of NGF with I_{Ca}. These results show that ion channels in adult neurons are subject to regulation by neurotrophic factors.

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List of Abbreviations

a.p.	action potential
Ara-C	cytosine arabinoside
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid
BDNF	brain-derived neurotrophic factor
BFSG	bullfrog sympathetic ganglion
cAMP	cyclic adenosine 3, 5-monophasphate
cDNA	complementary deoxyribonucleic acid
CNTF	ciliary neurotrophic factor
CSF-I	colony-stimulating factor I
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
DRG	dorsal root ganglion
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tertraacetic acid
EGF	epidermal-growth factor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FPTase	farnesyl-protein transferase
GAP	GTPase activating protein
GDNF	glial-derived neurotrophic factor
Grb2	growth-factor-receptor binding protein 2
GTP	guanosine 5'-triphosphate

HEPES	N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
α-HFA	α-hydroxyfarnesylphosphonic acid
I_A	A current
I_{AHP}	slow, voltage-insensitive, Ca^{2+}-sensitive K^+ current
I_C	fast, voltage-sensitive, Ca^{2+}-sensitive K^+ current
I_{Ca}	Ca^{2+} channel current
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
I_K	delayed rectifier potassium current
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
I_M	voltage- and time-dependent muscarine-sensitive K^+ current
LIF	leukemia inhibitory factor
IP_3	inositol trisphosphate
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NMG	N-methyl-D-glucamine
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NT-6	neurotrophin-6

PA	perillic acid
PDGF	platelet-derived growth factor
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3 kinase
PI-4-P	phosphatidylinositol-4-monophosphate
PI-3-P	phosphatidylinositol-3-monophosphate
PI-3,4-P2	phosphatidylinositol-3,4-bisphosphate
PI-3,4,5-P3	phosphatidylinositol-3,4,5-trisphosphate
PI-4,5-P2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC-γ	phospholipase C-γ
RPSG	Rana pipiens sympathetic ganglion
SH2	src homology 2
SNT	suc-associated neurotrophic factor-induced tyrosine-phosphorylated target
SOS	Son of sevenless
TEA	tertraethylammonium
Trk	tropomyosin receptor kinase
TTX	tetrodotoxin

Chapter 1

General Introduction

Neurotrophins and Neurotrophic Factors

The development of the vertebrate nervous system is characterized by programmed cell death. The survival of developing neurons depends on the supply of a neurotrophic factor synthesized in limiting amounts in their target fields (Davies, 1996). Neurotrophic factors constitute a class of protein molecules that are now considered critical for the development, maintenance and regeneration of the nervous system. Nerve growth factor (NGF) is the most extensively studied neurotrophic factor and is a member of a family of closely related neurotrophic molecules known as neurotrophins that include brain-derived neurotrophic factor (BDNF) (Leibrock *et al.*, 1989), neurotrophin-3 (NT-3) (Hohn *et al.*,1990; Maisonpierre *et al.*, 1990), neurotrophin-4/5 (NT-4/5) (Hallböök *et al.*, 1991; Berkemeier *et al.*, 1991) and neurotrophin-6 (NT-6) (Gotz *et al.*, 1994). Some hematopoietic cytokines, such as ciliary neurotrophic factor (CNTF) (Barbin *et al.*, 1984; Lin *et al.*, 1989; Stockli *et al.*, 1989) and cholinergic differentiation factor/leukemia inhibitory factor (LIF) (Yamamori *et al.*, 1989; Nawa *et al.*, 1991; Kurzrock *et al.*, 1991), fibroblast growth factor (FGF) (Morrison *et al.*, 1986; Unsicker *et al.*, 1987; Stemple *et al.*, 1988; Walicke, 1988; Ferrari *et al.*, 1989) and glial-derived neurotrophic factor (GDNF) (Lin *et al.*, 1993) are also considered as neurotrophic factors. More recently, another neurotrophic factor, cysteine-rich neurotrophic factor (CRNF) has been purified from the mollusk, *Lymnaea stagnalis*, by use of a binding assay on the p75 neurotrophin receptor (Fainzilber *et al.*, 1996). Major functions of these neurotrophic factors are mediated by membrane receptors that have intrinsic tyrosine kinase (receptor tyrosine kinase) activity. In addition, one group of growth factor receptors,

such as epidermal-growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor I and II (IGF-I, II) and colony-stimulating factor I (CSF-I) receptor, also contain intrinsic tyrosine kinase activity (Yarden *et al.*, 1988). These growth factors are important in the regulation of cell growth and differentiation. Here, tyrosine kinase receptors and their signal transduction mechanisms will be discussed first. Trophic regulation of the voltage-gated ion channels and ion channels in bullfrog sympathetic ganglion (BFSG) B-cells will also be reviewed to provide a background for this thesis.

Neurotrophin Receptors

Two pharmacologically distinct neurotrophin-binding sites have been found within the nervous system (Meakin and Shooter, 1992), a low affinity binding site (K_D=10^{-9} M) and a high affinity binding site (K_D=10^{-11} M). High affinity binding of the neurotrophins along with cell survival and phenotypic responses is conferred by the Trk (for tropomyosin receptor kinase) family of receptor tyrosine kinases. The Trk receptors display neurotrophin selectivity, with the Trk A (also known as Trk or gp^{140trk}), Trk B and Trk C serving as the receptors for NGF, BDNF and NT-3, respectively, and Trk A and Trk B can also act as receptors for NT-3 and NT-4/5 (Meakin and Shooter, 1992; Chao, 1992). Receptor specificities for NT-6 have not been defined. The Trk subfamily is distinguished by IgG-C2 domains and repeats that are rich in leucine and cysteine residues in the extracellular domain, and a consensus tyrosine-kinase domain with a small interruption and a short cytoplasmic tail (Chao and Hempstead, 1995). Cells that are responsive to NGF are restricted to sympathetic

neurons, subpopulations of neural crest-derived neurons and some neurons in the central nervous system (CNS) (cholinergic neurons in the basal forebrain, and cholinergic interneurons in the striatum) (Lindsay, 1996), as opposed to the larger neuronal population that are dependent upon BDNF, NT-3 and NT-4/5. During development, expression of Trk A is limited to sensory and sympathetic neurons in the peripheral nervous system (PNS) and cholinergic neurons of the forebrain (Martin-Zanca *et al.*, 1990; Holtzman *et al.*, 1992), while more extensive CNS expression is found for TrkB and TrkC (Barbacid, 1994).

The low affinity binding site is most likely accounted for by a transmembrane protein known as p75 or the low affinity NGF receptor (Chao *et al.*, 1986; Radeske *et al.*, 1987). Recently, this site has been termed low affinity neurotrophin receptor because all neurotrophins are able to bind competitively to this receptor (Squinto *et al.*, 1991), although association and dissociation rate constants of binding differ among the neurotrophins (Rodriguez-Tébar *et al.*, 1990; 1992). The p75 neurotrophin receptor belongs to a family of transmembrane molecules which also serve as receptors for the tumour necrosis factor family of cytokines (Chao, 1994). The p75 receptor contains four negatively-charged cysteine-rich extracellular repeats that are responsible for ligand-binding, and a unique cytoplasmic domain that is highly conserved among species (Chao and Hempstead, 1995). The p75 receptor has a much wider distribution and is expressed on numerous cell types (Bothwell, 1991; Thomson *et al.*, 1988), including Schwann cells, motor neurons, meningeal, dentalpulp cells, hair-follicle cells, and cerebellar Purkinje cells. The wide spread pattern of expression of p75 is consistent with its role as a potential receptor for BDNF, NT-3 or NT-4/5, in addition to NGF. Furthermore, the majority of neurotrophin-responsive cells express

both p75 and Trk family receptors, suggesting both receptors might play roles in the biological functions of the cells (Chao, 1994).

Signal transduction

I. Signal transduction pathways mediated by Trk

Although different forms of NGF (7S NGF, 2.5S NGF or βNGF) can be obtained by different purification methods, the three-dimensional structure of NGF has been resolved by X-ray crystallography and shown to consist of two identical subunits (McDonald *et al.*, 1991). Because of its two-fold symmetry, the active NGF dimers have two potential Trk A binding sites which may facilitate the formation of Trk A receptor homodimers, a step required for receptor activation (Jing *et al.*, 1992). Ligand-mediated dimerization is followed by autophosphorylation of the tyrosine residues, a mechanism common to other tyrosine kinase receptors (Schlessinger and Ullrich, 1992). Tyrosine autophosphorylation mediated by an intermolecular process (Schlessinger, 1988) is crucial for normal receptor signaling. The tyrosine-autophosphorylated regions in receptors represent specific binding sites for cytoplasmic target proteins involved in transmission of the biological signal. These proteins encode in their noncatalytic regions a 100 amino acid domain termed SH2 (for src homology 2) responsible for binding to specific receptor sequences containing phosphorylated tyrosine residues (Pawson and Gish, 1992; Songyang *et al.*, 1993). SH2 domains bind tyrosine phosphorylated receptors with different affinities, indicating that it is this region that determines the binding specificity of proteins with receptors (Cantley *et al.*, 1991; Pawson

and Gish, 1992; Songyang *et al.*, 1993). Together with the SH2 domain, some signaling proteins also contain a SH3 domain. The SH3 domain may modulate interactions with the cytoskeleton and membrane (Koch *et al.*, 1991).

There are at least four signal transduction pathways for NGF: phosphatidylinositol-3 kinase (PI3K), phospholipase C-γ, Ras and SNT (for suc-associated neurotrophic factor-induced tyrosine-phosphorylated target).

1. PI3K

PI3K may be an important signal protein for many surface receptors including growth factor, cytokine and G-protein-coupled receptors. Purified PI3K phosphorylates phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PI-4-P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) on the D-3 position, producing phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2), and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3), respectively (Auger *et al.*, 1989; Whitman *et al.*, 1988; Carpenter *et al.*, 1990). In response to stimulation of receptor tyrosine kinases, PI-3 kinases mediate increases in these phosphoinositides (Carter and Downes, 1992; Soltoff *et al.*, 1992).

1.1 Structure

PI3K was originally purified as a heterodimer of a 85 kDa regulatory subunit (p85) and 110 kDa catalytic subunit (p110) (Carpenter *et al.*, 1990; Kapeller and Cantley, 1994).

cDNA cloning of the subunits has revealed five forms of the regulatory subunit (Otsu *et al.*, 1991; Skolnik *et al.*, 1991., Escobedo *et al.*, 1991; Inukai *et al.*, 1996) and five forms of catalytic subunits (Hu *et al.*, 1993; Hiles *et al.*, 1992; Volinia *et al.*, 1995; Stoyanov *et al.*, 1995; MacDougall., 1995). Diversity of PI3K isoforms may be due to the alternate splicing of the two kinds of subunits.

The regulatory p85 subunit contains, in the amino terminal half, an SH3 domain, a region homologous to the GTPase activating protein (GAP) domain of the breakpoint cluster region (BCR) protein and two proline rich regions that can bind SH3 domains. The carboxy-terminal half has two SH2 domains and a region for binding to p110 (Lam *et al.*, 1994). Three recently-described forms of the regulatory subunit have only the carboxy-terminal SH2 domains and the p110-binding domain (Pons *et al.*, 1995; Inukai *et al.*, 1996). The recruitment of PI3K to the receptor is mediated by SH2 domains of p85 which bind to phosphotyrosine motifs on the Trk receptor complex (Kapeller and Cantley, 1994).

The catalytic p110 subunit is homologous to protein kinases and itself, has intrinsic protein serine/threonine kinase activity, as well as phosphoinositide kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). p110 also appears to be the binding site for wortmannin, a highly potent inhibitor of PI3K (Yano *et al.*, 1993).

There are some novel species of PI3K. The first one is the G-protein-activated PI3K. Purified from myeloid-derived cells (Stephens *et al.*, 1993a, 1994b) and neutrophils (Stephens *et al.*, 1993b), this isoform can be activated by both α and $\beta\gamma$ subunits of the G-protein transducin, but it lacks the p85 adaptor subunit (Stoyanov *et al.*, 1995). The activity of this isoform can be inhibited by wortmannin and its major product is PI-3,4,5-P3 (Stephens *et al.*,

1993a,b).

The second isoform was defined in studies of yeast protein sorting (Horazdovsky *et al.*, 1995). *Saccharomyces cerevisiae* VPS34 gene encodes a protein required for the sorting of proteins into the yeast vacuole: a compartment similar to the lysosome in higher eukaryotes. The vps34p is a PI3K which phosphorylates only PI on the D3 position to produce PI-3-P (Schu *et al.*, 1993). Correspondingly, one kind of mammalian PI3K which is highly homologous to vps34p was cloned and found to phosphorylate PI exclusively as well (Volinia *et al.*, 1995). These enzymes do not use p85, but appear to be regulated by an associated protein-serine kinase, vps 15p.

Finally, a recently-described C2 domain-containing catalytic subunit phosphorylates primarily PI and PI-4-P (MacDougall *et al.*, 1995). This enzyme probably provides an independent pathway for regulated production of PI-3,4-P2.

1.2 Functions

Studies using the PI3K inhibitors, wortmannin and Ly294002, dominant-negative mutations of PI3K, or a constitutively active PI3K, implicate distinct cellular functions of PI3K: mitogenic signaling, inhibition of apoptosis, intracellular vesicle trafficking/secretion, regulation of cytoskeleton function and neurite outgrowth. PI3K could affect cell function in three ways: (1) through the production of phosphoinositides, (2) through phosphorylation of proteins on serine or threonine via the endogenous protein kinase activity, or (3) by acting as an adaptor, not requiring enzymatic activity. However, for the second possibility, thus far, the only good protein substrate for PI3K is the p85 subunit and phosphorylation of this

subunit (which results in inhibition of lipid kinase activity) is orders of magnitude slower than phosphorylation of phosphoinsitides (Carpenter *et al*, 1993; Dhand *et al*, 1994). The third possibility is raised because the p85/p110 type PI3K has been shown to bind to a wide variety of molecules *in vivo* (e. g. protein-tyrosine kinases, Grb2, Ras, tubulin) and it would potentially act to assemble signaling complexes that do not require the PI3K activity.

1.2.1 Mitogenesis.

Several lines of evidence suggest that PI3K is involved in cell cycle G0 to S progression. Inhibitory antibodies to the p110 subunit of PI3K block the DNA synthesis that normally occurs in response to platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), but do not block DNA synthesis in response to colony-stimulating factor (CSF)-1, bombesin or lysophosphatidic acid (Roche *et al*, 1994). Microinjection of the SH2 domains of the p85 subunit of PI3K or antibodies against p85 block insulin-dependent DNA synthesis in rat fibrobasts (Jhun *et al.*, 1994).

Wortmannin treatment of CTTL-2 cells led to an accumulation of cells at G0/G1 (Karnitz *et al.*, 1995). Wortmannin suppressed thymidine incorporation in these cells, but did not completely block it. These findings all suggest PI3K plays a role in mitogenesis.

1.2.2 Apoptosis

Some developing neurons undergo programmed cell death (apoptosis). Evidence that PI3K activity is necessary to prevent apoptosis was provided by the ability of PDGF to prevent apoptosis in PC12 cells transfected with wild-type PDGF receptor but not in cells

transfected with a PDGF receptor mutant that lacks the PI3K binding site (Yao and Cooper, 1995). PI3K inhibitors, wortmannin and LY 294002 cause apoptosis in PC12 cells maintained in NGF, EGF, insulin or serum (Yao and Cooper, 1995). Those two compounds also block the ability of interleukin-4 (IL-4) and interleukin-3 (IL-3) to prevent apoptosis in haemopoietic cells (Scheid *et al.*, 1995). In contrast, granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-5 (IL-5) prevent apoptosis in the presence of wortmannin (Scheid *et al.*, 1995). Wortmannin can block the ability of insulin-like growth factor-1 (IGF-1), but not IL-3 to prevent apoptosis in hematopoetic precursors (Minshall *et al.*, 1996). Whether PI3K is necessary for prevention of apoptosis in other cells remains to be determined.

1.2.3. Vesicle trafficking

The importance of PI3K for vesicle trafficking was first demonstrated by the essential role of Vps34p, a PI3K in yeast, for Golgi to vacuole trafficking (Herman and Emr, 1990). Studies in mammalian cells suggest a similar role for PI3K. In mammalian cells, an isoform of PI3K homologous to vps34p was cloned and shown to be inhibited by wortmannin (Stephens *et al.*, 1994a). Wortmannin (at concentration of 100 nM) blocks the trafficking of vesicles from Golgi to lysosome, and cells accumulate large vesicle-like structures which seem to be prelysosomes (Brown *et al.*, 1995; Davidson, 1995). The best evidence for the importance of PI3K activity in trafficking to the lysosome comes from studies of PDGF receptor mutants (Joly *et al.*, 1994; 1995). Both wortmannin-treated cells and cells expressing PDGF receptor mutants that do not activate PI3K fail to endocytose the PDGF

receptor. It may be that transport to an intracellular location is important in either signaling cell division or preventing apoptosis. By preventing transport of the activated PDGF receptor, further signaling would be impeded.

Both wortmannin and a dominant-negative p85 subunit of PI3K block GLUT4 (a glucose transportor) translocation to the plasma membrane in response to insulin (Cheatham *et al.*, 1994).

1.2.4. Others

Some lines of evidence suggest that PI3K may be implicated in cell motility and adherence. Cells expressing PDGF receptor mutants that do not bind PI3K do not ruffle or undergo chemotaxis in response to PDGF (Wennstrom *et al.*, 1994; Kundra *et al.*, 1994). Both PDGF receptor mutants that do not bind PI3K and wortmannin inhibit binding of GTP to Rac, a monomeric GTP-binding protein which has been shown to be involved in cell ruffling response (Ridley *et al.*, 1992), in response to PDGF (Hawkins *et al.*, 1995). These data place PI3K upstream of Rac in fibroblasts, and consistent with this idea, injection of fibroblasts with V12 Rac circumvents inhibition of ruffling by wortmannin (Nobes *et al.*, 1995). Finally, although the biochemical mechanism is obscure, there is growing evidence both from receptor mutants and from inhibitors that PI3K is required for stimulus-dependent activation of integrins and cell adherence (Kinash *et al*, 1995; Serve *et al.*,1995).

Furthermore, continuous inhibition of PI3K by wortmannin can suppress neurite outgrowth in PC12 cells (Kimura *et al.*, 1994). But mutagenesis of the specific binding sites for PI3K reveals a non-essential function of PI3K in neurite outgrowth (Obermeier *et al.*,

1994).

1.3. Interaction of PI3K with other signaling proteins

1.3.1 Ras

Ras is a small, monomeric G-protein. PI3K binds to the GTP form of ras *in vitro* and this occurs via a domain of the p110 subunit of PI3K (Rodriguez *et al.*, 1994). Transfection of activated PI3K and activated Ras into PC12 cells resulted in elevated levels of PI-3,4-P2 and PI-3,4,5-P3. Dominant-negative Ras (type N17, in which Ser 17 is mutated to Asn) blunted the increase in levels of PI-3,4-P2 and PI-3,4,5-P3 in response to EGF and NGF. These data suggest that PI3K is down stream of Ras. Conversely, expression of a constitutively active PI3K results in *fos* induction, which is blocked by N17 Ras and a dominant-negative Raf. In addition, levels of GTP-bound Ras in cells transfected with constitutively active PI3K have also been elevated (Hu *et al.*, 1995). Furthermore, injection of the amino-terminal SH2 domain of PI3K as a dominant-negative blocker blocks *fos* induction in response to insulin (Jhun *et al.*, 1994). This block is rescued by activated Ras. Inhibition of *fos* induction by an antibody raised to the amino-terminal SH2 domain of p85 was also rescued by Ras. These data suggest that PI3K is upstream of Ras. Cell-type differences, or the possibility that Ras is both upstream and downstream of PI3K, may reconcile these disparate data.

It is possible that PI3K, in some cells, assists in the activation of Ras (e.g. the lipid products may help to bring SOS (for Son of sevenless) to the membrane) and itself further activated by GTP-Ras. Whichever model proves to be correct, the presence of PI3K in the

ras pathway places it in a position to promote cell division and perhaps avert apoptosis.

1.3.2. Protein kinase C

In vitro experiments suggest several protein kinases as potential downstream targets of PI3K. The calcium-independent protein kinase C isoforms, PKC-δ, PKC-ε and PKC-ζ were shown to be activated by PI-3,4-P2 and to a lesser extent by PI-3,4,5-P3 (Toker *et al.*, 1994). Since these PKC isoforms can also be activated by diacylglycerol, they could be downstream effectors of multiple signaling pathways. There was also a report that brain-derived PKC-ζ is activated by PI-3,4,5-P3 (Nakanishi *et al.*, 1993). This activation was not observed using recombinant PKC-ζ (Toker *et al.*, 1994), raising the possibility that contaminating PKC-ε in the preparation was responsible for the observed effects.

Evidence that a PKC is activated *in vivo* by a PI3K product was first provided by the observation that in thrombin-stimulated platelets, sustained phosphorylation of pleckstrin (the major PKC substrate in platelets) is blocked by PI3K inhibitor (Toker *et al.*, 1995; Zhang *et al.*, 1995). Addition of PI-3,4-P2 or PI-3,4,5-P3 to permeabilized platelets circumvented the inhibition of pleckstrin phosphorylation by wortmannin.

In addition, wild-type PDGF receptors caused the translocation of PKC-ε to the membrane and mutants that failed to bind PLC-γor PI3K did not. Adding back either the PI3K binding site or the PLC-γ binding site to the PDGF receptor restored the PDGF-dependent translocation of PKC-ε. Wortmannin blocked the PDGF-dependent translocation of PKC-ε in the mutants containing the PI3K binding site, but failed to block translocation in the mutants that bind only PLC-γ (Moriya *et al.*, 1996), suggesting that PI3K plays an

important role in the activity of PKC-ε.

Finally, evidence provided by using both dominant active and dominant negative PI3K constructs demonstrates that the activation of a phorbol ester response element in cells overexpressing PKC-λ is dependent on PI3K. The combination of *in vitro* and *in vivo* data suggests that the lipid products of PI3K activate the novel and the atypical protein kinase C isoforms. How activation of these PKC isoforms might be involved in preventing apoptosis or promoting cell division is not yet known.

1.3.3. Akt

Akt, a proto-oncoprotein, was originally isolated as a retrovirus-encoded oncogene (Bellacosa *et al.*, 1991). It is a protein-serine/threonine kinase. Some lines of evidence suggest that growth factor-dependent activation of cellular Akt is dependent upon activation of PI3K (Burgering and Coffer, 1995; Franke *et al.*, 1995). Akt contains a pleckstrin homology (PH) domain at the amino-terminus whose presence is critical for activation since other PH domains have been shown to bind phosphoinositides, the PH domain of Akt is a likely site for regulation via the lipid products of PI3K. Evidence that Akt is directly regulated by PI3K products was provided by the ability of this enzyme to be activated *in vitro* by a mixture of phosphoinositides that had been incubated with PI3K and ATP (Franke *et al.*,1995).

Activation of Akt may explain some of the downstream responses to PI3K. $p^{70\text{-}S6}$ kinase, a protein kinase, has been found to be stimulated by an activated form of Akt (Burgering *et al.*, 1995). $p^{70\text{-}S6}$ Kinase is activated by most growth factors and cytokines and

is responsible for phosphorylating the ribosomal S6 subunit. PI3K, protein kinase C and FRAP/RAFT all have a role in the activation of S6 kinase (Cheatham *et al.*, 1994; Chung *et al.*, 1994; Weng *et al.*, 1995). The PI3K dependence could be explained either by its ability to activate a PKC family member or by its ability to activate Akt, or both.

Attempts to demonstrate direct activation of $p^{70\text{-}S6}$ kinase by the protein kinase endogenous to PI3K or by the lipid products of PI3K have failed. $p^{70\text{-}S6}$ kinase, by phosphorylating ribosomes is thought to regulate translation of a subgroup of messages. However, the role of $p^{70\text{-}S6}$ kinase in cell cycle progression is not yet clear.

1.3.4. Mitogen activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK)

In some cell types and in response to some stimuli, PI3K activation is required for stimulation of MAPK (Kamitz *et al.*, 1995; Cross *et al.*, 1994; Knall *et al.*, 1996; Urich *et al.*, 1995). Different results have been obtained from different laboratories, but the PI3K dependence is probably either at the level of Raf activation or mitogen activated protein kinase kinase (MAPKK or MEK) activation. Isoforms of protein kinase C that are activated by PI3K are candidates for mediating activation of this pathway. Activation of MAPK could affect both apoptosis and promote cell division since the relative levels of MAPK and JNK/p38 kinase activities seem to affect whether a cell undergoes apoptosis.

1.3.5. Interaction with SH2 domains

PI-3,4,5-P3 binds to several SH2 domains *in vitro* and this binding is in competition

with phosphotyrosine-protein binding (Rameh *et al.*, 1995). Evidence was also provided that this occurs *in vivo* since the SH2-mediated interaction between the insulin receptor and PI3K could be modulated by changing the level of PI-3,4,5-P3 in the cells. These results suggest that PI-3,4,5-P3 may act to recruit SH2-containing proteins to the membrane independent of the need for tyrosine phosphorylation. In this way, PI3K could contribute to protein kinase signaling cascades by assembling protein at the membrane.

2. PLC-γ

PLC-γ catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate to the potent second messengers, diacylglycerol (DAG) and inositol trisphosphate (IP3) (Rhee and Choi, 1992). DAG is an activator of protein kinase C, while IP3 transiently increases the levels of intracellular calcium (Rhee and Choi, 1992).

Among the metabolic changes in NGF-treated PC12 cells that have been suggested to result from elevation of PLC-γ activity are changes in ion fluxes and intracellular pH, cytoskeletal rearrangements, and induction of certain cellular genes (Traynor *et al.*, 1982; Cremins *et al.*, 1986; Hama *et al.*, 1986; Contreras and Guroff, 1987; Heasley and Johnson, 1989; Lazarovici *et al.*, 1989). Mutation of the site in Trk responsible for inducing the tyrosine phosphorylation and receptor association of PLC-γ results in a selective loss of the NGF-mediated increase in the transcription of peripherin, a gene encoding an intermediate filament protein (Loeb *et al.*, 1994). Moreover, only mutation of the binding sites for both PLC-γ and Shc in growth factor receptor can block NGF-induced cell differentiation (Obermeier *et al.*, 1994) and neurite outgrowth (Stephens *et al.*, 1994), suggesting redundant

signal transduction pathways exist.

3. Ras

Ras (or $p21^{ras}$) is a small, monomeric G-protein. Like other G-proteins, Ras is inactive in the GDP-bound form and active in the GTP-bound form. The interconversion between these two forms is regulated by the balanced action of guanine nucleotide releasing factors (also known as mammalian Son of sevenless (mSOS)) and GTPase activating proteins (GAP). mSOS release Ras-bound GDP molecules, which are rapidly replaced by cellular GTP molecules. GAP accelerates the intrinsic GTPase activity of Ras. The predominant mechanism by which receptor tyrosine kinase controls Ras activation is by stimulation of guanine nucleotide exchange activity (McCormick, 1994).

Activation of Ras requires the interaction of some adaptor proteins: Shc and Grb2 (for growth-factor-receptor binding protein 2). Tyrosine-phosphorylated Shc associates with Grb2 which in turn binds SOS, Ras guanine nucleotide exchange factors (Suen *et al.*, 1993; Stephens *et al.*, 1994; Ohmichi *et al.*, 1994). Receptor phosphorylation of SOS to membrane-localized Ras, thus activates the Ras signaling pathway (Suen *et al.*, 1993).

Once in the active, GTP-bound state, Ras influences cell behaviour by interacting with target proteins (effectors). The best characterized effectors for Ras are the serine/threonine kinases of the Raf family, A-Raf, B-Raf and c-Raf-1 (Rapp, 1991). They interact with Ras-GTP, but not Ras-GDP both *in vitro* (Moodie *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993), and in intact cells (Finney *et al.*, 1993; Hallberg *et al.*, 1994). As a result of this interaction, Raf is brought to the plasma membrane where Ras is localized because of its post-

translational modification with farnesyl and palmityl moieties (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Whereas the interaction of Ras-GTP with Raf does not appear to cause activation of its kinase activity directly, it appears to be responsible for presenting Raf to another signaling component, probably localized in the plasma membrane, which is able to further modify it resulting in increased kinase activity. Once Raf has been activated, it phosphorylates and activates mitogen activated protein (MAP) kinase kinase (MAPKK) or MAP kinase (MAPK)/extracellular signal-regulated kinase (ERK)-activating kinase (MEK), which in turn phosphorylates and activates MAPK or ERK. Target proteins regulated by MAPK include cytoskeleton, protein kinase, phospholipase and transcriptional factors (Johnson and Vaillancourt, 1994).

In addition to the classical pathway described above, there are some exceptions for this pathway. First, MAPK pathway can be activated by G-protein-coupled receptors in yeast and higher eukaryotes (Johnson and Vaillancourt, 1994). In *S. cerevisiae*, the βγ-subunit of the pheromone receptor-coupled G protein activates MAPK pathway, whereas in *S. pombe* the α-subunit of the G protein regulates the MAPK pathway (Neiman *et al.*, 1993). In higher eukaryotes, both α- and βγ-subunits can mediate activation of MAPK pathways. The thrombin (Van Corven *et al.*, 1993), muscarinic acetylcholine M_2 (Winitz *et al.*, 1993) and α_2 adrenergic (Alblas *et al.*, 1992) receptors activate the MAPK pathway by a mechanism that also involves Ras activation. Protein kinase C (PKC) has also been found to be involved in the activation of MAPK by G protein-coupled receptors (Granot et., 1993). Consistent with these results is the finding that PKCα can phosphorylate Raf-1 upon serine residues *in vitro* (Kolch *et al.*, 1993). However, some agonists such as thrombin and the mitogenic

phospholipid, lysophosphatidic acid (LPA), have been shown to activate the MAPK pathway (Kahan *et al.*, 1992; Cook *et al.*, 1993; van Corven *et al.*, 1993; Hordijk *et al.*, 1994) by a mechanism independent of changes in PKC activation.

Second, some studies have indicated the existence of Raf-independent activation of MAP kinase (Burgering *et al.*, 1993). In NRK cells, expression of c-Raf-1 antisense does not affect MAP kinase activation by both PDGF and EGF (Kizaka-Kondoh and Okayama, 1993). Raf-independent MEK activators (Zheng *et al.*, 1994) and direct-activation of MEK by Ras (Lange-Carter and Johnson *et al.*, 1994) have also been found in some cells.

Third, cyclic AMP-dependent protein kinase A (PKA) regulates MAPK activity in some cell types. Manipulations leading to the activation of PKA can inhibit (Burgering *et al.*, 1993; Cook, 1993; Graves *et al.*, 1993; Sevetson *et al.*, 1993; Wu *et al.*, 1993; Chuang *et al.*, 1994), activate (Frodin *et al.*, 1994; Young *et al.*, 1994) or have no effect (Sevetson *et al.*, 1993; Burgering *et al.*, 1993; Mclees *et al.*, 1995; Vaillancourt *et al.*, 1994) on MAPK activity.

The importance of the Ras pathway in the control of cellular functions can be summarised as the follows:

(1). Apoptosis The role of Ras in apoptosis has been investigated and quite inconsistent results have been obtained. In chick embryonic neurons, the NGF-induced survival of dorsal root ganglion (DRG) neurons was inhibited by anti-Ras antibodies or their Fab fragments in a specific and dose-dependent fashion (Borasio *et al.*, 1993). The effect could be reversed by saturating the epitope-binding sites with biologically inactive Ras before microinjection. However, Ras did not promote the survival of NGF-dependent chick

sympathetic neurons, and the NGF-induced survival in these cells was not inhibited by the Fab-fragments even though the formation of Ras-GTP in these neurons markedly increased by NGF (Carter *et al.*, 1995).

On the contrary, neutralizing anti-p21^{ras} Fabs suppress rat sympathetic neuron survival induced by NGF, LIF, CNTF and cAMP (Nobes and Tolkovsky, 1995). Consistent with this result, introduction of active p21^{ras} into the cells is sufficient for rescue of NGF-dependent rat sympathetic neurons (Nobes *et al.*, 1996). These results indicate that Ras is a crucial anti-apoptotic mediator of survival in rat sympathetic neurons. However, the downstream target of Ras, MAP kinase, does not seem to be required for the actions of either cAMP or NGF on neuronal survival, since PD098059, an inhibitor of MAP kinase kinase did not block the effects of NGF on neuronal survival (Creedon *et al.*, 1996) and activation of MAPK is neither necessary nor sufficient for neuronal survival (Virdee and Tolkovsky, 1995).

These results are further complicated by an observation from PC12 cells. PC12 cells transfected with dominant-inhibitory Ras show long-term survival in the absence of NGF or other trophic factors. Moreover, introduction of dominant-inhibitory Ras into the cells also rescued non-dividing, neuronally differentiated PC12 cells from death caused by NGF withdrawal (Ferrari and Greene, 1994), suggesting that Ras serves as a mediator of apoptosis. One explanation for these contradictory results is that neurotrophin signaling may be differentially regulated depending on the neuronal context and the developmental stage of the cells.

(2). Differentiation or proliferation The possible role of Ras in cell differentiation or proliferation process signaling by growth factors have been investigated through different

approaches. Microinjection studies utilizing neutralizing anti-Ras antibody, and studies using a dominant inhibitory mutant of Ras have demonstrated that the function of the cellular Ras protein is required for NGF, FGF- and v-src-induced differentiation of PC12 cells (Hagag *et al.*, 1986; Szeberenyi *et al.*, 1990; Kremer *et al.*, 1991) as well as for growth and transformation of NIH 3T3 cells by activated tyrosine kinases (Mulcahy *et al.*, 1985; Smith *et al.*, 1986; Feig and Cooper, 1988). Moreover, expression of the activated Ras oncogene is sufficient to induce the neuronal differentiation of PC12 cells, as well as the proliferation and transformation of NIH 3T3 cells (Barbacid, 1987). However, experimental observations with PC12 cells expressing a variety of Trk SH2 protein binding site mutants demonstrate that NGF-induced neuronal differentiation is mediated through redundant signal transduction pathways involving Shc and PLC-γ (Obermeier *et al.*, 1994; Stephens *et al.*, 1994).

Studies have also been conducted to investigate whether the down-stream targets of Ras are involved in NGF-induced cell differentiation. Transfection of the PC12 cells with constitutively activated mutants of MAPKK stimulated neuronal differentiation of PC12 cell and transformation of NIH 3T3 cells. The interfering mutants inhibited growth factor-induced PC12 cell differentiation, growth factor stimulation of proliferation, and reverted v-src- and ras-transformed cells (Cowley *et al.*, 1994). Furthermore, the role of each component of MAPK cascade in cell differentiation has been examined by injecting constitutively active forms of enzymes into PC12 cells (Fukuda *et al.*, 1995). All these results show that, depending on cellular context, activation of MAPK pathway is necessary and sufficient for cell differentiation or proliferation.

4. SNT

SNT (for suc-associated neurotrophic factor-induced tyrosine-phosphorylated target) is a 78-90 kDa species that is rapidly phosphorylated on tyrosine in NGF-treated PC12 cells and neurotrophic factor-treated primary neurons (Rabin *et al.*, 1993). SNT is an independent pathway parallel to the Ras pathway. The tyrosine phosphorylated form of the protein is predominantly nuclear.

However, SNT-like proteins have also been detected in fibroblast, myoblast and lymphoid cell lines and upon treatment with FGF, they undergo tyrosine phosphorylation (Wang *et al.*, 1996). These proteins are associated with the Grb-2 adaptor and are the major tyrosine phosphorylated proteins associated with the Ras guanine nucleotide exchange factor Sos in FGF-stimulated fibroblasts, suggesting that SNT-like proteins, Grb2 and Sos complexes modulate the activity of Ras protein.

The function of SNT inside the cell is unknown. SNT has been proposed to serve as a co-pathway to Ras in NGF-promoted neuritogenesis, since deletion of a conserved juxtamembrane sequence in the Trk NGF receptor resulted in impaired neurite outgrowth and the mutated receptor mediated unimpaired tyrosine phosphorylation of the signaling proteins other than SNT (Peng *et al.*, 1995).

II. Signals mediated by p75

While the mechanism by which receptor tyrosine kinases effect intracellular signaling has become well-understood, the precise functions of the p75 receptor have been the subject

of considerable controversy and interest. Many studies suggest that p75 facilitates the interaction of NGF with the extracellular domain of Trk. When p75 and Trk are co-expressed in proper ratio, substantial numbers of high-affinity sites are observed. Deletion mutation in p75 have been found to eliminate high-affinity binding (Hempstead *et al.*, 1990; Battleman *et al.*, 1993), suggesting that an intact p75 protein co-expressed with Trk A is required for high-affinity site formation. Over-expression of Trk A in PC12 cells led to increases in the number of high- and low-affinity sites (Hempstead *et al.*, 1992) and decreases in p75 levels, or inhibition of binding of NGF to p75 decreased both high- and low-affinity binding sites (Weskamp and Reichardt, 1991; Benedetti *et al.*, 1993).

Biological responsiveness to trophic factors depends upon the relative levels of p75 and Trk family members. Co-expression of p75 with Trk A in MAH (human natural killer 1) cells led to an eight-fold increase in autophosphorylation of Trk A tyrosine, and an accelerated differentiative response, as manifested by a more rapid mitotic arrest and neuronal maturation (Verdi *et al.*, 1994). These effects were dependent upon a high ratio of p75 to Trk A in these cells, and required binding of NGF to p75.

Consistent results have been obtained from primary-culture experiments that used sensory neurons from mice carrying a homozygous mutation in the p75 gene. Trigeminal neurons that lack p75 were found to require nearly four times more NGF than required by wild type neurons to achieve comparable levels of survival (Davies *et al.*, 1993). Sympathetic neurons from neonatal p75-null mice require more NGF for survival than required by neurons from normal mice at earlier developmental stages (Lee *et al.*, 1994).

In addition, manipulation of p75 levels with antisense oligonucleotides in primary

sensory-neuron culture produced differential effects upon cell survival that are dependent upon developmental age (Barrett and Bartlett, 1994).

While p75 increases sensitivity of Trk to neurotrophins, co-existence of these two receptors may endow the cells with greater discrimination in neurotrophin responsiveness (Benedetti *et al.*, 1993). Expression of p75 with Trk A enhances NGF-mediated survival but also limits the responsiveness to neurotrophins other than NGF. When Trk A is expressed in fibroblast cells in the absence of p75, NGF, NT-3 and NT-4/5 can stimulate autophosphorylation of Trk A, and lead to a proliferative response. However, similar concentration of NT-3 and NT-4/5 failed to induce increased autophosphorylation of Trk A when p75 was co-expressed (Ip *et al.*, 1993). The presence of p75 appears to restrict the ability of NT-3 to activate Trk A.

The production of some second messengers has also been found to be related to p75. In PC12 cells, both NGF and BDNF elevate cAMP levels within a subsecond time period (Knipper *et al.*, 1993). The cAMP formation induced by NGF and BDNF was reduced by GDP-β-S and pertussis toxin, but not K252a which is active against the Trk receptor. In p75 expressing, but Trk-deficient PCNA cells (a mouse fibroblast cell line), both NGF and BDNF induced cAMP formation. These results suggest that cAMP formation induced by NGF and BDNF is mediated via a p75/G-protein mechanism.

Furthermore, a potential signaling pathway for p75 in the hydrolysis of sphingomyelin has been proposed from studies in T9 glioma cells (Dobrowsky *et al.*, 1994). The production of ceramide was increased in NGF treated T9 cells, which express low levels of p75, but do not express Trk receptors. This increase was also observed in EGF/p75 chimeric receptors

(Yan *et al.*, 1991). Since ceramide is regarded as a potential mediator of cell growth (Saba *et al.*, 1996) and is capable of influencing protein-phosphorylation events (Hannun, 1994; Kolesnick and Golde, 1994), the ability of NGF to produce ceramide through the p75 receptor offers an additional signaling mechanism for neurotrophins.

Finally, unliganded p75 may mediate signal transduction in apoptotic pathways (Rabizadeh *et al.*, 1993; Barrett and Bartlett, 1994). NGF binding appears to block this apoptotic activity, perhaps contributing to the well-known effect of NGF in neuronal survival.

However, these results remain to be reconciled with a number of contradictory findings. First, some experimental results suggest that p75 does not modify Trk sensitivity to NGF (Jing *et al.*, 1992; Glass *et al.*, 1991; Ip *et al.*, 1993). Dose-response studies performed in fibroblasts have shown that similar concentrations of NGF are required to elicit responses in cells that express both p75 and Trk A, compared with cells that express only Trk A (Jing *et al.*, 1992; Ip *et al.*, 1993). Also, antibodies to p75 do not interfere with the ability of NGF to promote cell survival and neurite outgrowth (Weskamp and Reichardt, 1991). In addition, NGF mutants that are defective in binding to p75 are nearly fully functional (Ibanez *et al.*, 1992), and Trk receptors can function independently in a variety of cell types. These results question the role of the p75 receptors in neurotrophin signaling.

Trophic regulation of voltage-gated ion channels

I. Calcium (Ca^{2+}) channels

The possibility that NGF is involved in the regulation of Ca^{2+} channel properties

derived from the experimental result that inhibition of neurotransmitter release by dihydropyridines on NGF-differentiated PC12 cells was pronouncedly attenuated (Takahashi *et al.*, 1985; Kongsamut and Miller, 1986). In NGF-treated PC12 cells, the total whole-cell Ca^{2+} channel currents increased significantly compared with the untreated cells (Streit and Lux, 1987; Usowicz *et al.*, 1990; Furukawa *et al.*, 1993; Lewis *et al.*, 1993; Cavalié *et al.*, 1994). Since NGF also induced growth of somata and neurites, the current density (current normalized to the cell surface area) in NGF-treated PC12 cells was reported to be unchanged (Streit and Lux, 1987; Garber *et al.*, 1989) or increased (Furukawa *et al.*, 1993; Lewis *et al.*, 1993; Cavalié *et al.*, 1994).

Attempts have been made to see which type of Ca^{2+} channels was influenced by NGF in PC12 cells. Although Streit and Lux (1987, 1989) suggested that only one type of high-voltage-activated (HVA) Ca^{2+} channel was present and induced in NGF-treated PC12 cells, Plummer *et al.* (1989) found the co-existence of N- and L-type channels with mainly the N-type currents being increased during NGF-induced differentiation. This conclusion was confirmed in other clones of PC12 cells (Usowicz *et al.*, 1990; Lewis *et al.*, 1993; Furukawa *et al.*, 1993). By contrast, Garber *et al.* (1989) reported a significant increase in T-type Ca^{2+} currents after NGF-treatment of their cell line. Some possible reasons for the discrepancy among these results may be that different laboratories used distinctive clones of PC12 cell lines or the culture environment was disparate in those experiments.

Radioligand binding studies with ^{125}I-ω-conotoxin and [^{3}H](+)-isradipine suggest that NGF treatment in PC12 cells can enhance expression of Ca^{2+} channel proteins, especially ω-conotoxin-sensitive N-type Ca^{2+} channel proteins (Usowicz *et al.*, 1990; Lewis *et al.*, 1993).

These results suggest the enhanced whole-cell Ca^{2+} channel currents are due to the increased number of Ca^{2+} channel in the cell membrane.

The inactivation of Ca^{2+} current during NGF-induced differentiation of PC12 cells was investigated (Streit and Lux, 1987, 1990). A shift of the steady-state inactivation curve towards more negative potentials, as well as an increase in the strength of time-dependent inactivation, was observed in differentiating PC12 cells with NGF compared to undifferentiated cells. The increase in the strength of inactivation preproceeded the increase in the peak calcium current amplitude observed in PC12 cells during NGF treatment. In differentiating cells, regional differences in the strength of inactivation paralleled differences in current density. Barium currents recorded from growth cones, where the current density was high, showed 30% more inactivation than soma currents. The possible explanation from these authors is that the redistribution of Ca^{2+} channels following NGF treatment rather than the appearance of a second type channel causes the alterations in the strength of inactivation observed during NGF-induced PC12 cells differentiation. Similar results were obtained from another laboratory (Rane and Pollock, 1994). The steady-state inactivation curve was shifted to the more negative potentials in PC12 cells treated with basic fibroblast growth factor (bFGF) than untreated cells. Since the inactivation time constants for ω-conotoxin sensitive and insensitive components in bFGF treated cells were in the same order as those in untreated cells, one suggestion from the results of this experiment was that the greater increase in the ω-conotoxin sensitive component (N type) relative to the other components may be the reason for the significant negative shift of the midpoint for steady-state inactivation and for the more rapid rate of inactivation of the whole cell current. However, Plummer *et al.* (1989)

did not report a significant increase in inactivation during the differentiation of PC12 cells when they cut off all neurites before recording. Since the mechanism underlying the inactivation of Ca^{2+} currents is very complicated, the effects of NGF on Ca^{2+} channel inactivation are worth further investigation.

Few studies have been carried out on the effects of growth factors or neurotrophins on Ca^{2+} channel in neurons. Levine *et al.* (1995) investigated the effects of both NGF and BDNF on voltage-gated Ca^{2+} channel currents in cultured embryonic basal forebrain neurons using the whole-cell patch-clamp technique. Neurons grown in the presence of 100 ng/ml NGF for 4-6 days had qualitatively similar properties to those grown under serum-free conditions. The characteristics of the action potentials, firing patterns, and inward and outward currents of the NGF-treated cells were not strikingly different from those of the serum-free cells. There was, however, a considerable increase in the magnitude of both the L-type and N-type components of the whole cell Ca^{2+} channel currents in the NGF-treated cells when compared to serum-free cells. There was no obvious change in the kinetics of the currents, as the rates of activation and inactivation in NGF-treated cells were similar to that of the serum-free cells. Comparison of the results on the effects of NGF on Ca^{2+} channel from PC12 cells with those from the forebrain neurons suggests that regulation of Ca^{2+} channel kinetics by NGF may be dependent on the development stage of the cells or the cellular context. In addition, there was no obvious change in cell size, neurite outgrowth length or complexity in NGF-treated basal forebrain neurons compared with untreated cells, suggesting that ion channel expression and neurite outgrowth in neurons may be controlled by different signals.

In contrast to the effects of trophic factors on Ca^{2+} channel expression which takes several days to occur, there are some lines of evidence suggesting that trophic factors may acutely modulate Ca^{2+} channels. Koike *et al.* (1993) reported that basic fibroblast growth factor (bFGF) at a concentration of 10-100 ng/ml can augment L-type current in acutely isolated neurons from rat ventromedial hypothalamus. But more convincing evidence for the acute effects of neurotrophic factors on Ca^{2+} channels comes from the work of Wildering *et al.* (1995). NGF at doses ranged between 1 and 10,000 pg/ml can reversibly increase high-voltage-activated calcium currents in molluscan neurons and the effects can observed within 2 min of NGF application. Acute modification of Ca^{2+} channel properties may be the means whereby neurotrophins modulate synaptic efficacy (Kang and Schuman, 1995; Knipper *et al.*, 1994; Girod *et al.*, 1994; Lessmann *et al.*, 1994; Lohof *et al.*, 1993).

The signaling pathways involved in the regulation of Ca^{2+} channels in response to neurotrophic or other growth factors are still elusive. Some lines of evidence suggest that tyrosine kinase is involved in the enhanced expression of Ca^{2+} channels by growth factors. Transfection of PC12 cells with $pp^{v\text{-}src}$, a tyrosine kinase gene, can induce neuronal differentiation and 3-4 fold enhancement in Ca^{2+} channel currents (Lewis *et al.*, 1993). Treatment of PC12 cells with genistein, a tyrosine kinase inhibitor, can block bFGF-induced Ca^{2+} channel current (Rane and Pollock, 1994). This result can not, however, distinguish between a role for receptor tyrosine kinase and a role for intracellular, downstream tyrosine kinase(s).

Cavalié *et al.* (1994) examined the involvement of the immediate early genes, *c-fos* and *c-jun* in the expression of Ca^{2+} channel currents using Northern blot analysis and cell

transfection in conjunction with whole-cell current recordings. The levels of *c-fos* and *c-jun* mRNAs increased transiently during each daily exposure to NGF. Naive PC12 cells were transiently co-transfected with expression plasmids that contained the full length of *c-fos* and *c-jun* cDNA. After 2 days following transfection, the PC12 cells could be grouped according to the size of Ca^{2+} channel current. In 56% of cells, Ca^{2+} current was similar to control currents. In the remaining 44% of cells, Ca^{2+} current showed a 2.2-fold enhancement with respect to control cells. Transfection of only *c-fos* had no effects on Ca^{2+} current but, in 24% of cells transfected with *c-jun*, Ca^{2+} current was slightly enhanced. These results suggest that induction of immediate early genes might be involved in the expression of Ca^{2+} channel by NGF.

More recently, constitutive expression of the dominant negative Ras mutant in PC12 cells blocks growth factor-induced increases in ω-conotoxin GVIA-sensitive, nimodipine-sensitive, and ω-conotoxin GVIA/nimodipine-resistant Ca^{2+} currents, but it does not inhibit sodium current induction (Pollock and Rane, 1996). However, manipulations that produce sustained activation of the $p21^{ras}$ signaling pathway and the neurite extension characteristic of morphological differentiation fail to increase Ca^{2+} channel current densities. These results also indicate the existence of distinct signaling pathways for neurite outgrowth and induction of ion channels.

II. Sodium (Na^+) channels

Voltage-gated Na^+ channels are responsible for the generation and propagation of action potential (Hodgkin and Huxley., 1952b), and ultimately play a key role in the coding

and transfer of information in the nervous system. The PC12 cell line is a widely used model system in which to study the expression of Na^+ channels by growth factors. Prior to the treatment with NGF, PC12 cells cannot sustain action potentials, while after exposure to NGF they become capable of producing Na^+-dependent action potentials (Dichter *et al.*, 1977; Rudy *et al.*, 1982). Rudy *et al.* (1982) have shown that long-term NGF exposure increased the number of Na^+ channels in PC12 cells by 15- to 25-fold per cell and by 6- to 9-fold/mg protein, as determined by binding of the channel blocker saxitoxin. This increase appeared to be sufficient to account for the induction of excitability by NGF. Using ^{22}Na uptake to measure the Na^+ permeability through Na^+ channel activated by alkaloid toxins (veratridine, batrochatoxin and scorpion toxin) of PC12 cells before and after long-term NGF treatment, Rudy *et al.* (1987) demonstrated that NGF increased the number of functional Na^+ channels that otherwise behave similarly to those present before NGF treatment, and that it induces the presence of TTX-resistant Na channels.

Using the whole-cell patch clamp method, Pollock *et al.* (1990) further confirmed that NGF produces a 5- to 6-fold increase in Na^+ channel density. The Na^+ channels induced by NGF are not different from those in cells not treated with NGF and are similar to those in other cell types. Basic fibroblast growth factor (bFGF), another growth factor that causes PC12 cells to differentiate into sympathetic-like neurons, also produces a 5- to 6-fold increase in Na^+ current density with channels indistinguishable from those in PC12 cells treated with or without NGF. In this experiment, cyclic AMP decreased Na^+ current and Na^+ current density.

Furthermore, NGF-induced enhancement of Na^+ channel current or density in PC12

cells was also reported from other laboratories (Kalman *et al.*, 1990; Ginty *et al.*, 1992; D'Arcangelo *et al.*, 1993; Fanger *et al.*, 1993; Ifune and Steinbach, 1990; Toledo-Aral *et al.*, 1995).

In addition to PC12 cells, NGF also increased Na^+ channel density in chromaffin cells. Current density increased progressively over time, until an apparent plateau (3.5-fold increase) was reached by the end of the second week (Islas-Suàrez *et al.*, 1994). The kinetics and voltages for half-activation and half-inactivation of Na^+ currents in NGF-treated cells were not significantly different from those seen in control cells.

In contrast to the wealth of knowledge about the action of NGF in PC12 cells, little is known about the effects of trophic factors on neurons. Using *in situ* hybridization cytochemistry, Zur *et al.* (1995) has shown that Na^+ channel αI, II, III, βmRNAs are up-regulated by NGF in cultured embryonic DRG neurons and that the extent of regulation varies for those subunits.

Some attempts have been made to elucidate the signal transduction mechanism for NGF-mediated induction of Na^+ channel expression in PC12 cells. There were some controversial results as to whether cAMP was involved in the signal transduction pathways. cAMP itself decreases Na^+ current and current density (Pollock *et al.*, 1990). A cAMP analogue, 8-bromocyclic AMP does not change Na^+ current in PC12 cells, but it reduces the efficacy of NGF at increasing Na^+ currents (Ifune and Steinbach, 1990). However, using single channel recording, Kalman *et al.* (1990) showed that dibutyryl cAMP, a membrane permeable analogue of cAMP, increased the percentage of patches with Na^+ channels as effectively as NGF. A specific inhibitor of the protein kinase A catalytic subunit blocked the

increases in the percentage of the patches with Na^+ channels by NGF or cAMP, suggesting NGF increases Na^+ channel number in PC12 cells, in part, by activating PKA.

The role of PKA in the induction of Na^+ channel expression by NGF and bFGF has been further examined in PC12 cell lines deficient in cAMP-dependent PKA activity (Ginty *et al.*, 1992). Both NGF and bFGF do not elicit an increase in the density of functional Na^+ channels in PC12 cell lines deficient in PKA, as determined from whole-cell patch clamp recordings. Despite the inability of the neurotrophic factors to induce functional Na^+ channel expression in the PKA-deficient cells, Northern blot hybridization studies and saxitoxin binding assays of intact cells indicate that NGF and bFGF are still capable of eliciting increases in both Na^+ channel mRNA and Na^+ channel protein in the membrane. Thus PKA activity appears to be necessary at a posttranslational step in the synthesis and expression of functional Na^+ channels.

However, results based on combined whole-cell voltage-clamp and molecular hybridization suggest that expression of the type II/IIA Na^+ channel requires activation of cAMP-dependent PKA, whereas induction of the peripheral neuron type Na^+ channel occurs through a PKA-independent signal transduction pathway (D'Arcangelo *et al.*, 1993).

Some other intracellular proteins activated by NGF, such as ras (Fanger *et al.*, 1993; Kalman *et al.*, 1990) and protein kinase C (Kalman *et al.*, 1990) are excluded in the regulation of Na^+ channel number by NGF, even though transfection of activated ras into an excitable cell line (AtT-20) modifies voltage-dependent Na^+ current (Flamn *et al.*, 1990). Recently, platelet-derived growth factor (PDGF)-mediated induction of Na^+ channels has been shown to require redundant intracellular pathways including phosphatidyl-inositol-3-kinase (PI3K),

phosphalipase C-γ, GAP (for GTPase activating protein) and Syp (a tyrosine phosphatase) in PC12 cells (Fanger *et al.*, 1997).

III. Potassium (K^+) channels

Disparate results regarding the effects of neurotrophic factors on K^+ channels have been obtained from different laboratories. Garber *et al.* (1989) reported that NGF failed to affect K^+ channel currents measured by patch-clamp recording in PC12 cells. However, Sharma *et al.* (1993) reported that levels of kv2.1 (a delayed rectifier K^+ channel) polypeptide in PC12 cell membranes increased four-fold in response to NGF treatment. This increase in polypeptide levels could be seen within 12 h and elevated levels were maintained for at least 6 days of continuous NGF treatment. RNase protection assays indicate that the increase in kv2.1 protein occurs without an increase in steady state levels of kv2.1 mRNA following NGF treatment, implying that mechanisms other than regulation of gene expression can affect ion channel expression in PC12 cells. The discrepancy between the results from these two laboratories can be reconciled by the speculation that the increased expression of K^+ channel polypeptide by NGF might not be functional.

In NIH3T3 and C3H10T1/2 cell lines, treatment of the cells with EGF and PDGF increases the induction of Ca^{2+}-activated K^+ channel (Huang and Rane, 1994). These effects are mediated through $p21^{ras}$ and its immediate downstream target, the Raf kinase.

Expression of K^+ channel, kv1.5, and receptors for PDGF or FGF simultaneously in *Xenopus oocytes* shows that receptor activation mediated a decline in the kv1.5 current

amplitude, with a half-time of about 20 min (Timple and Fantl, 1994). The reduction in K^+ current amplitude occurred with little change in the kinetics or voltage sensitivity of activation. A mutant FGF receptor, which does not activate PLC-γ, but retains several of its other functions, did not modulate the kv1.5 current. Simultaneous injection of IP3 and superfusion of phorbol 12-myristate 13-acetate reproduced the modulation of the kv1.5 current. These results demonstrate that the PDGF and FGF receptors can modulate a voltage-activated K^+ channel by increasing PLC-γ activity.

In all, variation in cell types and K^+ channel subtypes may complicate the effects of neurotrophic or other growth factors on K^+ channels.

Ion channels on bullfrog sympathetic ganglion B-cells

Bullfrog sympathetic ganglia (BFSG) enjoy a high level of popularity as an experimental preparation because of their simple structure, robust and spherical neurons. The reasons that we have chosen this preparation to investigate the effects of NGF on voltage-dependent ion channels are described in detail in Chapter 2. There are two types of principal neurons in BFSG, the larger B-cells which project to the targets of the skin and the smaller C-cells which are primarily vasomotor (Horn *et al.*, 1988; Ivanoff an Smith, 1995). These cells are easily identified by their relative sizes. The various ionic conductances present on the membrane of BFSG B-neurons are well understood. Properties of voltage-dependent Na^+, Ca^{2+} and a variety of K^+ channels have been characterized in these neurons.

I. Ca^{2+} channels

Ca^{2+} channels in BFSG B-cells were first studied by Adams (1981) and their properties have been increasingly detailed (Jones and Marks, 1989a,b; Jones and Jacobs, 1990; Sala, 1991; Werz *et al.*, 1993; Delcour *et al.*, 1993). These channels undergo rapid activation and deactivation (τ<3 ms) when either Ba^{2+} or Ca^{2+} is used as a charge carrier (Jones and Marks, 1989a). The activation kinetics can be fitted reasonably well by a Hodgkin-Huxley-type model with a single gating particle of charge +3. However, more rapid kinetics were obtained with the use of the faster voltage clamping procedures (Sala, 1991). These faster kinetics fit better with a "m^2" model in which two gating particles control the channels.

In contrast to the rapid activation and deactivation process, the Ca^{2+} channels inactivate slowly and incompletely during 0.5-1 sec depolarizing voltage steps (Jones and Marks, 1989b). Because inactivation occurs with either Ca^{2+} or Ba^{2+} as the charge carrier, is not prevented by strong buffering of intracellular Ca^{2+} with 10 mM BAPTA, and varies little as the peak current is changed 10-fold by changing the divalent ion concentration, simple versions of voltage, Ca^{2+}- or current-dependent inactivation models can not explain the inactivation process. Moreover, multiple components, termed "fast", "intermediate" and "slow", of Ca^{2+}current inactivation in these neurons have been observed (Jones and Marks, 1989b; Werz *et al.*, 1993; Jassar *et al.*, 1993). In contrast to the "fast" and "intermediate" inactivation processes which occur in less than 1 sec, the "slow" inactivation lasts several minutes with repeated application of voltage pulses (Jassar *et al.*, 1993). Furthermore, phosphorylation has been shown to enhance inactivation of Ca^{2+} channels in these neurons (Werz *et al.*, 1993).

In BFSG B-neurons, ninety percent of the total Ca^{2+} currents are ω-conotoxin GVIA sensitive N-type channel current (Jones and Elmslie, 1992). The dihydropyridine antagonist, nifedipine, has little blocking effect on the total current when applied alone, but it can partially reverse the shift of the activation kinetics induced by BAY K8644 (Jones and Marks, 1989a).

Two types of single Ca^{2+} channel currents have been recorded from BFSG neurons: L-type channels, characterized by a 28 pS slope conductance, sensitivity to dihydropyridine Ca^{2+} channel agonist, and availability even with depolarizing holding potentials; and N-type channels, characterized by a 15 pS slope conductance, resistance to dihydropyridines and inactivation with depolarized holding potentials (Lipscombe *et al.*, 1988).

However, a different current which dominates at more negative voltage (-30 to +10 mV) has been found in BFSG neurons (Elmslie *et al.*, 1994). The novel current is not sensitive to selective blockers of L- and N-type channels (respectively, dihydropyridines or ω-conotoxin) and is inhibited weakly by norepinephrine. It is selectively inactivated at -40 mV and is selectively blocked by Ni^{2+}, whereas Cd^{2+} is slightly more potent against the main current. The novel current is associated with a 19 pS channel (0.6 pA at 0 mV).

Ca^{2+} channels on BFSG neurons are subject to modulation by many substances or neurotransmitters (Smith, 1994). A variety of agonists such as adrenaline, adenosine triphosphate (ATP), leutinizing hormone releasing hormone (LHRH), neuropeptide Y (NPY) and substance P inhibit Ca^{2+} channels in BFSG neurons (Schofield and Ikeda, 1988; Jones and Marks, 1989a; Bley and Tsien, 1990; Jones, 1991). The effect of (nor)adrenaline are not mediated via phosphorylation of membrane proteins (Elmslie *et al.*, 1993). NPY inhibits Ca^{2+} channel currents in C-cells but not in B-cells of BFSG (Schofield and Ikeda, 1988) whereas

LHRH probably affects the current in both cell types (Elmslie *et al.*, 1990). The agonist-induced inhibition of Ca^{2+} may be due to the transition of the channels from an 'willing' state to a 'reluctant' state (Bean, 1989; Elmslie *et al.*, 1990; Jones and Elmslie, 1992).

II. Na^+ channels

The fast, voltage-activated sodium channel currents which contribute to the upshoot of the action potential have been characterized in BFSG neurons (Jones, 1987). Both activation and inactivation of Na^+ channel currents occur at unusually positive voltages, with half-maximal activation at +2 mV and half-maximal steady-state inactivation at -35 mV. Both fast (in the order of milliseconds) and slow (in the order of seconds) inactivation processes have been observed.

The total Na^+ channel currents consist of two pharmacologically and kinetically distinct components. A larger portion of the total current is blocked by tetrodotoxin (TTX) and saxitoxin (STX) and activates and inactivates relatively rapidly. A smaller amount of the current (about 25% of the peak current) is sensitive to STX and Cd^{2+}, but resistant to TTX. The smaller component activates rapidly, but inactivates about 3-fold more slowly than the larger current.

In contrast to Ca^{2+} channels which are modulated by agonists or neurotransmitters, regulation of Na^+ by extracellular ligands has not been reported.

III. K^+ channels

1. M-current (I_M)

I_M is a muscarine-sensitive, slowly activating and non-inactivating K^+ current (Brown and Adams, 1980; Adams *et al.*, 1982a, 1986; Marrion *et al.*, 1992). In addition to muscarinic agonists, I_M is inhibited by peptidergic agonists (Adams and Brown, 1982; Adams *et al.*, 1982b, 1983; Jones, 1985; Pfaffinger, 1988; Pfaffinger *et al.*, 1988; Bosma and Hille, 1989; Bley and Tsien, 1990), synaptically-released acetylcholine (Ach) and luteinizing hormone releasing hormone (LHRH) (Adams and Brown, 1982; Jones *et al.*, 1984; Jones, 1985), adrenoceptor agonists (Akasu, 1988; Selyanko *et al.*, 1990a) and nucleotides (Groul *et al.*, 1981; Adams *et al.*, 1982b; Tokimasa and Akasu, 1990). Furthermore, I_M is blocked by millimolar concentrations of Ba^{2+}, but not by tetraethylammonium (TEA) or by aminopyridines.

The threshold membrane potential for the activation of I_M is about -60 mV (recorded by microelectrode, Adams *et al.*, 1982a) or -75 mV (recorded by patch-electrode, Jones, 1989; Selyanko *et al.*, 1990a). Since the activation threshold for I_M is close to the normal resting potential of BFSG cells and its activation by depolarization exerts a hyperpolarizing influence, I_M may serve as a physiological voltage-clamp by which cells resist the influence of a depolarizing stimulus (Adams *et al.*, 1982a). Agonist-induced I_M suppression leads to membrane depolarization and increased possibility of spontaneous action potentials (Jones, 1985, 1989). In the intact ganglion, suppression of the I_M by synaptically-released Ach and LHRH contributes to the electrogenesis of the slow excitatory postsynaptic potential (EPSP)

and the late-slow EPSP, respectively (Smith, 1994).

2. Delayed rectifier potassium current (I_K)

The I_K of BFSG exhibits a similar kinetics to those of the classical delayed rectifier identified in squid axon (Hodgkin and Huxley, 1952a,b). The current activates when the membrane potential is positive to -25 mV and inactivates during very long depolarizing commands (30 sec to 1 min; Adams *et al.*, 1982a).

I_K in BFSG is blocked by 3,4-diaminopyridine (Goh *et al.*1989), but it is not modulated by muscarinic (or other) agonists (Selyanko *et al.*, 1990b).

The physiological role of I_K has not been defined. The current is much too slow to be involved in the repolarization of a single action potential or in the generation of its afterhyperpolarization (Lancaster and Pennefather, 1987). One possible role for I_K is to control repetitive discharge when other K^+ currents are compromised or antagonized (Goh *et al.*, 1989).

3. Fast, voltage-sensitive, Ca^{2+}-sensitive K^+ current (I_C)

I_C is a rapidly activating, Ca^{2+}-sensitive, voltage-sensitive K^+ current (Adams *et al.*, 1982a). Inclusion of Cd^{2+} and exclusion of Ca^{2+} from the extracellular medium can block I_C, suggesting its activation depends on the prior influx of Ca^{2+}.

I_C is mediated by 100 pS channels (Adams *et al.*, 1982c) which resemble high

conductance, 'maxi-$G_{K\text{-}Ca}$' channels (Smart, 1987) and is selectively blocked by the extracellular application of TEA or by charybdotoxin (Goh and Pennefather, 1987).

Since I_C activates rapidly, it is responsible for the repolarization of the action potential and the generation of part of the afterhyperpolarization (Adams *et al.*, 1982c; Pennefather *et al.*, 1985; Lancaster and Pennefather, 1987).

4. Slow, voltage-insensitive, Ca^{2+}-sensitive K^+ current (I_{AHP})

I_{AHP} is a slow, non-inactivating, voltage-insensitive, Ca^{2+}-dependent K^+ current. In intact ganglia, I_{AHP} can be evoked by depolarizing command potentials which activate Ca^{2+} channels to produce Ca^{2+} influx when using sharp microelectrodes. However, in dissociated cells, I_{AHP} can not be recorded by patch-pipette (Jassar *et al.*, 1995).

I_{AHP} is much more sensitive to intracellular Ca^{2+} than I_C and its activation is proportional to the square of the intracellular Ca^{2+} (Yamanda *et al.*, 1989). I_{AHP} can be blocked by d-tubocurare and apamin (Goh and Pennefather, 1987).

I_{AHP} is responsible for the generation of the latter part of the action potential afterhyperpolarization.

5. A current (I_A)

I_A, a fast, transient outward K^+ current, was characterized in BFSG B-cells by Adams *et al.* (1982a). To activate I_A, a hyperpolarizing prepulse must be applied to remove the inactivation which occurs at the normal resting membrane potential. Activation is voltage-

dependent and rapid with a threshold of -60 mV and a time constant less than 5 ms (at -30 mV). Activated I_A declines exponentially to zero with a time constant of about 50 ms at -30 mV. The time constant for removal of inactivation is about 150 ms and half inactivation occurs at -110 mV for hyperpolarizing prepulse between -90 and -120 mV.

The physiological significance of I_A in BFSG has not been defined. Since the activation and the inactivation curves exhibit little overlap, I_A is unlikely to contribute to the resting membrane potential. In current clamp experiments, after long hyperpolarizing current commands, I_A is activated and this results in a hyperpolarizing 'notch' on the repolarizing voltage trajectory. This 'notch' prevents or delays the occurrence of 'anodal break' spikes in BFSG.

Statement of the problem

Almost all the studies of the regulation of ion channel properties by neurotrophic or growth factors have been limited to PC12 or other responsive cell lines. While those cell lines are good models to study the effects of neurotrophic factors on differentiation, there are several limitations to the use of cell lines:

(1) Different clones of PC12 cells have different and sometimes unstable phenotypic characteristics which may lead to inconsistent results.

(2) The role of trophic factors in the regulation of ion channels during differentiation or proliferation is likely to be different from their role in the maintenance of normal physiological function of the neurons.

(3) The results from neurotrophic factor-responsive cell lines must be further examined in primary neurons.

Therefore, one purpose of this thesis is to test whether NGF, one of the neurotrophic factors, regulates voltage-dependent ion (Ca^{2+}, Na^{+}, K^{+}) channel properties in adult primary neurons.

In contrast to the plethora of information regarding the signal transduction mechanisms of trophic factors in neuronal differentiation, little is known about the signal transduction mechanisms underlying the regulation of ion channels by trophic factors. If NGF regulates ion channel properties in adult neurons, the second aim of this thesis is to investigate the signal transduction mechanisms involved by using many specific inhibitors or antibodies.

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Chapter 2

Regulation of N- and L-type Ca^{2+}-channels in Adult Frog Sympathetic Ganglion B-cells by Nerve Growth Factor *in vitro* and *in vivo*

A version of this Chapter is in revision for publication in the Journal of Neurophysiology.

Introduction

Nerve growth factor (NGF) is mandatory for the survival of sympathetic neurons and small nociceptive, sensory neurons at certain critical periods in the development of the peripheral nervous system (Levi-Montalcini and Angelletti, 1968; Davies, 1996; Lewin, 1996). These neurons lose their absolute dependence on NGF for survival as they mature. After this, NGF becomes involved in the selection, specification and maintenance of differentiated neuronal phenotypes (Lewin *et al.*, 1992; Lewin 1996; Lindsay 1996). Chalazonitis *et al.*, (1987) showed that long-term exposure to NGF *decreases* action potential (a.p.) duration of neurons in organotypic cultures of dorsal root ganglia (DRG) from 13d fetal mice. They proposed that this effect may reflect modulation of Ca^{2+} channel current (I_{Ca}) in neurons that no longer require NGF for survival. By contrast, Ritter and Mendell (1992) showed that treatment of neonatal rats with NGF *in vivo* from birth to 5 weeks of age produced essentially the reverse effect; a.p. duration was *increased.* This effect was confined to high threshold mechanoreceptor (HTMR) neurons and other types of sensory neuron were unaffected. It therefore remains to be determined *i)* whether NGF acts to increase or to decrease I_{Ca} in mature peripheral neurons *ii)* whether findings in culture can be extrapolated to the *in vivo* situation and *iii)* whether NGF affects the number, the type or the properties of Ca^{2+} channels. We have used biophysical and pharmacological techniques to address these questions and to analyze in detail the effects of NGF on Ca^{2+} channels in mature sympathetic neurons both in tissue culture and *in vivo*.

Because disconnection of DRG neurons from their target tissues alters the expression of cell type-specific proteins (Zhang *et al.*, 1993), cell-surface markers (Persson *et al.*, 1995) and neurotransmitter receptors (Abdulla and Smith, 1997), there is no reliable method of

identifying one specific subtype of sensory neuron in culture. Dissociated or cultured cells cannot, of course, be classified on the basis of the conduction velocity of their axons (Villiere and McLachlan 1996). Since as many as 43 different neuronal subtypes are found in mammalian DRG (Lewin, 1996), the use of DRG cultures is precluded when exactly the same neuronal subtype is to be studied under different experimental conditions; in the present case, in the presence or absence of NGF. The problem is compounded by progressive changes in ion channel properties as neurons are maintained in culture (Scott and Edwards, 1980; Traynor *et al.*, 1992) and by the fact that NGF affects only one subtype of neuron in mammalian DRG *in vivo* (HTMR's; Ritter and Mendell, 1992).

Unlike DRG neurons, all sympathetic neurons respond to NGF. We therefore analyzed its actions on the properties of Ca^{2+} channels by using B-cells from the paravertebral sympathetic ganglia of bullfrogs. These cells were selected for several reasons:- *i)* They can be readily identified in culture or after acute dissociation simply on the basis of their size. Unlike rat DRG (Rose *et al.*, 1986; Scroggs and Fox, 1992; Villiere and McLachlan, 1996), bullfrog sympathetic ganglia (BFSG) contain just two types of neuron; the larger B-cells and the smaller C-cells (Horn *et al.*, 1988; Ivanoff and Smith, 1995); *ii)* Ca^{2+} channel kinetics are better characterized in BFSG B-cells than in any other vertebrate neuron (Lipscombe *et al.*, 1988; Jones & Marks, 1989a and b; Sala, 1991; Elmslie *et al.*, 1992; Werz *et al.*, 1993; Jassar *et al.*, 1993). This enabled us to investigate actions of NGF on specific, well-characterized kinetic components of channel function and thereby to gain a unique insight into its mechanism of action; *iii)* the electrophysiological characteristics of B-cell somata are remarkably consistent (Adams *et al.*, 1986; Jassar *et al.*, 1993; Smith 1994) so that data from a control population of B-cells can be compared with that from an NGF-treated population;

iv) the duration of the a.p. of B-cells in explant culture is increased by murine NGF (Traynor *et al.*, 1992), an effect which is reminiscent of its action on mammalian HTMR neurons *in vivo* (Ritter and Mendell, 1992); *v)* B-cells dissociated and cultured from the sympathetic ganglia of *adult* bullfrogs can be maintained for at least 2 weeks in NGF-free, defined-medium, serum-free culture (Lei *et al.*, 1995a; 1995b). These cells are functionally intact at the end of a 15d experimental period and there is a significant increase in a.p height ($P<0.001$) at this time (S. Lei, unpublished observations). Progressive changes in Ca^{2+} channel properties in the B-cell population can therefore be studied and used as a reliable baseline against which to document and analyze the actions of NGF.

The use of an amphibian system to study the action of NGF is further justified by:- *i)* The observation that serum-supplemented explant cultures of whole BFSG exhibit a 'classical' growth response to murine NGF (Kelly *et al.*, 1989); *ii)* The effects of NGF on I_{Ca} in BFSG B-cells that are to be described in this paper are mimicked by a bivalent antibody that activates mammalian *TrkA*, *TrkB* and *TrkC* receptors. Frog neurons therefore express neurotrophin receptors that are immunologically-similar to mammalian *Trk* receptors (S. Lei, Unpublished observations); *iii)* the gene coding for amphibian NGF has been cloned from *Xenopus laevis* and the predicted aminoacid sequence of the mature peptide bears significant similarity to mammalian NGF (Carriero *et al.*, 1991); and *iv)* antibodies raised against mouse NGF can inhibit a neurotrophic action of denervated frog sciatic nerve on frog DRG neurons (Kuffler and Megwinoff, 1994). The use of mammalian antibodies to block actions of amphibian NGF is justified by the observation that they bind to mature *Xenopus laevis* NGF (Carriero *et al.*, 1991).

Although the low-density, defined-medium culture system that we have developed

provides a simple and reliable experimental system in which to analyze the regulation of Ca^{2+}-channel function by NGF in mature peripheral neurons, it is also necessary to test whether Ca^{2+}-channels are regulated by similar mechanisms *in vivo*. This is important because, as mentioned above, NGF *decreases* a.p. duration of all types of mammalian DRG cells in culture (Chalazonitis *et al.*, 1987) yet has essentially the reverse effect *in vivo* where it selectively *increases* a.p. duration of HTMR neurons (Ritter and Mendell, 1992). *In vivo* effects were therefore examined by injecting NGF or NGF antiserum into the thighs of another species of smaller frog (*Rana pipiens*). The sympathetic ganglia of these frogs were then removed and the neurons dissociated so that the properties of Ca^{2+}-channels in B-cells could be examined. After acute dissociation, the somata of B-cells can be readily distinguished from those of C-cells in *Rana pipiens* sympathetic ganglia (RPSG; Selyanko *et al.*, 1990) as they can in BFSG. It is assumed that RPSG B-cells also project to mucous glands in the skin of the thighs (Horn *et al.*, 1988), so that subcutaneous injections of NGF or NGF antiserum would have access to B-cell axons and terminals.

Some of the results have been published in abstract form (Lei *et al.*, 1995a; 1995b).

Methods

Tissue culture

Medium-sized adult bullfrogs (10-15cm; body weight 250-350g) were purchased from a biological supply house and kept in running water at room temperature. Dissection, dissociation and culture procedures were performed under aseptic conditions. After pithing, the ventral portion of the frog was sterilized with 9% iodine in ethanol. The rectus abdominis

muscle was removed and the animal was carefully eviscerated to expose the paravertebral sympathetic chains. The VIIth-Xth paravertebral sympathetic ganglia were removed from both sides and dissociated using a trypsin/collagenase procedure (Selyanko *et al.*, 1990). The dissociated cells were resuspended in 3ml of a defined, serum-free culture medium containing 73% L-15 medium (GIBCO) in water supplemented with 10mM glucose, 1 mM $CaCl_2$, 100 units/ml penicillin and 100μg/ml streptomycin. To minimize interactions among cells, the dissociated cells from one frog were evenly distributed into 30 plastic Petri dishes (35mm diameter) containing 3ml of medium to yield 5 to 10 neurons per dish. In some experiments, neuron-enriched cultures were used to limit the influence of neurotrophic substances from non-neuronal cells. These were prepared by preplating the dissociated ganglion cells from one frog into two 35mm culture dishes. After 1-2h, the non-neuronal cells adhered to the bottom of the dish. The nonadherent cells, which were primarily neurons, were collected, redistributed to 30 dishes and cultured in medium supplemented with 10μM cytosine arabinoside (Ara-C). This antimetabolite inhibits proliferation of non-neuronal cells in amphibian embryos (Peralta *et al.*, 1995). When used, NGF was added to the medium to a concentration of 200ng/ml or (in a few experiments) 50ng/ml. The dishes were placed in a humidified glass chamber and maintained at room temperature (22°C) for up to 15d. Unless otherwise stated, the culture medium was replaced on the 7th day. The growth of neurons was observed under a phase-contrast microscope.

NGF Injections

In the interests of economy, medium-sized leopard frogs (*Rana pipiens*; 60-80g body weight) were used in preference to bullfrogs and were given subcutaneous injections of 2.5S

NGF (1μg/g body weight) into the right thigh. Animals were injected on days 1, 3, 5, and 7 of the experimental period and the somata of acutely-dissociated neurons from the ganglia of the right sympathetic chain were examined electrophysiologically on day 9 or 10. Control data were obtained from B-cells from animals that were injected with saline according to the same schedule.

NGF Antiserum Injections

Four leopard frogs received 4 injections of rabbit anti-2.5S NGF fractionated antiserum (33μg protein/g body weight) into their right thighs over a 7d period using the same schedule as was used for NGF. Control animals for this group received comparable injections of rabbit serum (33μg protein/g body weight) in 7d. The electrophysiological properties of the somata of acutely-dissociated B-cells were examined on day 10 of the experimental period.

Electrophysiology

Ca^{2+} channel current was recorded from both acutely-dissociated and from cultured cells using the whole-cell recording technique (Jassar *et al.*, 1993). Ba^{2+} was the charge carrier. The external solution contained (mM): N-methyl-D-glucamine (NMG) chloride, 117.5; NMG-HEPES, 2.5; $BaCl_2$, 2.0; (pH 7.2). Internal solution consisted of (mM): NMG-Cl, 76.5; HEPES acid, 2.5; Tris-BAPTA, 10; Tris-ATP, 5; $MgCl_2$, 4; (pH 7.2). For recording, tissue culture medium was replaced with external solution at a flow rate of 2 ml/min. This flow rate allowed exchange of solutions within about 2 min. Medium to large-sized cells with input capacitance (C_{in}) > 40pF from either BFSG (Jassar *et al.*, 1993) or

RPSG (Selyanko *et al.*, 1990) were selected for recording. Since C_{in} of BFSG C-cells has been found to be <20pF (Kurenny *et al.*, 1994) and that of RPSG C-cells has been found to be <18pF (Selyanko *et al.*, 1990), we are confident that all recordings were made from B-cells. An Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA, USA) in the single-electrode, discontinuous voltage-clamp mode was used to record I_{Ca}. The use of a "switching amplifier" minimizes series-resistance problems associated with large, rapidly activating currents because the amplifier headstage is clamped to the recorded membrane voltage (Jones, 1987). Low-resistance electrodes (d.c. resistance 1-2 MΩ in the external solution) were used to permit switching frequencies between 40 and 50 kHz and a clamp gain between 8 and 16nA/mV. During data acquisition, the corner frequency of the filter was set to 10kHz. Whole-cell recordings were first obtained in the current-clamp "bridge" mode and the amplifier switched to the single-electrode, discontinuous voltage-clamp mode for the study of I_{Ca}. The holding potential (V_h) was -80mV unless otherwise stated. C_{in} was calculated by integrating the capacitive transient which accompanied a 10mV depolarizing command from -80mV. For those measurements involving peak currents, capacity transients were cancelled and leak current was subtracted by applying 1/4 amplitude hyperpolarizing pulses, multiplying responses by four and addition (Jones and Marks, 1989a). For tail current and kinetic analysis, neither linear leakage nor capacitive currents were subtracted from the recorded traces. Currents were recorded from acutely-isolated spherical cells and there was no space-clamp problem. However, since the cultured cells grew processes, about 5-10% cells were subject to space-clamp error which was apparent from distortion of the current traces. Recordings from those cells were discarded. Data were digitized using a Labmaster DMA interface and processed on an IBM-compatible computer (Northgate 386) using the

pClamp suite of programmes. Experimental records were digitally filtered at 4kHz prior to making permanent records with an X-Y plotter. All data are presented as means ± S.E.M and Student's two-tailed T-test or Analysis of Variance has been used to assess statistical significance. In graphs where no error bars are visible, the error bars are smaller than the symbols used to designate the data points.

2.5s NGF (β-NGF) was either purchased from Alomone Labs., Jerusalem, Israel or kindly provided by Dr R. B. Campenot (Department of Cell Biology and Anatomy, University of Alberta) and shown to increase the neurite growth in cultured rat superior cervical ganglion neurons. ω-conotoxin GVIA was dissolved in the external solution to make a final concentration of 300nM. Nifedipine was dissolved in dimethyl sulphoxide (DMSO) to make 10mM stock solution. This solution was diluted with external solution to obtain a final concentration of 10μM for application from light-proof reservoirs to ganglion cells under subdued lighting conditions. Control experiments with the same concentration of DMSO in the external solution showed no effects on I_{Ca}. Rabbit anti-2.5sNGF-fractionated antiserum (Sigma lot# 065H8950) was provided as a white powder and reconstituted with sterile de-ionized water. All other drugs and chemicals were from Sigma (St Louis, MO USA).

Results

The BFSG B-cells are unipolar neurons when they are inside the animals. After dissociation, the cells were usually devoid of neurites, but began to generate them after 2-3d in culture even in the absence of NGF (Figure 2-1A). The neurites grew longer, thicker and more branched as the time in culture increased. Cells survived for at least 15d in the defined medium, serum-free system. The production of neurites was reflected in marked increases

in C_{in}. Figure 2-1B shows that C_{in} increased about two-fold after 6d in culture and then remained relatively constant for the duration of the experiment (15d). Inclusion of NGF in the culture medium scarcely enhanced the growth of the somata or the neurites (Figure 2-1A) and therefore produced no significant increase in C_{in} (Figure 2-1B). It would therefore seem that exogenous NGF is neither necessary for neurite extension nor for survival of adult BFSG cells under our culture conditions.

Changes in I_{Ca} as B-cells are maintained in culture

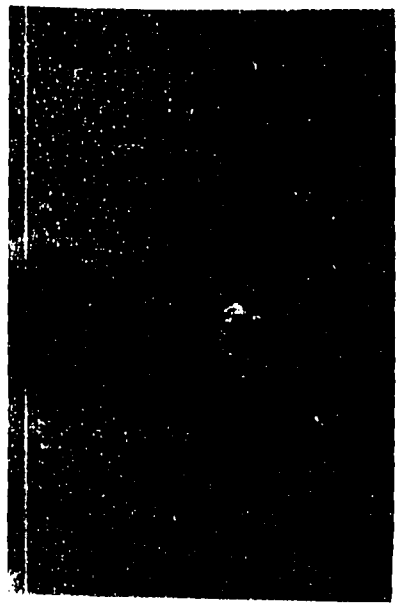
Figure 2-2 illustrates typical recordings of I_{Ca} from an acutely-dissociated cell, a cell maintained in defined medium for 12d and a cell maintained in the presence of NGF for 12d. Currents were evoked by a series of depolarizing voltage commands (V_h= -80mV). Tail currents following the depolarizing pulses were recorded at -40mV to slow their kinetics and facilitate their analysis (see legend to Figure 2-4D).

The time course of changes in I_{Ca} as neurons were maintained in culture was analyzed in two ways.

First, the change in *total* current with time was examined. Figure 2-3A shows that there was a transient increase in *total* I_{Ca} over the first 3d in culture ($P<0.01$) and that the current then steadily declined until that present after 15d in culture was similar to that in acutely-dissociated cells ($P>0.05$). Since at least 20 cells were recorded for each of the data points shown in Figure 2-3, it may be concluded that the *total* amount of I_{Ca} does not decrease as BFSG B-cells are maintained in culture without NGF. Inclusion of 200ng/ml NGF in the culture medium almost doubled the *total* current. This effect was clearly apparent after 6d in culture and was maintained throughout the whole experimental period (15d; Figure 2-3A).

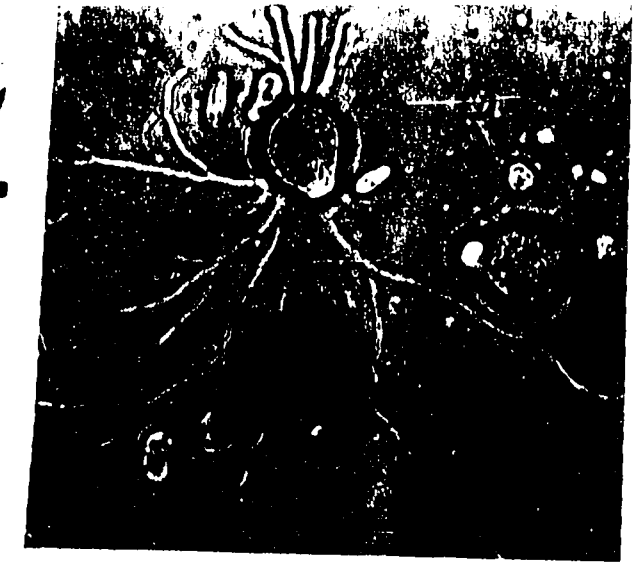
Figure 2-1. A. Photomicrographs of an acutely-isolated BFSG B-cell, a 12d cultured cell and a cell cultured for 12d in the presence of 200ng/ml NGF. Calibration bar is 100μm and refers to all three pictures. **B.** Changes in the input capacitance (C_{in}) of B-neurons as they grow in `normal' (defined-medium, serum-free) culture in the presence or absence of NGF **C.** Changes in C_{in} of B-neurons as they grow in neuron-enriched (Ara-C treated) culture (n>20 for all observations).

A. Acute

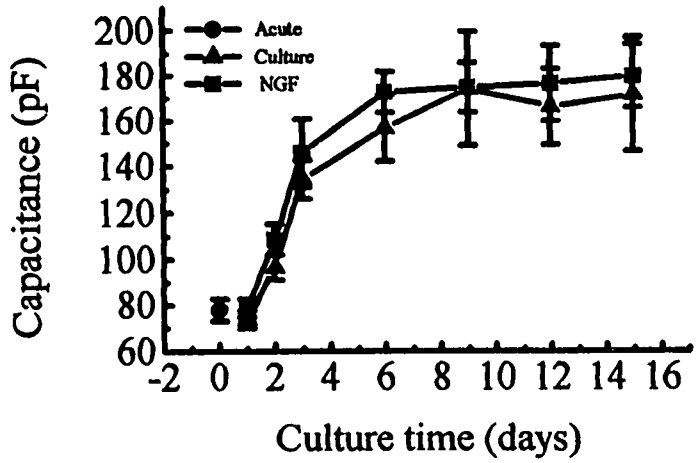


Culture (12d)

Culture 12d + NGF



B. Normal Culture



C. Neuron-enriched Culture

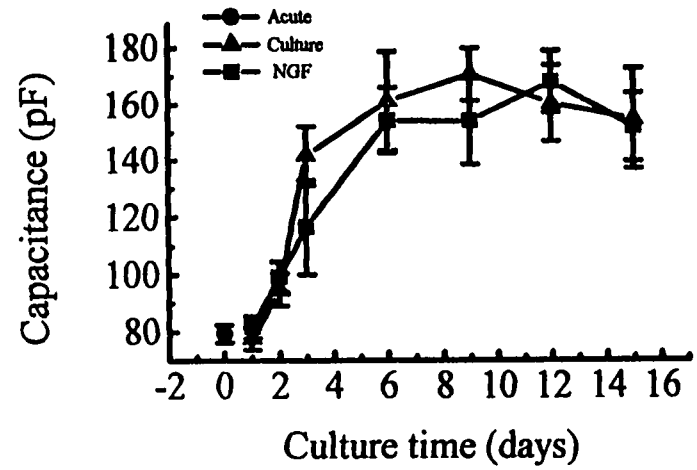


Figure 2-2. Typical Ca^{2+}channel currents (I_{Ca}) recorded from **A.** an acutely isolated BFSG B-cell and **B.** and **C.** from cells maintained for 12d in `normal' (defined-medium, serum-free) culture with or without NGF.

(Figure 2-2)

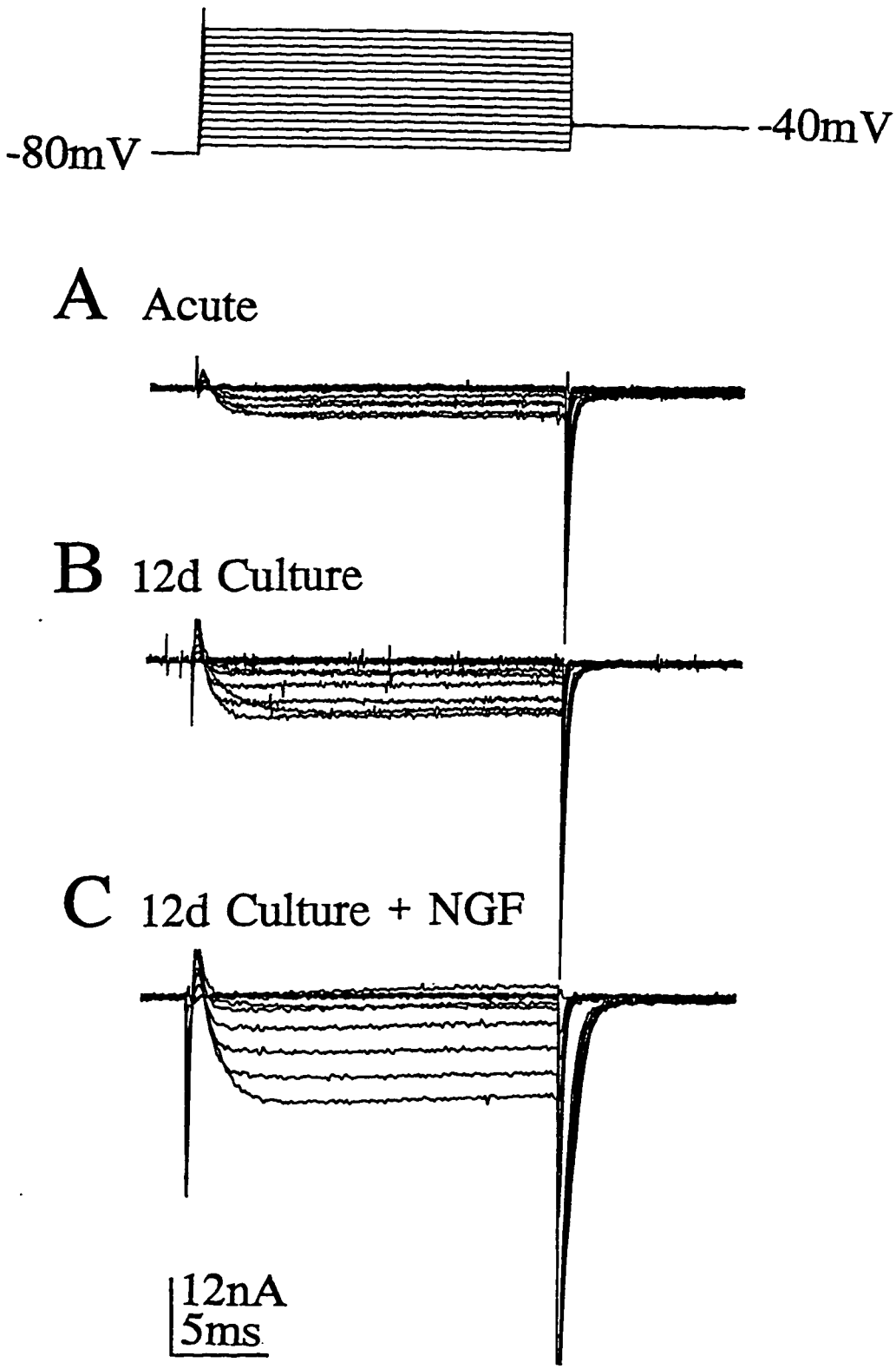
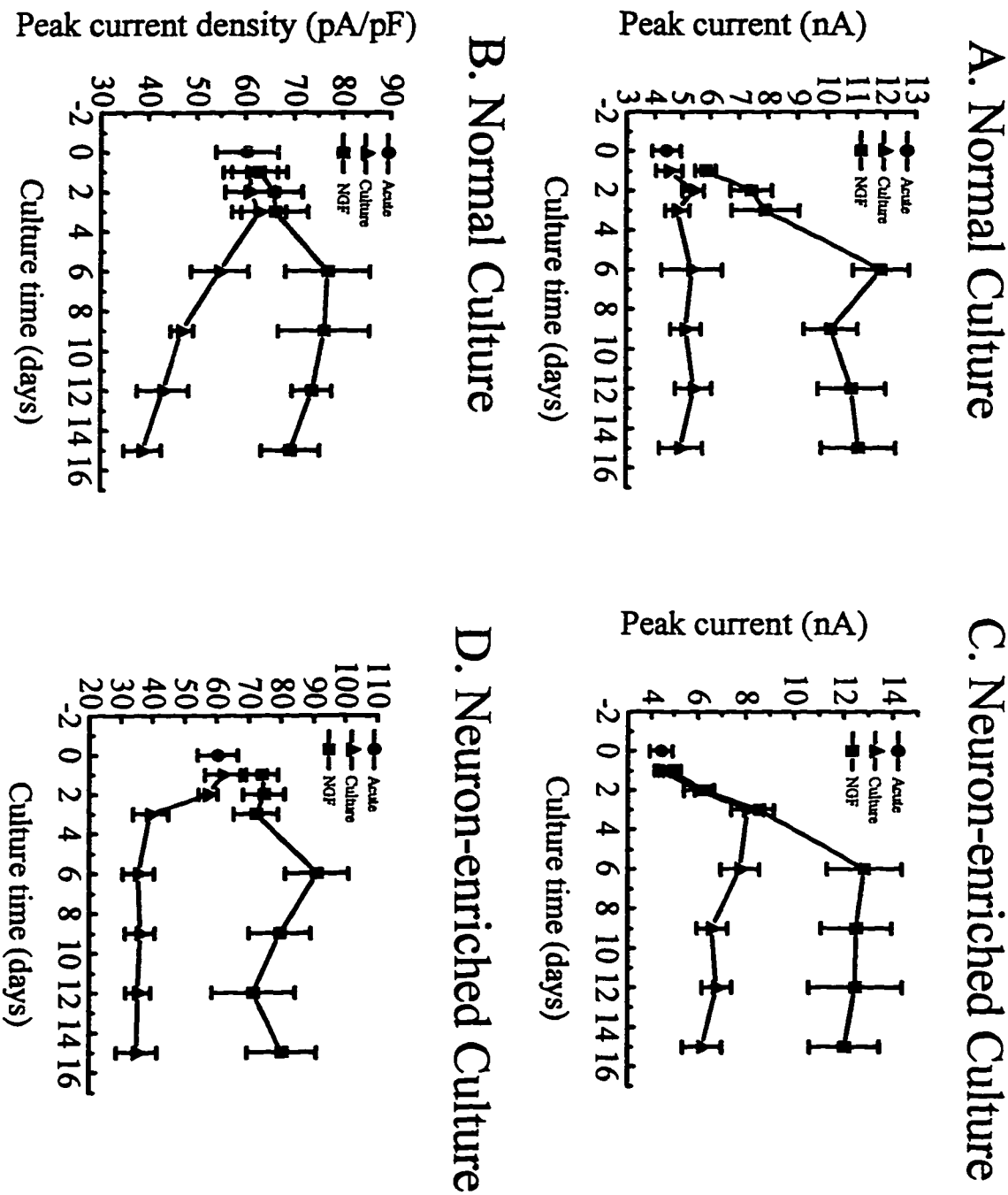


Figure 2-3. Time course of the change in I_{Ca} recorded at 0mV from a V_h of -80mV as B-neurons grow in culture with or without NGF. **A** and **B.** Changes in peak (total) current or current density for cells in `normal' (defined-medium, serum-free) culture. **C** and **D**. Changes in peak (total) current or current density for cells in neuron-enriched (Ara-C treated) culture (n>20 for all observations).

(Figure 2-3)


A. Normal Culture
Peak current (nA)
Culture time (days)
B. Normal Culture
Peak current density (pA/pF)
Culture time (days)
C. Neuron-enriched Culture
Peak current (nA)
Culture time (days)
D. Neuron-enriched Culture
Culture time (days)
Acute
Culture
NGF

When a lower concentration of NGF (50ng/ml) was used, I_{Ca} seemed larger than that of control neurons yet displayed considerable variability, it was therefore decided to use 200ng/ml of NGF for the remainder of the study.

Secondly, changes in current *density* (I_{Ca} normalized to cell size; C_m) were examined. This analysis seeks to account for differences in cell size amongst populations of neurons because the largest cells in any given population would be expected to have the largest currents. Because C_m increased dramatically during the culture period (Figure 2-1B), the *density* of I_{Ca} in cells cultured without NGF decreased to about 36% of its initial value within 15d (Figure 2-3B). By contrast, the *density* of I_{Ca} changed little in the presence of NGF, the slight increase at 6d (Figure 2-3B) was not significant ($P>0.05$) and current *density* was similar to the control value ($P>0.2$) after 15d. Because NGF increased *total* I_{Ca} (Figure 2-3A) this must have been balanced by the increase cell size (Figure 2-1B) so that there was no appreciable increase in current *density* (Figure 2-3B).

Although the above experiments were carried out on isolated B-cells in serum-free defined medium, the results pose two additional questions; *i)* can the survival of neurons under our culture conditions be attributed to the trophic support of glial cells (Assouline *et al.*, 1987; Lewin and Mendell, 1993), macrophages (Guenard *et al.*, 1991) or fibroblasts (Unsicker *et al.*, 1987; Koike *et al.*, 1993) in the culture and *ii)* could the effects of NGF be mediated via the release of other trophic substances from these non-neuronal cells? To address these questions, the effect of NGF was re-examined in neuron-enriched cultures with media supplemented with Ara-C (10μM). Figure 2-3C shows that *total* I_{Ca} remains relatively constant throughout the 15d experimental period and that inclusion of NGF more than doubled the *total* current as it did in the absence of Ara-C (Figure 2-3A). Figure 2-3D

illustrates that I_{Ca} *density* in the neuron-enriched cultures was maintained or slightly increased in the presence of NGF yet declined in its absence. Because both *total* I_{Ca} and I_{Ca} *density* responded similarly in normal (Figure 2-3A and B) and in neuron-enriched cultures (Figure 2-3C and D), the possible presence of non-neuronal cells had little or no effect on the response of cultured neurons to NGF. It is possible however that some trophic support may have been derived from non-neuronal cells because the transient increase in *total* I_{Ca} seen after 3-4d in cultured cells (Figure 2-3A) was not seen in neuron-enriched cultures (Figure 2-3C)[1]. This was reflected in a more rapid rate of decrease in I_{Ca} *density* in neuron-enriched cultures (Figure 2-3D) compared to normal cultures (Figure 2-3B). Nevertheless, the possible presence of non-neuronal cells had little or no effect on neurite production because cell capacitance of cells in neuron-enriched culture increased (Figure 2-1C) as did that of neurons in regular culture (Figure 2-1B).

Effects of NGF on Ca^{2+}-channel Kinetics

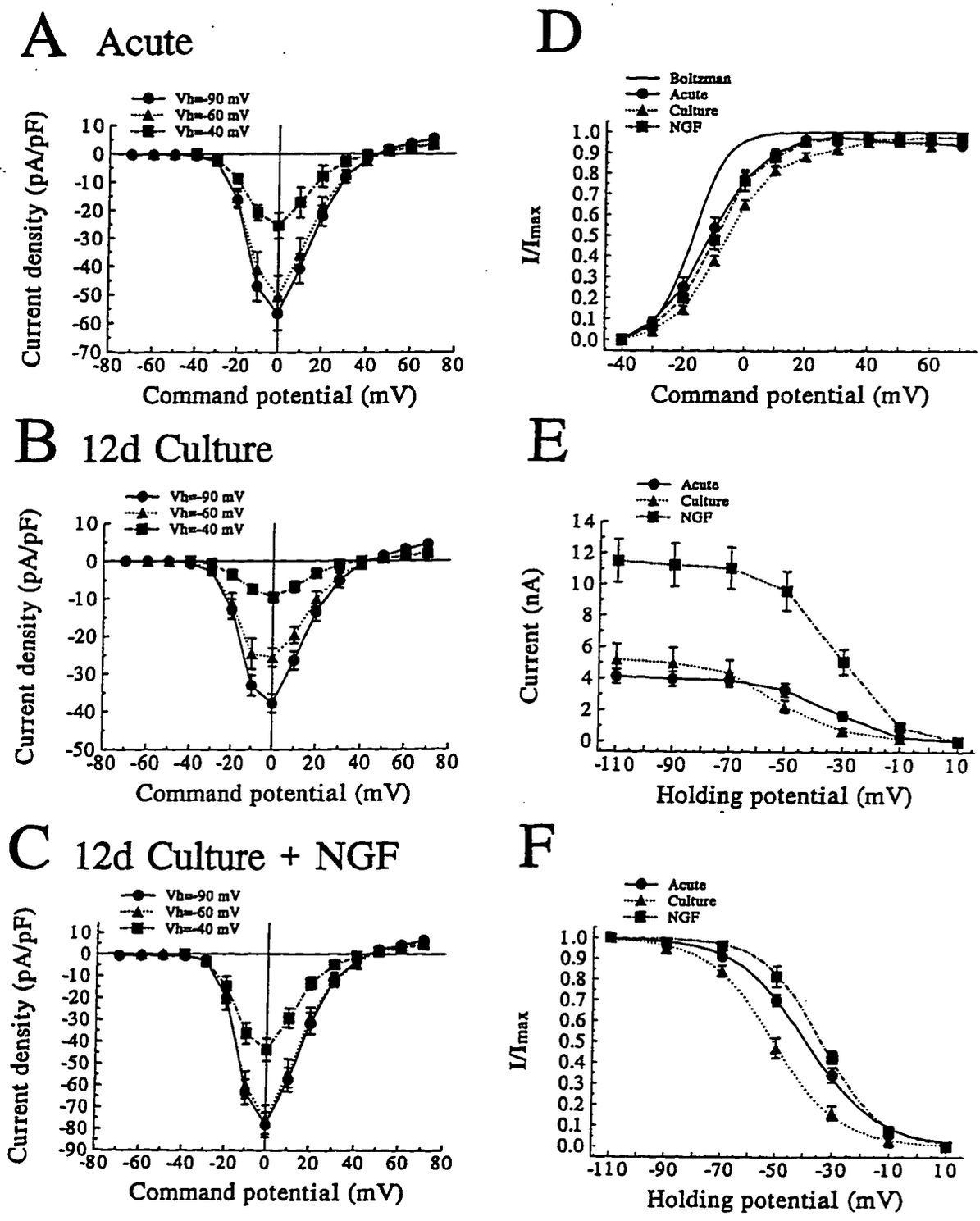
Having established that NGF increased I_{Ca} in bullfrog sympathetic neurons, the next major objective was to analyze the mechanism(s) whereby its effects were exerted. Because 80-90% of the I_{Ca} in dissociated B-cells is of the N-type ($I_{Ca,N}$; Elmslie *et al.*, 1992), NGF could increase total $I_{Ca,N}$ and/or induce the appearance of other types of current. Alternatively, there could be changes in activation/deactivation and/or inactivation kinetics of the current. These possibilities were examined using a variety of biophysical and pharmacological techniques.

Figures 2-4A, B and C illustrate the current-voltage relationships of acute cells and 12d cultured cells with and without NGF. Experiments were carried out using three different

values of V_h (-90, -60 and -40mV) and current expressed as I_{Ca} *density*. Several points emerge from the data; peak current density generally occurred at 0mV for acutely-dissociated cells and for cells cultured with or without NGF. This implies that the manipulations have little or no effect on the voltage-dependence of g_{Ca} activation and hence on its activation kinetics. This observation is confirmed by the activation curves obtained from tail current amplitudes (Figure 2-4D see figure legend for further details). There is no 'shoulder' on the I-V relationship for acutely-isolated (Figure 2-4A), 12d cultured (Figure 2-4B) or for NGF-treated (Figure 2-4C) cells within the -70 to -20mV range. This confirms that low-voltage activated T-type channel current ($I_{Ca,T}$; Fox *et al.*, 1987) is not expressed in acutely-isolated BFSG B-cells (see Jones and Marks 1989a; Jassar *et al.*, 1993) and that it does not appear during culture or in response to NGF (see Garber *et al.*, 1989). More significantly, however, shifting V_h altered the I-V relationships in different ways in the acute, cultured and cultured plus NGF groups. This implies that steady-state inactivation of I_{Ca} is changed as cells grow in culture and that inactivation of I_{Ca} is altered by NGF. Changing V_h from -90mV to -60mV has minimal effect on I_{Ca} of acutely-isolated cells whilst changing to -40mV reduces peak I_{Ca} to 43% of that recorded from -90mV (Figure 2-4A). By contrast, shifting V_h of cells cultured for 12 days in the absence of NGF from -90mV to -60mV reduces current to 67% of the -90mV value and a further shift to -40mV produces an additional reduction to 23% of that available from -90mV (Figure 2-4B). The situation in NGF-treated cells is similar to that in acutely-isolated cells; shifting V_h to -60mV has little or no effect on peak current and shifting to -40mV reduces I_{Ca} to 55% of that available from -90mV. These results are reflected in the steady-state, normalized inactivation plots shown in Figure 2-4F. V_h was set to a range of prepulse potentials from -110 mV to +10 mV for 20s before applying a test pulse to 0mV.

Figure 2-4. A, B and C. Current-density-voltage plots for I_{Ca} in acutely isolated B-cells, cells cultured for 12d and cell cultured for 12d in the presence of NGF. Three different holding potentials (V_h); -90,-60 and -40mV were used. (n>20 for data points). **D.** Activation curves for g_{Ca} expressed as I/I_{max} in the three experimental situations (n>20 for all data points). I_{Ca} was evoked at a series of different command potentials using 20ms commands. The tail currents at the end of each pulse were recorded at -40mV. Tail current amplitude was estimated by fitting the data to a monoexponential function to extrapolate the original amplitude. The solid line represents a plot of a Boltzmann expression of the form $I_{t(v)}/I_{t(max)} = g_v/g_{max} = \{1 + exp^{ze(Vo-V)/kT}\}^{-1}$ where $I_{t(v)}$ is the amplitude of the I_{Ca} tail following a command to a voltage, v; $I_{t(max)}$ is the maximum amplitude of I_{Ca} tails following commands to positive voltages. The ratio of these currents is equal to g_v/g_{max} *i.e.* the ratio of maximum g_{Ca} to that following a command to voltage, v or the fraction of Ca^{2+} channels open; z is the valency of the gating particle; e is the elementary electronic charge (1.602×10^{-19}C), k = Boltzmann's constant (1.381×10^{-23}VCK^{-1}), T=absolute temperature and V_o is the potential for half-maximal activation. Deviation of the experimental data from this line probably reflects rapid inactivation (Sala, 1991; Jassar *et al.*, 1993). **E.** Inactivation curves for the three experimental conditions. I_{Ca} was evoked at 0mV from various holding potentials. **F.** Data from **E** normalized and re-plotted (n=22 for acutely isolated cells, n=16 for cells cultured without NGF, n=25 for cells cultured with NGF).

(Figure 2-4)


A Acute
Vh=-90 mV
Vh=-60 mV
Vh=-40 mV
Current density (pA/pF)
Command potential (mV)
B 12d Culture
Vh=-90 mV
Vh=-60 mV
Vh=-40 mV
Current density (pA/pF)
Command potential (mV)
C 12d Culture + NGF
Vh=-90 mV
Vh=-60 mV
Vh=-40 mV
Current density (pA/pF)
Command potential (mV)
D
Boltzman
Acute
Culture
NGF
I/Imax
Command potential (mV)
E
Acute
Culture
NGF
Current (nA)
Holding potential (mV)
F
Acute
Culture
NGF
I/Imax
Holding potential (mV)

The normalized current evoked during the test pulse is plotted against V_h. Compared with acutely dissociated cells, the inactivation curve for cells cultured without NGF for 12 days was shifted to the left. This means that currents evoked from V_h of -70 to -30mV would generate less I_{Ca} as a result of increased steady-state inactivation. By contrast, the inactivation curve for NGF-treated cells was shifted to the right of that for acute cells so that more current was available as a result of reduced inactivation.

These changes in inactivation cannot however explain *all* of the changes in I_{Ca} that occur as the neurons grow in the presence of NGF. Figure 2-4E shows the inactivation curves replotted as absolute currents. The current attained in NGF-treated cells could never be attained in cultured cells no matter how negative a V_h were to be used because in these cells, the available current reaches a maximum when the holding potential is between -90 and -110mV. Thus, NGF causes an increase in the total amount of I_{Ca} by decreasing inactivation and by a second process (or processes, see below) that is/are independent of the effect on inactivation.

Analysis of Changes in Inactivation

Because the processes underlying g_{Ca} inactivation in BFSG B-cells are complex (Jones and Marks, 1989b; Werz *et al.*, 1993; Jassar *et al.*, 1993) and four kinetically-distinguishable processes; 'fast', 'intermediate' and 'slow' and 'very slow' inactivation are involved, we examined which aspects of the inactivation process were affected by cells maintained in culture and how these changes were affected by NGF.

The upper panel in Figure 2-5 illustrates the voltage protocol used to investigate the voltage dependence of the rate of g_{Ca} inactivation. The cells were clamped by a series of

500ms conditioning pulses to various potentials prior to application of a standard 75ms test pulse to 0mV. Typical current records from acutely-isolated cells, cells cultured for 12d and cells cultured for 12d in the presence of NGF are illustrated in Figures 2-5A, B and C respectively. Inactivation of conductance during the conditioning pulse was greater and more rapid in the 12d cultured cells compared with control or NGF-treated cells. The increased (rate of) inactivation observed in cultured cells can be clearly seen by examining the time course of the conditioning current flowing at 0mV in the sample data record shown in Figure 2-5B and comparing it to that seen in control (Figure 2-5A) or NGF-treated cells (Figure 2-5C). Data for acutely-dissociated, 12d cultured and NGF-treated cells are summarized in Figures 2-6A, 6B and 6C which display the variation of peak and end-of-pulse current density for conditioning pulses at various potentials and peak current density recorded in the test pulse to 0mV. In agreement with Jones and Marks, (1989b) and Jassar *et al.* (1993), minimal current flows in the test pulse following conditioning pulses to 0 or -10mV where I_{Ca} is maximal. Maximal inactivation (minimal size of test pulse current) for acute, cultured and NGF cells occurs between -10 and 0mV. From Figure 2-6B, I_{Ca} (at -10 mV) inactivates by 50.9 ± 1.9% (n = 32) during a 500 ms depolarizing pulse in cultured cells in the absence of NGF. This is significantly greater than inactivation observed for both acutely dissociated cells (29.1 ± 1.6%, n =24, $p<0.01$; Figure 2-6A) and cultured cells in the presence of NGF for 12d (25.9 ± 2.1%, n = 27, $p<0.01$; Figure 2-6C). The voltage-dependence of inactivation of g_{Ca} in acute, 12d cultured and 12d NGF-treated cells are normalized, replotted and compared in Figure 2-6D (see Jones and Marks, 1989b). The difference between the maximal and minimal test current (maximal amount of inactivation) was defined as 1 and the difference between the maximal test current and other test current at different conditioning command potentials was

normalized to this value. Comparison of the data shows that g_{Ca} inactivation in 12d cultured cells was more pronounced at positive potentials ($p<0.01$ from +10 mV to +70 mV). Inclusion of NGF in the culture medium attenuated inactivation in the cultured cells at both negative potentials ($p<0.01$ from -60mV to -20mV) and at positive potentials ($p<0.01$ from +10 mV to +70mV).

Figure 2-7A illustrates typical recordings from acute, 12d cultured and NGF treated cells that were used to study the detailed kinetics of inactivation at 0mV and the rate of recovery at -80mV. Long duration (4s) voltage commands were applied once every 15s and increasing delays were introduced between this initial 4s pulse and subsequent brief pulses that were used to measure the rate of recovery from inactivation. Because there is a `very slow' component of $g_{Ca}$ inactivation BFSG B-cells, it was necessary to normalize the raw data records in Figure 2-7A to the first response prior to plotting (Jassar *et al.*, 1993; Smith, 1994). The onset of inactivation during the pulse could be fit by `intermediate' (τ_2) and `slow' (τ_3) exponential rate constants of amplitudes A_2 and A_3, respectively. A full numerical description of the current required the inclusion of a non-inactivating component of amplitude A_0 (Werz *et al.*, 1993; Table 2-1)[2]

In cells cultured for 12d without NGF, g_{Ca} exhibited 89±2% (n=24) inactivation within a 4s pulse. This is significantly greater than that seen in acutely-dissociated cells (77±2%, n=22, $p<0.01$) and in cells cultured with NGF for 12d (68±3%, n=21, $p<0.01$). These effects resulted from changes in the amplitude (A_2) of the `intermediate' component of inactivation ($\tau_2$). The values of $\tau_2$ and $\tau_3$ and the amplitude of the slowly inactivating component ($A_3$) were essentially unchanged (Table 2-1). The values of `total inactivation' shown in this table represent the total amount of inactivation that occurred in the 4s pulse.

Figure 2-5. Recordings of Ca^{2+}channel currents (I_{Ca}) to show voltage-dependence of rate of inactivation in BFSG B-cells. Cells were clamped by a series of 500ms conditioning pulses to various potentials prior to application of a standard 75ms test pulse to 0mV. Typical recordings from **A.** an acutely-isolated cell, **B.** and **C.** B-cells maintained for 12d in `normal' (defined-medium, serum-free) culture with or without NGF.

(Figure 2-5)

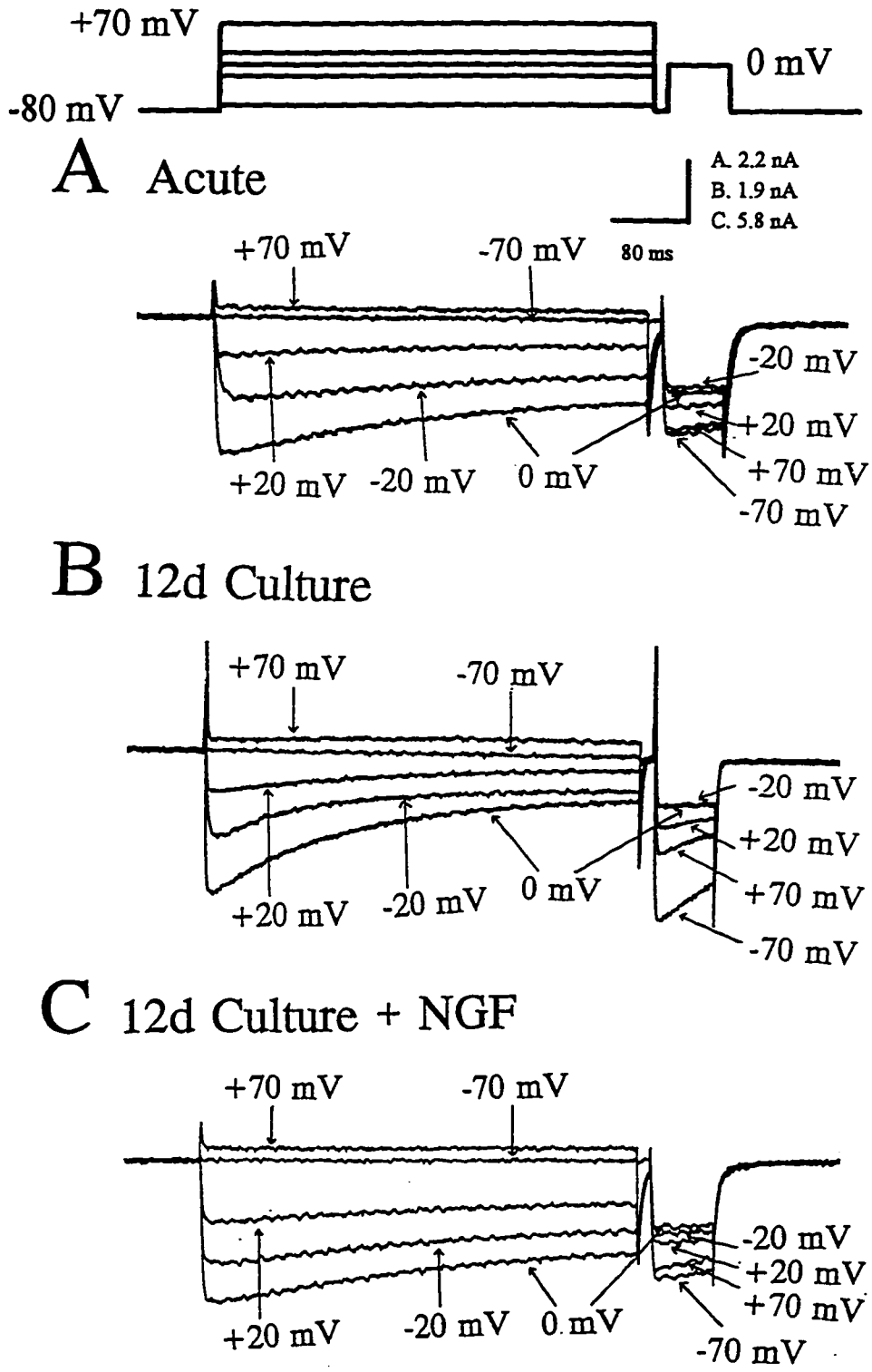
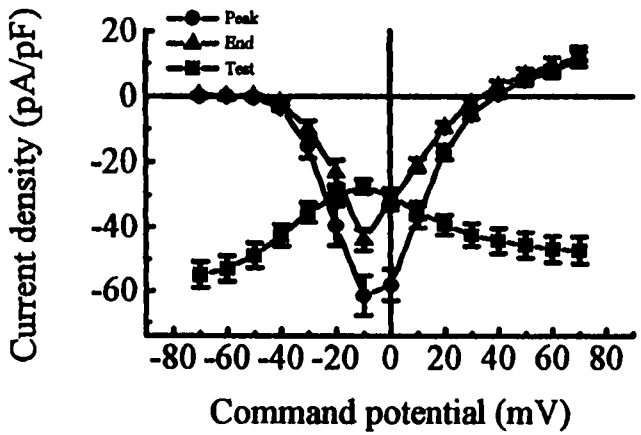
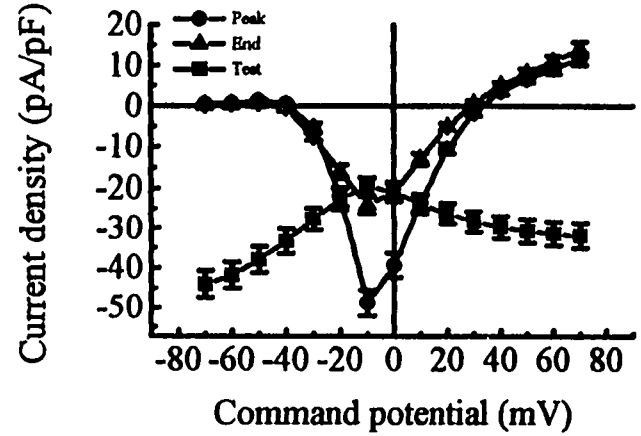

+70 mV
-80 mV
0 mV
A Acute
A. 2.2 nA
B. 1.9 nA
C. 5.8 nA
80 ms
+70 mV
-70 mV
-20 mV
+20 mV
+70 mV
-70 mV
+20 mV
-20 mV
0 mV
B 12d Culture
+70 mV
-70 mV
-20 mV
+20 mV
+70 mV
-70 mV
+20 mV
-20 mV
0 mV
C 12d Culture + NGF
+70 mV
-70 mV
-20 mV
+20 mV
+70 mV
-70 mV
+20 mV
-20 mV
0 mV

Figure 2-6. Voltage-dependence of rate of inactivation of I_{Ca} in **A.** acutely-isolated BFSG B-cells, **B.** and **C.** B-cells maintained for 12d in `normal' (defined-medium, serum-free) culture with or without NGF. Data were obtained from experiments such as those illustrated in Figure 2-5. Graphs in **A**, **B** and **C** show changes in peak and end-of-pulse current density recorded during 500ms conditioning pulses to potentials as indicated on the abscissa as well as peak current densities recorded in subsequent test pulses to 0mV (n>20 for all observations). **D.** Normalized summary of data. The difference between maximum currents in test pulses (that followed conditioning pulses to -80mV) and minimum currents in the test pulses (that followed conditioning pulses to -10mV) were defined as `1' for each of the plots in **A**, **B** and **C**. Test pulse currents recorded after conditioning pulses to other potentials are then expressed as fractions of this value.

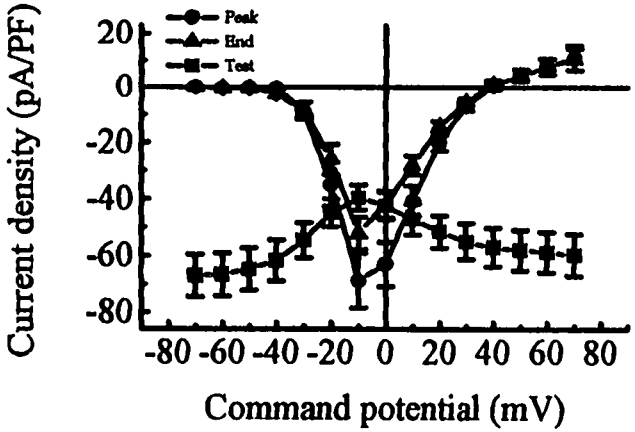
A. Acute



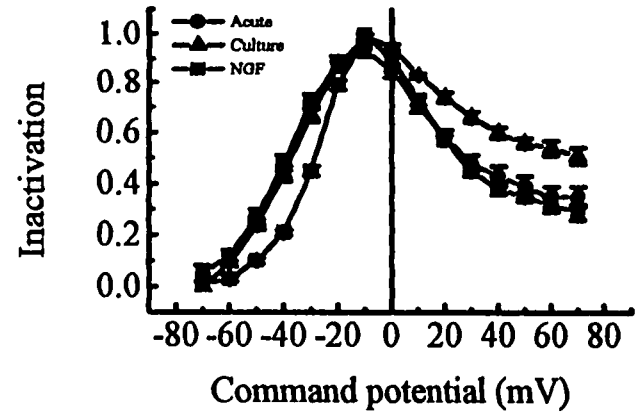
B. 12d culture



C. 12d Culture + NGF

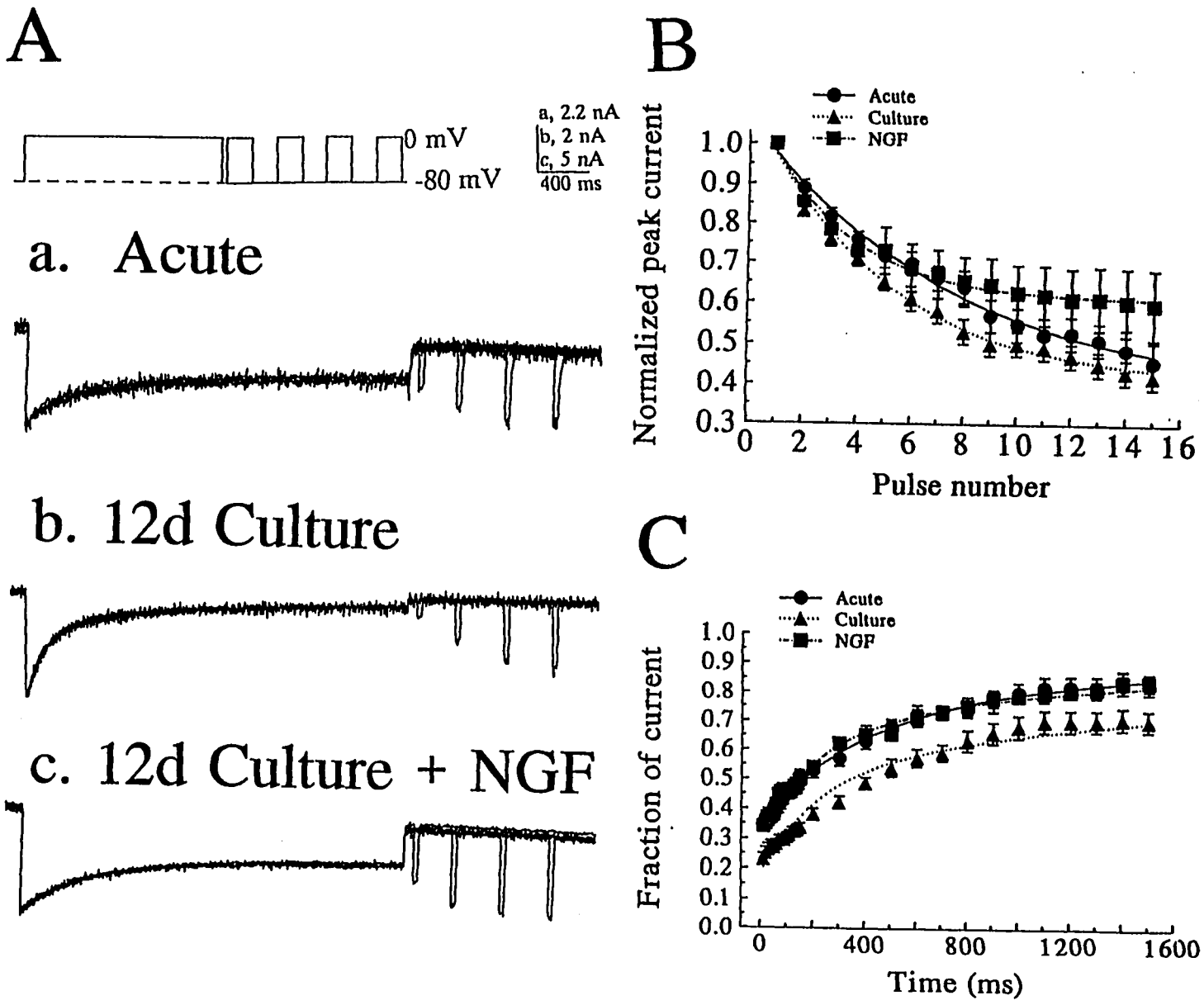


D



(Figure 2-6)

Figure 2-7. Kinetics of I_{Ca} inactivation. **A.** Currents evoked by 4s commands to 0mV from a holding potential of -80mV recorded from **a.** an acutely isolated BFSG B-cell and **b.** and **c.** from cells maintained for 12d in `normal' (defined-medium, serum-free) culture with or without NGF. Note differences in gain for currents in these three traces as indicated by the scale bar. 4s commands to 0mV were evoked once every 15s throughout each experiment. Each long pulse command was followed by a second brief (75ms) pulse to 0mV and the delay between the first and second pulses increased for each successive trial. The envelope of the small current amplitudes gave an index of the time course of recovery from inactivation. Because a slow inactivation process produces a progressive decline in successive 4s pulses, data records in **a**, **b** and **c.** are digitally rescaled to the amplitude of the first record. **B.** Graph to show time course of slow inactivation. Abscissa is pulse number (to 0mV for 4s once every 15s) and ordinate is normalized peak current amplitude. **C.** Time course of recovery from inactivation at -80mV obtained from envelope of amplitudes of brief (75ms) pulses to 0mV.


A
0 mV
-80 mV
a, 2.2 nA
b, 2 nA
c, 5 nA
400 ms
a. Acute
b. 12d Culture
c. 12d Culture + NGF
B
Acute
Culture
NGF
Normalized peak current
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0 2 4 6 8 10 12 14 16
Pulse number
C
Acute
Culture
NGF
Fraction of current
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 400 800 1200 1600
Time (ms)

(Figure 2-7)

Table 2-1. Components of inactivation of I_{Ca} in acutely-isolated BFSG B-cells (acute), cells cultured for 12d in serum-free defined medium (culture) and cells cultured for 12d in the presence of 200ng/ml NGF. A2 and A3 are amplitudes of the exponentials described by the time constants τ_2 and τ_3 (terminology of Werz *et al.*, 1993). A_0 represents the amplitude of the residual current that does not inactivate during the test pulse.

	Total inactivation in 4s pulse (%)	A_2 (%)	A_3 (%)	A_0 (%)	τ_2 (ms)	τ_3 (ms)
Acute	76.5±1.6	32.7±1.5	43.7±1.8	23.5±1.6	192.5±16.3	1770.4± 145.2
Culture	91.0±0.9**	49.3±1.5**	41.7±1.0	9.0±0.9**	191.5±9.4	1804.5± 89.1
NGF	75.3±1.6	32.8±2.0	42.5±1.2	24.7±1.6	222.4±15.7	1997.6± 135.6

** $p<0.01$

The rate of onset of 'very slow' inactivation (Jassar *et al.*, 1993) was assessed by measuring the size of peak currents induced by successive 4s commands to 0mV that were activated once activated every 15s. Figure 2-7B illustrates the change in current for a series of 15 such pulses and demonstrates that there is no significant difference for the onset of slow inactivation of I_{Ca} (at 0 mV) among acutely dissociated cells, cultured cells with or without

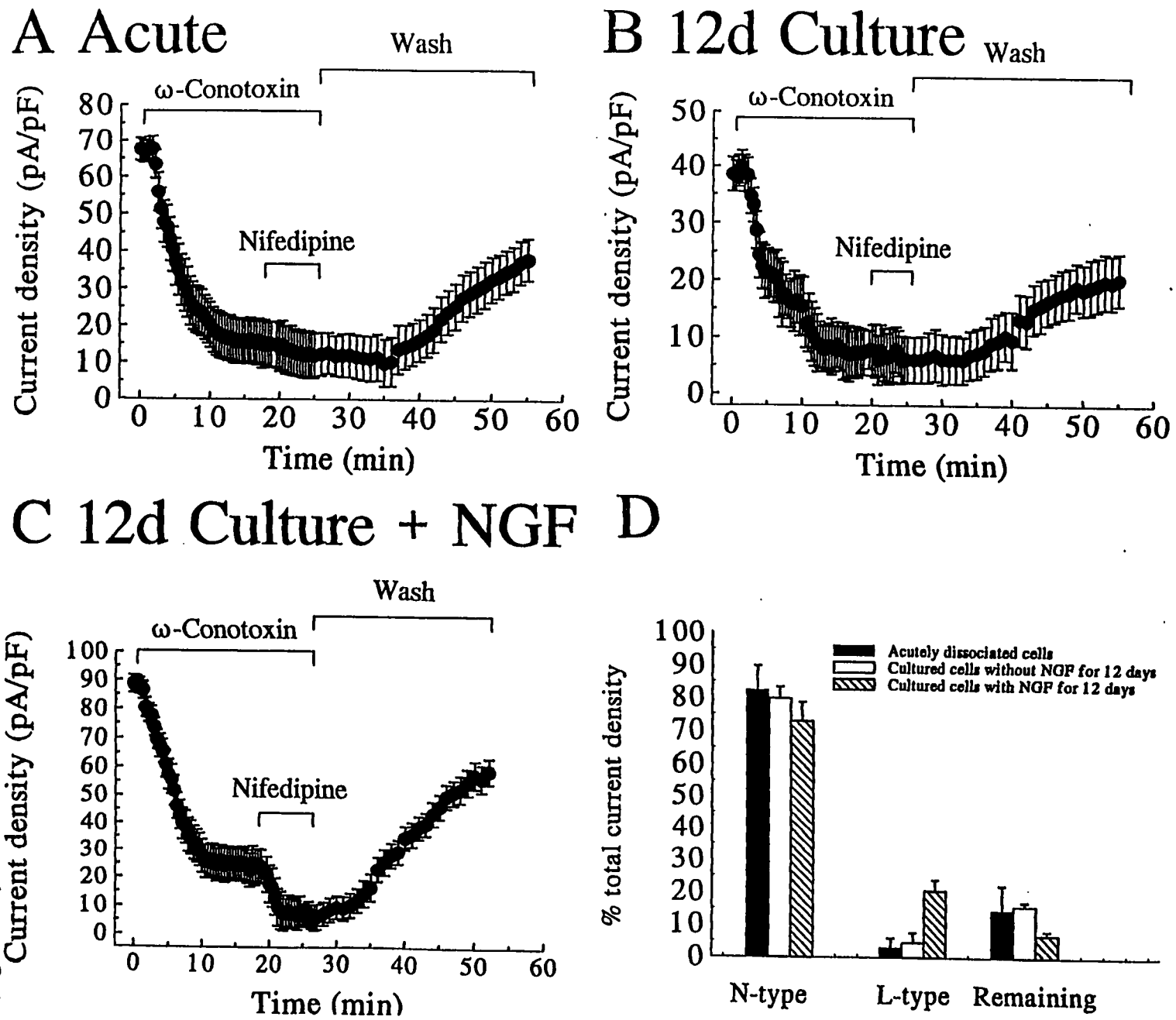
NGF for 12 days ($p>0.05$). In view of the lack of a significant difference, the kinetics of 'very slow' inactivation were not analyzed further.

Recovery from inactivation at -80mV is less complete for 12d cultured cells (without NGF) than for acutely isolated cells. Inclusion of NGF in the culture medium restores the recovery from inactivation to that observed in control cells (Figure 2-7C).

NGF-Induced Changes in L-type Ca^{2+}-channels

Studies in pheochromocytoma (PC12) cells (Usowicz *et al.*, 1990; Lewis *et al.*, 1993) and in rat embryonic forebrain neurons (Levine *et al.*, 1995) have shown that L-type current ($I_{Ca,L}$), $I_{Ca,N}$ and 'conotoxin/dihydropyridine' resistant current ($I_{Ca,other}$) are differentially regulated by NGF. We therefore compared the pharmacology of I_{Ca} recorded from control cells with that from 12d cultured and 12d NGF-treated cells. Figure 2-8A summarizes data from 12 control cells that were exposed to ω-conotoxin GVIA (ω-CgTx; 300nM) followed by nifedipine (10μM). Voltage steps to 0mV (from V_h= -80mV) were elicited every 25s during the perfusion of 300nM ω-CgTx and 10 μM nifedipine and once every 1 min during recovery until the current stabilized. Current density was reduced from 67.5±3.2pA/pF to 15.0±5.9pA/pF by ω-CgTx and subsequent addition of nifedipine reduced current by another 3.1±1.4pA/pF. The average current in control neurons was 82.4% conotoxin-sensitive $I_{Ca,N}$, 3% nifedipine sensitive $I_{Ca,L}$ and 14.6% $I_{Ca,other}$. Similar experiments on cells cultured for 12d in the absence (n=12) and presence of NGF (n=14) are shown in Figure 2-8B and Figure 2-8C. Whilst the pharmacological sensitivity of the current in 12d cultured cells (Figure 2-8B) resembles that seen in acutely-isolated cells (Figure 2-8A), there is clearly a greater portion of $I_{Ca,L}$ in NGF-treated cells. Under these conditions, 20.4% of the total I_{Ca} is L-type. The

Figure 2-8. Pharmacological properties of I_{Ca}. Graphs of I_{Ca} density versus time following exposure to ω-conotoxin GVIA (ω-CgTx; 300nM) followed by nifedipine (10μM). Data points represent mean current densities from 12-14 cells in each series of experiments. I_{Ca} was evoked at 0mV from a V_h of -80mV once every 20s before, during and after exposure to drugs. **A,B** and **C** are data from acutely isolated cells and from cells cultured for 12d in the presence or absence of NGF. **D.** Summary of data from **A,B** and **C** re-expressed as percentage of ω-CgTx-sensitive $I_{Ca,N}$, percentage of nifedipine-sensitive $I_{Ca,L}$ and percentage of ω-CgTx-/nifedipine-resistant $I_{Ca,other}$ in the three experimental situations.


A Acute
Wash
ω-Conotoxin
Nifedipine
Current density (pA/pF)
Time (min)
B 12d Culture
Wash
ω-Conotoxin
Nifedipine
Current density (pA/pF)
Time (min)
C 12d Culture + NGF
D
Wash
ω-Conotoxin
Nifedipine
Current density (pA/pF)
Time (min)
% total current density
Acutely dissociated cells
Cultured cells without NGF for 12 days
Cultured cells with NGF for 12 days
N-type
L-type
Remaining

(Figure 2-8)

relative proportions of $I_{Ca,L}$, $I_{Ca,N}$ and $I_{Ca,other}$ in cells in the three experimental situations are summarized in the histograms of Figure 2-8D.

Thus, in addition to decreasing inactivation of total I_{Ca} and increasing the total current by mechanisms other than decreased inactivation, NGF also appears to alter the proportion of nifedipine-sensitive $I_{Ca,L}$ in BFSG B-neurons.

It could be argued however, that the NGF-invoked increase in $I_{Ca,L}$ accounts for the observed decrease in the percentage of the total g_{Ca} that inactivates within a 4s pulse (*see* Figure 2-7A and Table 2-1). This is because $I_{Ca,L}$ may inactivate slowly (Fox *et al.*, 1987) so that the appearance of such a component would attenuate the observed inactivation of the total current in the presence of NGF. To address this, cells were studied in the presence of 10μM nifedipine. In cells cultured for 12d in the absence of NGF, the I_{Ca} recorded in the presence of nifedipine inactivated by 91.9±1.1% (n=22) after 4s at 0mV. By contrast, when NGF had been included in the cultures, the inactivation seen in the presence of nifedipine was reduced to 85.8±1.8% (n=22; $p<0.01$). NGF therefore promoted significant reduction of inactivation under conditions where $I_{Ca,L}$ was blocked. Thus, NGF-induced changes in $I_{Ca,L}$ do not account for the observed decrease in inactivation.

Time course of the action of NGF

There are now several reports of actions of neurotrophic factors on Ca^{2+}-channels that develop within minutes (Koike *et al* 1993; Selinfreund and Blair 1994; Shen and Crain, 1994; Toledo-Aral *et al.*, 1995; Wildering *et al.*, 1995). We therefore examined the time course of action of NGF on I_{Ca} in our system. The rate of decline in I_{Ca} *density* as cells were maintained in culture was unaffected by a 1d exposure to NGF (Figure 2-9A). With or without 1d

exposure to NGF, current density decreased to 61% of its control level in 5d. By contrast, exposure to NGF for 3 or 6d prevented the decline in current density (Figure 2-9A). The rate of decline of current density following NGF removal was similar for both 6 and 3d exposures. After 3d exposure, current declined to 80% of control within 5d of the withdrawal of NGF and after 6d exposure, current declined to 84% of control in 5d.

The rate of onset of the action of NGF was studied by examining its effect on cells which had been maintained in NGF-free medium for 6d. The maximal effect of NGF took at least 9d to develop (Figure 2-9B).

In vivo experiments

Frogs (*Rana Pipiens*) received 4 injections of NGF (1μg/g body weight) over a 7d period and I_{Ca} was examined 2-3d later. In general, I_{Ca} in RPSG B-cells was less than in BFSG B-cells. *Total* I_{Ca} (at 0mV from a V_h of -90mV) was 55.8% larger in cells from NGF-injected frogs than in cells from control animals; [5.30±0.42nA (n=41) compared with 3.40±0.29nA (n=34) $P<0.001$]. This difference was reflected as a difference in I_{Ca} *density*. Figures 2-10A and B show the averaged I-V relationships. I_{Ca} *density* (from all values of V_h) is greater in the B-cells from the NGF-treated animals. For example, I_{Ca} *density* (at 0mV; V_h=-90mV) in cells from NGF-treated animals is about 39% larger than that recorded from B-cells of saline-injected animals [64.8±5.2pA/pF (n=41) compared to 46.5±1.2pA/pF (n=34) $P<0.02$].

The situation with acutely-dissociated cells, that do not have neurites, differs from the situation in culture, where neurites are produced and C_{in} increases (Figure 2-1B). Whilst an increase in *total* I_{Ca} is seen in neurons that are cultured with NGF (Figure 2-3A) and in neurons that are isolated from NGF-treated animals, I_{Ca} *density* is only increased in the latter

situation. This is because current *density* is the ratio of *total* current to C_{in} and the C_{in} of B-cell somata from control animals [75.3±4.0pF (n=34)] is no different from that of cells from NGF-treated animals [84.6±3.9pF; (n=41;P>0.05)]. By contrast, the increase in C_{in} seen in culture eventually matches the increase in *total* I_{Ca} so that the I_{Ca} *density* measured at 15d is no different from the initial control value (see Figure 2-1B and Figure 2-3B).

Figure 2-10A also shows that altering the V_h of control cells from -90 to -40mV decreased peak I_{Ca} by 43%, from 46.5±4.2 to 27.5±2.7 pA/pF (n=34) whereas the same change in V_h for cells from NGF-treated animals changed peak current by about 34% from 64.8±5.2 to 44.9±4.2pA/pF (n=41). This difference and hence the difference in inactivation between the two groups is not significant (0.2<P<0.5).

Injection of NGF antiserum also failed to change the mean C_{in} of the acutely dissociated cell bodies of RPSG B-cells (P>0.5). Leopard frogs received 4 injections of 33μg protein/g body weight over a 7d period and I_{Ca} was examined 3d later. The control group received 4 injections of 33μg serum protein/g body weight over a similar time period. The C_{in} of B-cells from serum-injected (control) animals was 81.8±4.9pF (n=32) and that of B-cells from NGF antiserum-treated animals was 85.2±4.6pF (n=32). *Total* I_{Ca} (at 0mV from a V_h of -90mV) was smaller in acutely-dissociated cells from NGF antiserum-injected animals than in cells from control animals; [2.4± 0.21nA (n=32) compared with 3.4± 0.29nA (n=34) P< 0.05; a 29.4% decrease]. The effect of NGF-antiserum on I_{Ca} is therefore essentially the opposite of the effect of NGF.

Figure 2-10C and D show the averaged I-V relationships obtained from rabbit serum-injected (control) RPSG B-cells and from cells from animals treated with NGF antiserum. Peak current *density* (from all values of V_h) is smaller in the cells from the NGF antiserum-

Figure 2-9. Graphs to show **A** Effects of pretreatment of cultures for 1,3 or 6d with NGF on I_{Ca} density and **B** Rate of onset of increased I_{Ca} density following application of NGF to cultures that had been maintained for 6d in its absence. Peak I_{Ca} recorded at 0mV from a holding potential of -80mV.

(Figure 2-9)

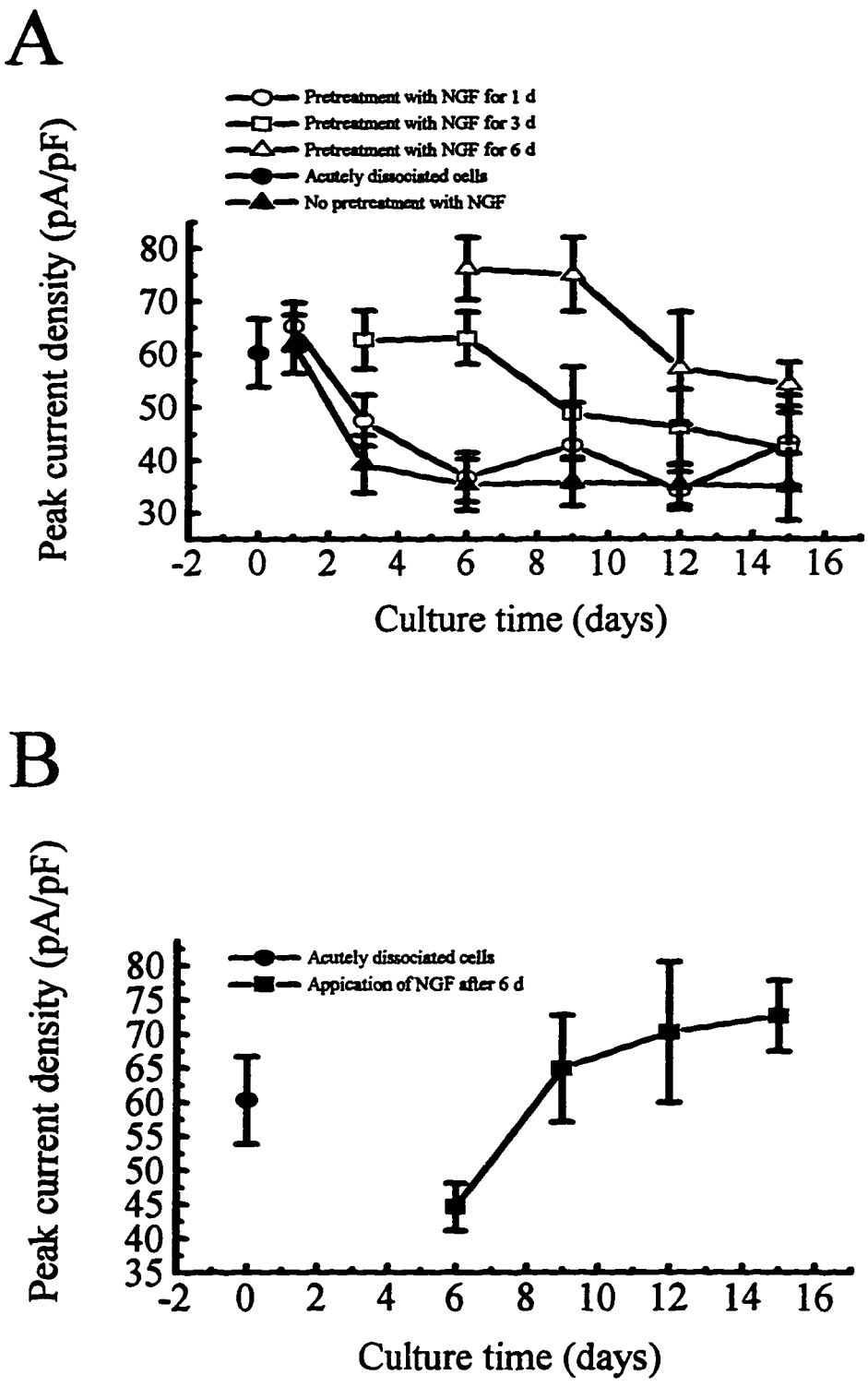
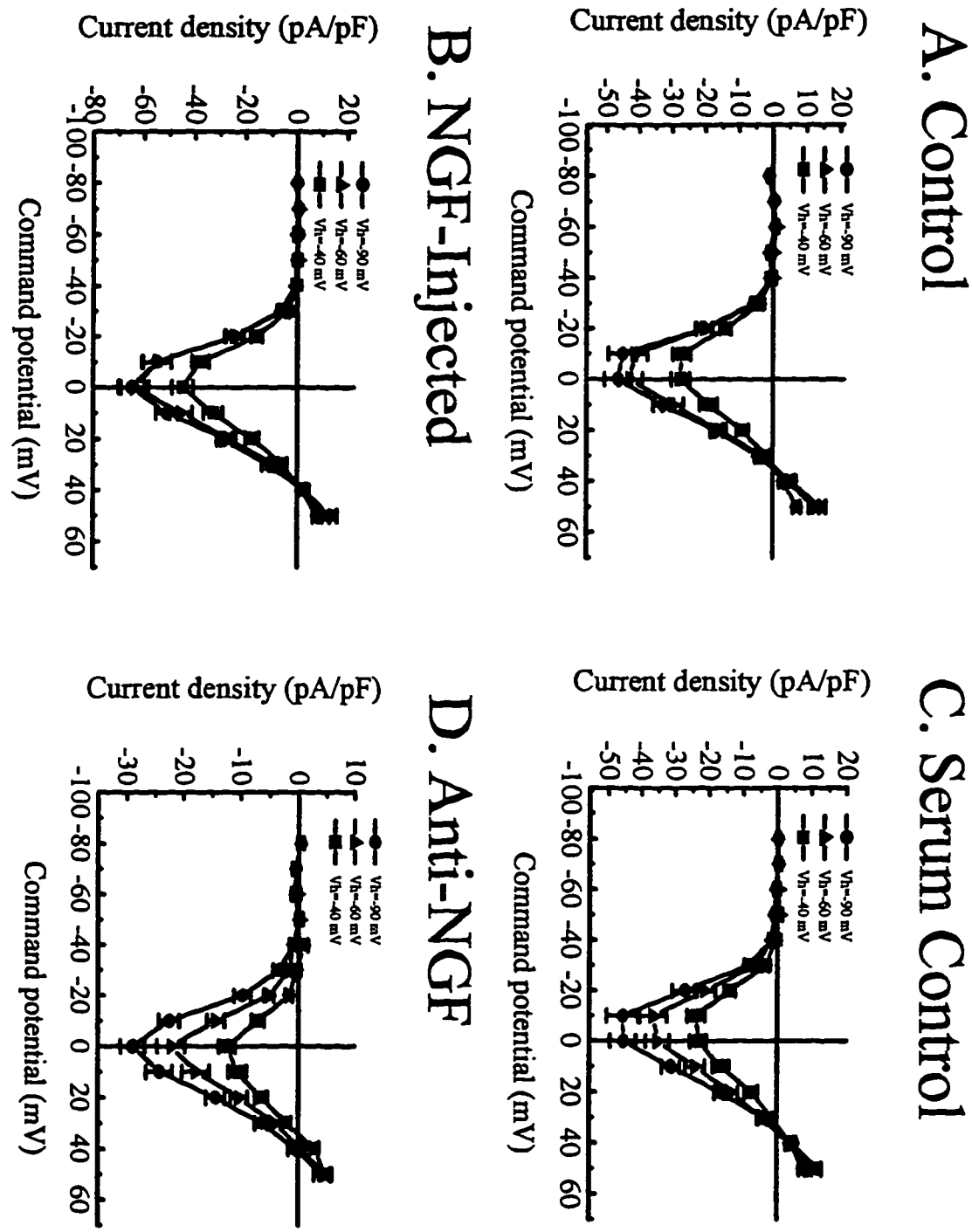

A
Pretreatment with NGF for 1 d
Pretreatment with NGF for 3 d
Pretreatment with NGF for 6 d
Acutely dissociated cells
No pretreatment with NGF
Peak current density (pA/pF)
80
70
60
50
40
30
-2
0
2
4
6
8
10
12
14
16
Culture time (days)
B
Acutely dissociated cells
Appication of NGF after 6 d
Peak current density (pA/pF)
80
75
70
65
60
55
50
45
40
35
-2
0
2
4
6
8
10
12
14
16
Culture time (days)

Figure 2-10. Current-density-voltage plots for I_{Ca} in B-cells acutely isolated from *Rana pipiens* sympathetic ganglia. Three different holding potentials (V_h); -90,-60 and -40mV were used. **A.** Data from cells from control (saline-injected) animals (n=41). **B.** Data from cells from frogs that had received NGF injections (n=34). **C.** Data from cells from frogs that had received rabbit serum (n=32). **D.** Data from cells from frogs that had received NGF antiserum (n=32).

(Figure 2-10)


A. Control
B. NGF-Injected
C. Serum Control
D. Anti-NGF
Current density (pA/pF)
Command potential (mV)
Vh=-90 mV
Vh=-60 mV
Vh=-40 mV

treated animals. Thus, the *density* of I_{Ca} (recorded at 0mV from a V_h of -90mV) in cells from NGF antiserum-treated animals is 28.9±2.1pA/pF (n=32) whereas that from control cells is 45.8±4.8pA/pF (n=32; P<0.005). The antiserum therefore decreases the I_{Ca} *density* at this potential by about 37%.

Figure 2-10D also shows that altering the V_h of cells from NGF-antiserum treated animals from -90 to -40mV decreased peak I_{Ca} *density* by about 58% from 28.9±2.1 to 12.3±1.4pA/pF (n=32) whereas the same change in V_h for cells from control (serum injected) animals changed peak current *density* by 49%, from 45.8±4.8 to 23.8±2.6 pA/pF (n=32). This difference and hence the difference in inactivation between the two groups is not statistically significant (0.2>P>0.1).

Comparison of g_{Ca} inactivation following NGF antiserum treatment [58% from 28.9±2.1 to 12.3±1.4pA/pF (n=32)] with that seen following NGF treatment [34% from 64.8±5.2 to 44.9±4.2pA/pF (n=41)] does however reveal a highly significant difference (P<0.001).

Discussion

The principal findings of this work are that *i)* NGF treatment potentiates I_{Ca} in a sympathetic neuron by three independent mechanisms, *ii)* one of these actions is novel in that it demonstrates that inactivation of I_{Ca} in an adult neuron can be modulated by an extracellular ligand, *iii)* NGF selectively alters one particular kinetic component of the inactivation process, *iv)* effects of NGF seen in culture resemble those seen *in vivo*. These findings, together with the *in vivo* effects of NGF-antiserum are consistent with the hypothesis that NGF or similar molecules are involved in the maintenance of Ca^{2+} channel function in adult sympathetic

neurons. We suggest that the presence of appropriate neurotrophic support allows the expression and activity of Ca^{2+}-channels to keep pace with the growth or size of the neuron. Further analysis of the data provides insights into how these effects may be achieved.

Potentiation of I_{Ca} by NGF

The three mechanisms that contribute to NGF-induced potentiation of I_{Ca} in BFSG are *i)* increases in $I_{Ca,L}$ *ii)* increases in $I_{Ca,N}$ and *iii)* decreases in inactivation. These three effects reflect independent phenomena rather than different experimental manifestations of a single effect. The inactivation curves shown in Figure 2-4E show that the NGF-induced increase in total I_{Ca} is not solely a consequence of decreased steady-state inactivation; if this were so, removal of inactivation by holding cells at -110mV would restore current seen in 12d cultured cells to the same value seen in NGF-treated cells. It is also unlikely that this NGF-induced increase in total current simply reflects the increase in $I_{Ca,L}$. NGF more than doubles the I_{Ca} evoked from -110mV (approximately a 120% increase; see Figure 2-4E) and if this were solely due to an increase in $I_{Ca,L}$, at least half of the current (minimum 55%) seen in NGF-treated cells would be L-type. The amount of $I_{Ca,L}$ in these cells is however much lower (20.4%; Figure 2-8D). We have also shown that the NGF-induced increase in $I_{Ca,L}$ cannot account for the overall decrease in I_{Ca} inactivation because NGF produced a decrease in inactivation of total current under conditions where L-channels were blocked with a supramaximal concentration of nifedipine.

NGF-induced decrease in I_{Ca} inactivation - a novel mechanism of modulation?

Treatment of BFSG B-neurons with phosphatase inhibitors increases the amplitude (A_2) of the 'intermediate' component of $I_{Ca,N}$ inactivation (Werz *et al.*, 1993) without major alteration of the other kinetic parameters; τ_2, τ_3 and A_3. This suggests that Ca^{2+}-channels are subject to tonic phosphorylation *in vivo* and that preservation of phosphorylation of a site on $I_{Ca,N}$ channels leads to increased inactivation. Because NGF attenuates inactivation by selectively reducing this same kinetic parameter (A_2), it may produce its effect by reducing phosphorylation of a site on the N-type Ca^{2+} channel. Indeed, Aoki *et al.*, (1996) recently reported that NGF can increase the mRNA for a novel tyrosine phosphatase in PC12 cells.

An alternative hypothesis for the action of NGF on g_{Ca} inactivation is that it favours the appearance of 'slowly-inactivating' $I_{Ca,N}$ channels as opposed to 'rapidly-inactivating' $I_{Ca,N}$ channels that may be characteristic of nerve terminals. These different subtypes of N-type Ca^{2+} channel may derive from different splice variants of the α_{1B} Ca^{2+}-channel subunit gene (Fisher and Bourque, 1996).

A third mechanism whereby NGF could alter inactivation of g_{Ca} in B-cells would involve alteration in expression of β-subunits of Ca^{2+}-channel proteins (Varadi *et al.*, 1991). This would not adequately explain the present results because β-subunits regulate both the inactivation and the activation kinetics of Ca^{2+}-channels whereas NGF affected only the inactivation process.

Although an *increase* in I_{Ca} inactivation has been seen during NGF-induced differentiation of PC12 cells (Streit and Lux, 1987;1990), to the best of our knowledge, regulation of g_{Ca} inactivation by an extracellular ligand has not previously been described in a differentiated, adult neuron.

Those actions of NGF that are not a consequence of decreased inactivation, *i.e.* the

increase in $I_{Ca,N}$ seen from a V_h of -110mV and its effects on $I_{Ca,L}$ may reflect alterations in channel expression at the translational or transcriptional level. Perhaps mechanisms similar to those activated during differentiation of PC12 cells (Lewis *et al.*, 1993; Cavalié, *et al.*, 1994) can be invoked in adult BFSG B-cells by NGF. The NGF-induced increase in $I_{Ca,L}$ may be relevant to the proposed involvement of changes in Ca^{2+} influx via L-type Ca^{2+} in the transduction process for some growth factors (Levine *et al.*, 1995) by activation of mitogen-activated protein kinase (Rosen *et al.*, 1994; Finkbeiner and Greenberg, 1996).

Maintenance of Ca^{2+} channels by NGF

Cultured BFSG B-neurons extend neurites either in the absence or in the presence of NGF. In the absence of NGF, the *total* I_{Ca} remains roughly constant but in its presence, *total* I_{Ca} increases such that current *density*[2] keeps up with the growth and increased cell size. NGF does not therefore appear to initiate growth *per se* but once neurite production occurs, it seems to regulate the properties of the neuron so that the demands of a greater neuronal size can be met. This is to be expected if NGF were to play a 'maintenance' role in adult peripheral neurons (Nja and Purves 1978; Korshing and Thoenen 1983; Gold *et al.*, 1991). NGF injection *in vivo* may promote sprouting of the terminal fields of BFSG neurones as it does in mammalian DRG (Diamond *et al.*, 1987), the increased I_{Ca} seen in cell bodies under these conditions may reflect increased channel synthesis in order to meet the demands of an enlarged neuron.

In serum-supplemented explant cultures of whole BFSG, addition of murine NGF promotes a pronounced enhancement of neurite outgrowth (Kelly *et al.*, 1989). The absence of an obvious growth response following treatment of single BFSG B-cells with NGF in

defined medium (Figure 2-1) may be a consequence of the lack of nutritional support and/or the absence of other types of trophic support that are required for an NGF-induced growth response. The present data show, however that the NGF-induced increase in g_{Ca} in BFSG is not secondary to NGF-induced neurite extension.

Physiological Significance of NGF's action

The main advantage of tissue culture experiments on BFSG B-cells is that one specific cell type can be identified and voltage-clamped so that the direct effects of NGF on a variety of aspects of Ca^{2+}-channel function can be analyzed. The problem with this approach is that adult neurons may be transformed by the culture process such that their response to NGF may no longer be relevant to the behaviour of adult neurons *in vivo*. Our data show however, that NGF increases *total* I_{Ca} *in vivo* and *in vitro*. Because of this similarity, the various indirect mechanisms whereby NGF might influence BFSG B-cells (Korsching and Thoenen, 1983; Assouline *et al.*, 1987; Lewin and Mendell, 1993; Guenard *et al.*, 1991; Unsicker *et al.*, 1987), do not seem to be invoked *in vivo*. These data, and the result that NGF antiserum decreases *total* I_{Ca} amplitude *in vivo* are consistent with the hypothesis that Ca^{2+}-channels are regulated *in vivo* by NGF. Injections of NGF *in vivo* do not seem to decrease g_{Ca} inactivation as NGF does *in vitro*. One possible reason for this is that amphibian symapthetic neurons have access to a certain amount of target-derived NGF *in vivo* in much the same way as mammalian neurons. Amphibians are known to synthesize NGF (Carriero *et al.*, 1991). It therefore may be more appropriate in the *in vivo* experiments to compare cells from animals treated with anti-NGF to cells treated with NGF. When this is done there is a highly significant difference in inactivation.

Axotomy of BFSG B-neurons promotes increased inactivation of N-type g_{Ca} ($g_{Ca,N}$; Jassar *et al.*, 1993) in much the same way as prolonged culture. It is of particular relevance to the present study that the amplitude of the 'intermediate' component (A_2) of inactivation is increased by both manipulations and that the other kinetic parameters (τ_2, τ_3 and A_3) are little changed. It will be recalled from Table 2-1 that A_2 is the component that is selectively decreased by NGF. One hypothesis suggests that the changes induced in BFSG B-neurons following disconnection with their target reflects loss of retrogradely-supplied NGF (Verge *et al.*, 1996). Indeed, the normal properties of $I_{Ca,N}$ seem to be re-established once regeneration has occurred and target contact is re-established (Kelly *et al.*, 1988; Petrov *et al.*, 1996). A selective effect of NGF on A_2 might therefore be predicted from the hypothesis that loss of target derived-NGF is involved in the effects of axotomy on N-type Ca^{2+} channels. This hypothesis cannot however explain all of the effects of axotomy on the electrophysiological properties of B-cells because Na^+ currents are increased both by axotomy (Jassar *et al.*, 1993) and by treatment of cultures with NGF (S. Lei, W.F. Dryden and P.A. Smith, unpublished observations). The data do however argue quite strongly against the contrary hypothesis that axotomy-induced increases in $g_{Ca,N}$ inactivation result from *increased* production and release of NGF as part of the inflammatory response that accompanies nerve injury (see Lewin and Mendell, 1993). If this were so, NGF would increase rather than decrease $g_{Ca,N}$ inactivation.

FOOTNOTES

1.

Because Ara-C failed to influence the current between 4 and 15d, Ara-C has no direct toxic effects on cultured neurons. For example, for cells cultured for 12 days, I_{Ca} density in the presence of both Ara-C and NGF is not significantly different from that of the cells cultured with NGF alone ($p>0.05$)

2.

The terminology of Werz *et al.* (1993) has ben used to describe the kinetics of I_{Ca} inactivation. The fastest component of inactivation (τ_1) of amplitude A_1 can only clearly be distinguished in cells that have been treated with phosphatase inhibitors.

3.

Because current density is expressed as the ratio of I_{Ca} to C_{in}, the present experiments provide no information as to where new channels are expressed. Ca^{2+} channels are normally thought to be expressed in cell bodies and growth cones and Lipscombe *et al* (1988) have shown that they appear on neurites of BFSG in culture. It is not known to what extent channels in neurites and/or growth cones of cultured cells contribute to the total current measured at the cell body.

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Chapter 3

Signal transduction mechanisms for the regulation of Ca^{2+} channels by NGF

Introduction

Neurotrophic factors are required for the development, survival, and maintenance of distinct population of neurons. One prototypic neurotrophic peptide, nerve growth factor (NGF) belongs to a family which includes brain-derived growth factor, neurotrophin-3, neurotrophin-4/5 and neurotrophin-6 (for a review, see Lewin, 1996). NGF is required for the differentiation and survival of sympathetic and some sensory neurons in the peripheral nervous system and provides trophic support for the cholinergic neurons of the basal forebrain (Lindsay, 1996).

NGF exerts its neurotrophic activity by binding to a receptor complex comprised of a low affinity component, $p75^{NGFR}$ (Chao *et al.*, 1986; Radeke *et al.*, 1987) and a high affinity component, Trk A (Kaplan *et al.*, 1991; Klein *et al.*, 1991). Trk A contains a cytoplasmic domain with tyrosine kinase activity (Kaplan *et al.*, 1991). The two identical subunits of NGF contain two potential Trk A binding sites which facilitate the formation of Trk A receptor homodimers, a step required for receptor activation (Jing *et al.*, 1992). Ligand-mediated dimerization is followed by autophosphorylation of the tyrosine residues on the receptors (Schlessinger and Ullrich, 1992). Binding of the specific phosphotyrosine residues of the ligand-activated receptor to the src homology 2 (SH2) domains on cytoplasmic effector proteins leads to the activation of intracellular signals (Koch *et al.*, 1991; Schlessinger and Ullrich, 1992). Intracellular signal proteins activated by this process include phosphatidylinositol-3-kinase (PI3K), phospholipase C-γ, ras adaptor proteins (Shc, Grb2 and SOS) and suc-associated nucleotide neurotrophic factor-induced tyrosine phosphorylated target (SNT) (Kaplan and Stephens, 1994).

PI3K is a heterodimeric enzyme composed of a p85 regulatory subunit and a p110 catalytic subunit. Activity of PI3K leads to the production of D-3-phosphorylated phosphoinositides (Whitman *et al.*, 1988; 1987). While the exact physiological roles of these products are unknown, the activity of PI3K has been implicated in the growth-factor-mediated mitogenesis (Jhun *et al.*, 1994; Karnitz *et al.*, 1995), apoptosis (Yao and Cooper, 1995; Scheid *et al.*, 1995; Minshall *et al.*, 1996) and neurite outgrowth of PC12 cells (Kimura *et al.*, 1994).

PLC-γ catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to the potent second messengers, diacylglycerol (DAG) which activates protein kinase C and inositol trisphosphate (IP_3) which transiently increases the levels of intracellular calcium (Rhee and Choi, 1992). Combined with Ras, the PLC-γ pathway is proposed to be responsible for NGF-induced cell differentiation (Obermeier *et al.*, 1994) and neurite outgrowth (Stephens *et al.*, 1994).

Ras, a G-protein of small molecular weight, can be activated with the help of several adaptor proteins (Shc, Grb2 and SOS) in response to NGF (Kaplan and Stephens, 1994). The activated Ras initiates a series of cascades of events that lead to the activation of mitogen-activated protein kinase (MAPK), which has a number of substrates, including transcription factors (Pulverer *et al.*, 1991; Gille *et al.*, 1992), phospholipase A_2 (Nemenoff *et al.*, 1993) and other kinases, such as ribosomal S6 kinase II, or $pp90^{Rsk}$ (Sturgill *et al.*, 1988). This pathway is important in growth factor-mediated apoptosis (Borasio *et al.*, 1993; Nobes and Tolkovsky, 1995; Nobes *et al.*, 1996), differentiation and proliferation (Hagag *et al.*, 1986; Szeberenyi *et al.*, 1990; Kremer *et al.*, 1991; Cowley *et al.*, 1994; Fukuda *et al.*, 1995).

While Trk A has been shown to be essential for the biological activity of NGF (Loeb *et al.*, 1991; Loeb and Greene, 1993; Weskamp and Reichardt, 1991; Ibanez *et al.*, 1992), the role of the p75 receptor in NGF-mediated signaling cannot been fully excluded. It may play a role in facilitating the function of Trk functions (Hantzopoulos *et al.*, 1994; Hempstead *et al.*, 1991; Battleman *et al.*, 1993; Benedetti *et al.*, 1993; Barker and Shooter, 1994; Verdi *et al.*, 1994), in interacting with G-proteins (Feinstein and Larhammer, 1990; Knipper *et al.*, 1993), in generating intracellular signals, such as ceramide (Dobrowsky *et al.*, 1994) and cAMP (Knipper *et al.*, 1993), in signaling apoptosis in the absence of NGF (Rabizadeh *et al.*, 1993; Barrett and Bartlett, 1994; MacEwan, 1996) and in activating some protein kinases (Volonte *et al.*, 1993a,b; Canossa *et al.*, 1996).

Voltage-gated Ca^{2+} channels modulate numerous cellular functions via regulation of Ca^{2+} influx. In the previous chapter, we have shown that Ca $^{2+}$channels in adult bull-frog sympathetic ganglia (BFSG) are regulated by NGF. The signal transduction mechanisms are unknown. In this chapter we will therefore use Trk and p75 antibodies to examine the roles of the two receptors in NGF-mediated Ca^{2+} channel regulation. Specific inhibitors for different pathways will also be used to identify which pathway(s) is/are necessary for the regulation of Ca^{2+} channel currents by NGF. Some of the results have been published as an abstract (Lei *et al.*, 1996).

Methods

Chemicals

NGF (2.5S) and anti-p75 antibody were purchased from Alomone Labs., Jerusalem, Israel. Anti-Trk Ig-G antibody and negative control Ig-G antibody (using Aspergillus niger glucose oxidase as an antigen) were supplied by Zymed Laboratories Inc., DAKO. Leibovitz's L-15 medium and penicillin-streptomycin antibiotics were provided by GIBCO BRL. The following chemicals were products of BIOMOL: genistein, daidzein, lavendustin A, wortmannin, LY29002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), U-73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), U-73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione), α-hydroxyfarnesylphosphonic acid, perillic acid, actinomycin D. The rest of the chemical reagents used in the experiments were purchased from Sigma (St. Louis, MO, USA).

Tissue culture

Tissue culture method was essentially the same as described in the "Methods" of Chapter 2. Neuron-enriched culture was used in all the experiments. The dissociated ganglion cells were preplated into two or three 35 mm culture dishes. After 1-2h, most of the non-neuronal cells were adherent to the bottom of the dishes and the nonadherent cells, which were primarily neurons, were harvested, redistributed to 30 dishes and cultured in 3 ml/dish of fresh medium. The basic culture medium consists of 73% L-15 medium supplemented with 10 mM glucose, 1 mM $CaCl_2$, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μM

cytosine arabinoside. Since all the inhibitors used to probe the signal transduction pathways by NGF are highly hydrophobic, they were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions. The stock solutions were diluted in the basic culture medium to make the desired final concentrations. The criteria used to select the concentration of inhibitors included published IC_{50} concentrations (concentrations at which half of the effects were inhibited) of the inhibitors, published dose-response curves or published effective concentration used by other investigators. The final DMSO concentration in the culture medium was controlled to be 0.1% and the same concentration of the DMSO was used in the blank or NGF control groups. DMSO at this concentration did not affect Ca^{2+} currents. Cells cultured with inhibitor plus NGF (200 ng/ml) were pretreated with a medium containing the same concentration of the inhibitor for one hour before replacement with the medium containing both inhibitor and NGF. The culture medium was changed daily to maintain the effective concentrations of the inhibitors, but for wortmannin and PD98059, which are hydrolyzed more rapidly, the culture media were exchanged every 5-8 hours. Continuous inhibition of PI-3-kinase and MAP kinase kinase activities could be achieved with this kind of treatment (Kimura *et al.*, 1994; Virdee and Tolkovsky, 1996). The cells were cultured at room temperature in a light-proof environment to avoid photolysis of potentially light-sensitive reagents. Electrophysiological analysis was then carried out after 6 days of culture since the effect of NGF was significant at that time (see Figure 2-3).

Electrophysiology

Whole-cell Ca^{2+} channel currents were recorded from cultured cells of different groups. For recording, tissue culture medium was replaced with external solution containing (mM): N-methyl-D-glucamine (NMG) chloride, 117.5; NMG-HEPES, 2.5; $BaCl_2$, 2.0; (pH7.2). Internal solution consisted of (mM): NMG-Cl,76.5; HEPES, 2.5; Tris-BAPTA, 10; Tris-ATP, 5; $MgCl_2$, 4; (pH7.2). Cell size was determined by integrating the capacitive transient current elicited by 10 mV depolarizing voltage from a holding potential of -80 mV. Currents were evoked by a series of depolarizing voltage steps (10 mV interval) from a holding potential of -80 mV. Peak current generally occurred at 0 mV for cultured cells with or without NGF. Current density (peak current at 0 mV normalized to the capacitance) was used as an indicator to evaluate the effects of the antibodies or inhibitors. Since some inhibitors might change the capacitance by influencing the cell sizes or neurite outgrowth and the current density alone might not authentically represent the expression of Ca^{2+} channels on the cell membranes, the capacitance and absolute current were also carefully analyzed. For comparative purposes, data are presented as current density, which is derived from absolute current and capacitance. In each experiment, at least 20 cells were examined. Culture dishes containing cells exposed to either DMSO alone or DMSO plus NGF were prepared and their currents measured to provide an assurance of batch quality. The data from all these experiments were pooled together and used as blank and positive control for each group, respectively. All the data are presented as means ± SEM and Student's two tailed t-test was used to assess statistical significance.

Results

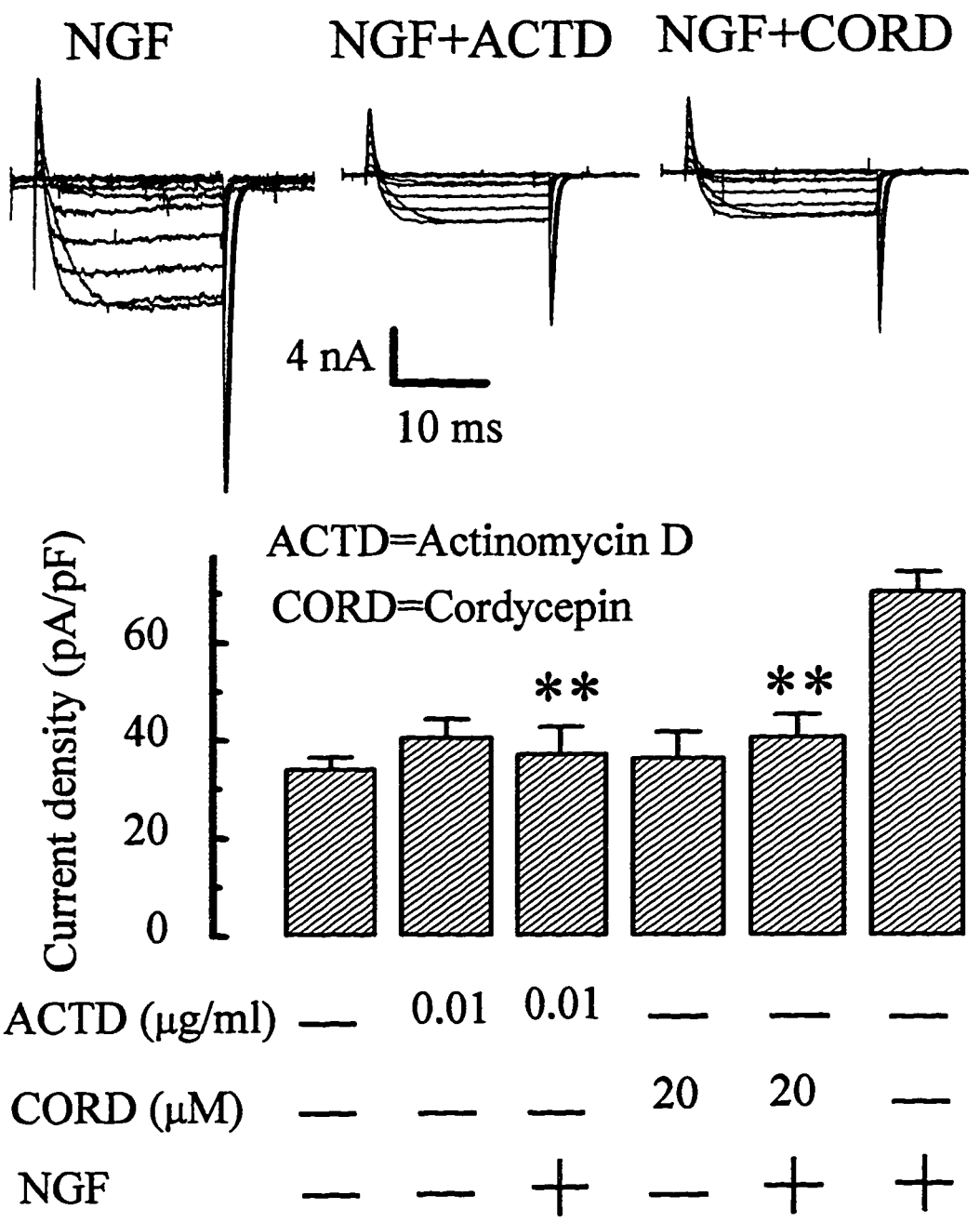
NGF response is transcription-dependent

While NGF attenuates the inactivation of Ca^{2+} channel currents, the up-regulation of Ca^{2+} channel currents in response to NGF cannot be fully explained by NGF-induced-reduction of the inactivation of Ca^{2+} channel currents (see Figure 2-4E). The possibility that NGF-mediated enhancement of Ca^{2+} channel gene expression is responsible for the increase of Ca^{2+} channel currents was therefore examined first. Cordycepin (20 μM), an RNA-synthesis inhibitor, inhibited the NGF-mediated enhancement of the current density (Figure 3-1). Actinomycin D, a DNA-transcription inhibitor, had similar effects; it blocked NGF-induced enhancement of Ca^{2+} channel current at a concentration of 0.01 μg/ml (Figure 3-1). These results suggest that RNA synthesis associated with NGF exposure, was involved in the increase in whole-cell Ca^{2+} currents, and thus is consistent with an up-regulation of gene expression.

Microscopic observation indicated that treatment of the cells with either cordycepin or actinomycin D decreased the outgrowth of the neurites of both cultured cells and cultured cells with NGF, suggesting neurite outgrowth was transcription-dependent. This was further confirmed by the analysis of the input capacitance (Table 3-1). Compared with the cells cultured without transcriptional inhibitors, the capacitance was 36% and 38% smaller for the cultured cells with cordycepin (from 172.8 ± 8.1 pF, n=107 to 107.1 ± 9.4 pF, n=20, $p < 0.01$) and actinomycin D (from 172.8 ± 8.1, n=107 to 109.1 ± 10.7 pF, n=20, $p < 0.01$), respectively (Table 3-1). NGF failed to counterbalance the decrease of the capacitance

Figure 3-1. Transcriptional inhibitors blocked enhancement of Ca^{2+} current density in response to NGF. Upper panel, currents invoked by a series of depolarization pulses (10 mV interval) from the holding potential of -80 mV. Lower panel, histogram of the current density. ** $p < 0.01$ compared with the current density of the cells treated with DMSO plus NGF. Both actinomycin D and cordycepin blocked NGF-mediated increase in Ca^{2+} current density.

(Figure 3-1)


NGF
NGF+ACTD
NGF+CORD
4 nA
10 ms
ACTD=Actinomycin D
CORD=Cordycepin
Current density (pA/pF)
60
40
20
0
**
**
ACTD (μg/ml) — 0.01 0.01 — — —
CORD (μM) — — — 20 20 —
NGF — — + — + +

significantly in the presence of the inhibitors (Table 3-1).

Extracellular Ca^{2+} is not required for NGF response

Influx of extracellular Ca^{2+} ($[Ca^{2+}]_o$) regulates gene expression in response to synaptic activation in primary cultures of cortical neurons (Murphy *et al.*, 1991), and it also mediates induction of gene expression in response to membrane depolarization of PC12 cells (Greenberg *et al.*, 1986; Morgan and Curran, 1986). $[Ca^{2+}]_o$ influx increases BDNF expression, enhancing neurite outgrowth by a BDNF-dependent mechanism in cultured rat cortical neurons (Ghosh *et al.*, 1994), and stimulates expression of $p140^{trkA}$, the NGF tyrosine kinase receptor, enabling cells to undergo NGF-stimulated neurite outgrowth in the sympathoadrenal cell line MAH (Birren *et al.*, 1992). Furthermore, $[Ca^{2+}]_o$ influx stimulates MEK and MAP kinase via activation of Ras (Rosen *et al.*, 1994; Rosen and Greenberg, 1996).

To investigate whether $[Ca^{2+}]_o$ was necessary for NGF-induced Ca^{2+} channel expression, we omitted the $[Ca^{2+}]_o$ in the culture medium and used 2 mM EGTA to chelate any possible source of Ca^{2+} in the culture medium. The results are shown in Figure 3-2. Depletion of $[Ca^{2+}]_o$ did not affect NGF-mediated increase of Ca^{2+} channel current density, suggesting $[Ca^{2+}]_o$ was required for neither the activation of NGF receptors nor the signal transduction mechanisms underlying the induction of Ca^{2+} channels by NGF.

Analysis of cell size or neurite outgrowth by means of capacitance measurements also denied a role for $[Ca^{2+}]_o$ in neurite outgrowth. Removal of $[Ca^{2+}]_o$ did not change the capacitance of the cells in the absence (168.55±18.01 pF, n=20; vs 182.7±15.31, n=20;

p>0.05) and presence (170.1±13.23 pF, n=20; vs 157.1±16.64 pF, n=21; p>0.05) of NGF.

NGF response is tyrosine-kinase-dependent

Tyrosine kinase-mediated phosphorylation is likely to be important in the signal transduction of NGF because Trk A receptors have tyrosine kinase activity and intracellular non-receptor tyrosine kinases such as c-Src, c-Yes and Fyn inside the cells may be activated by growth factors (Vaillancourt *et al.*, 1995; Kremer *et al.*, 1991). Two typical tyrosine kinase inhibitors, genistein and lavendustin A, were therefore used to test whether tyrosine kinase activity was necessary for NGF-mediated Ca^{2+} channel expression. In the presence of 20 μM genistein alone, Ca^{2+} current density was not influenced, compared with that of the cells cultured with daidzein, an inactive analogue. However, at this concentration, genistein completely blocked the increase of the current density induced by NGF (Figure 3-3).

At higher concentration (100 μM), genistein reduced the current density of the cells treated with daidzein by 79% and that of the cells treated with NGF plus daidzein by 78% (Figure 3-4). This result suggests that Ca^{2+} currents are tonically regulated by tyrosine kinase in sympathetic ganglion cells.

Lavendustin A, another inhibitor had similar effects. At 1 μM, lavendustin A blocked NGF-induced enhancement of current density, but it had no effects on the control current density. A higher concentration of lavendustin A (10 μM) reduced the current density of control cells and that of the cells treated with NGF by 58% and 70%, respectively (Figure 3-5).

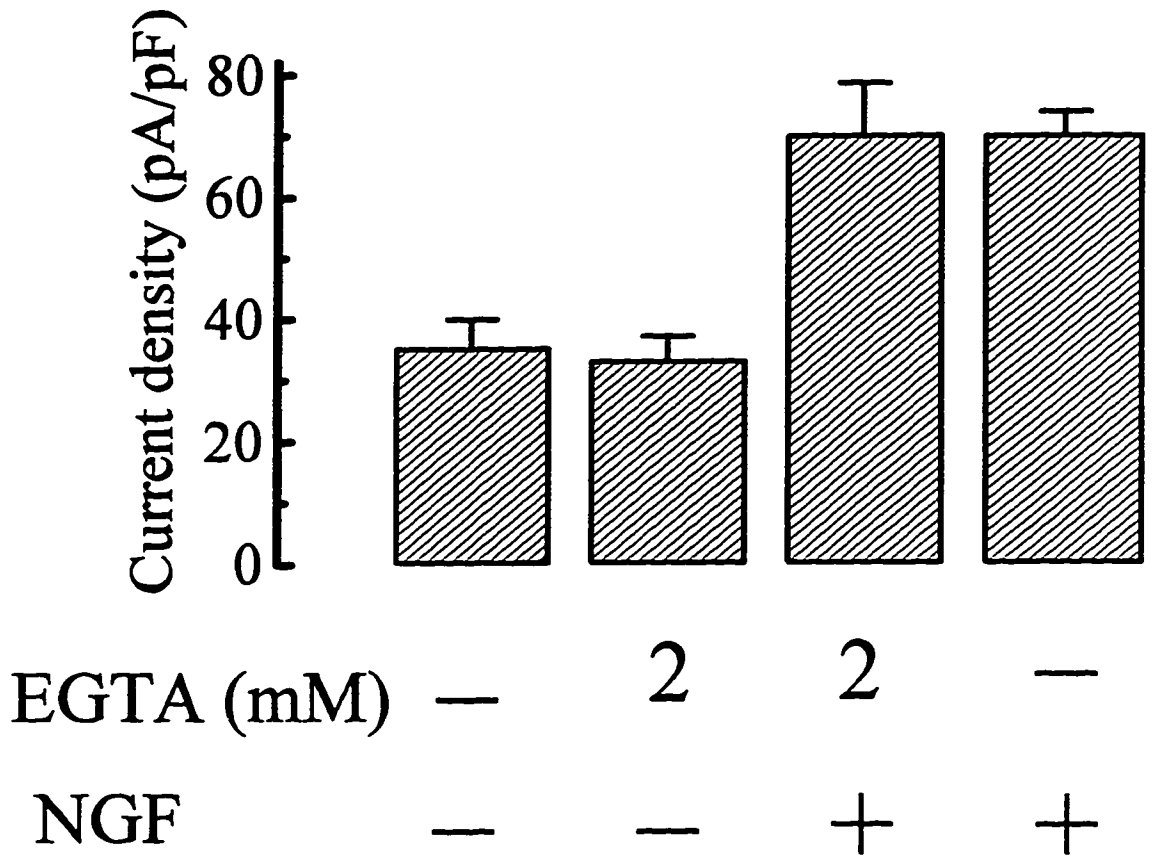
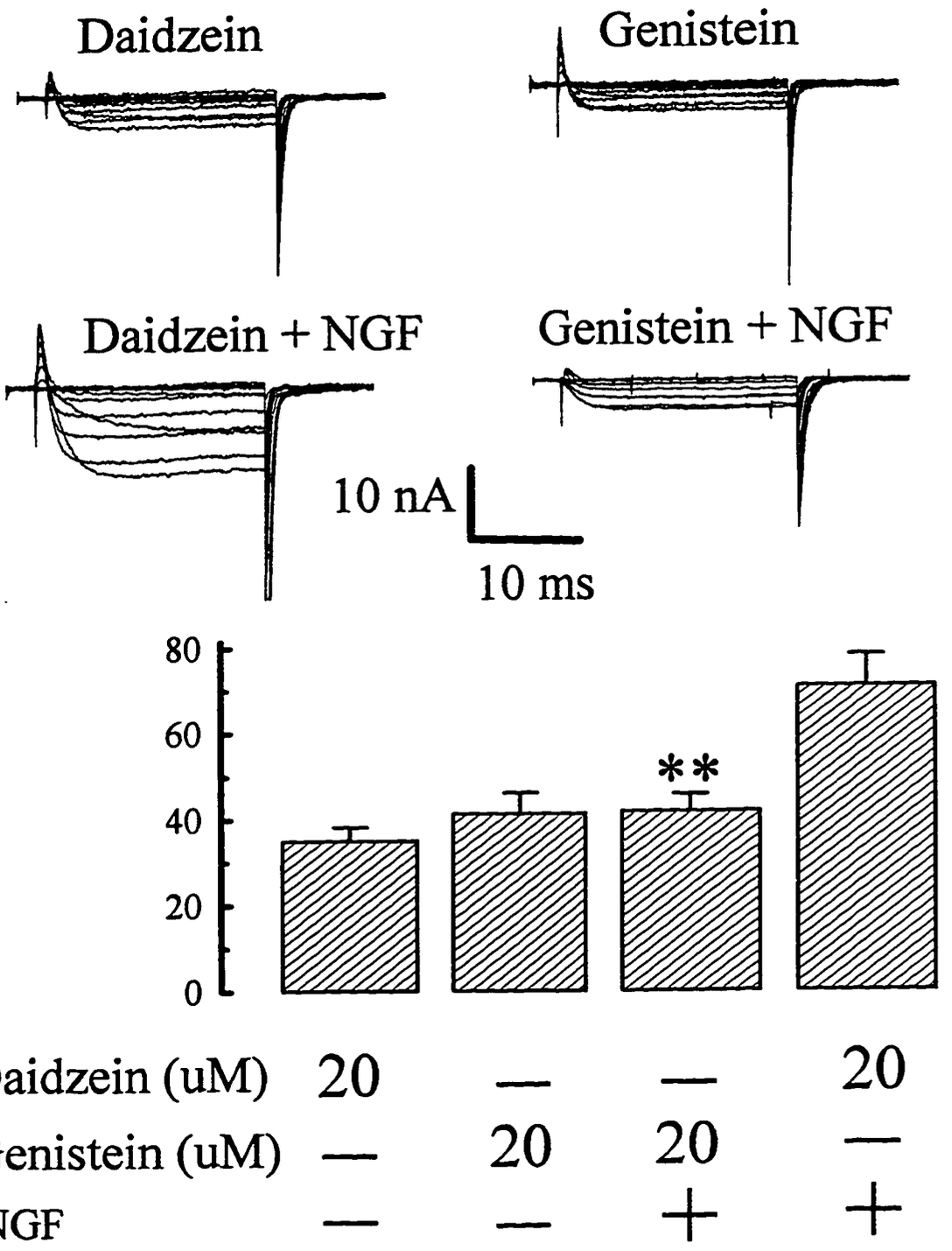


Figure 3-2. Extracellular Ca^{2+} was not required for NGF response. Cells were cultured in Ca^{2+}-free medium (the $[Ca^{2+}]_o$ was omitted and 2 mM EGTA was added into the medium). NGF still increased Ca^{2+} current density in the Ca^{2+}-free medium.

Figure 3-3. Enhancement of Ca^{2+} current density can be blocked by tyrosine kinase inhibitor, genistein. Upper panel, Ca^{2+} channel currents; Lower panel, histogram of the current density. ** $p < 0.01$ compared with the cells treated with both daidzein and NGF. Genistein at the concentration of 20 μM blocked NGF response, but it had no effects on the basal Ca^{2+} current density.

(Figure 3-3)


Daidzein
Genistein
Daidzein + NGF
Genistein + NGF
10 nA
10 ms
80
60
40
20
0
**
Daidzein (uM) 20 — — 20
Genistein (uM) — 20 20 —
NGF — — + +

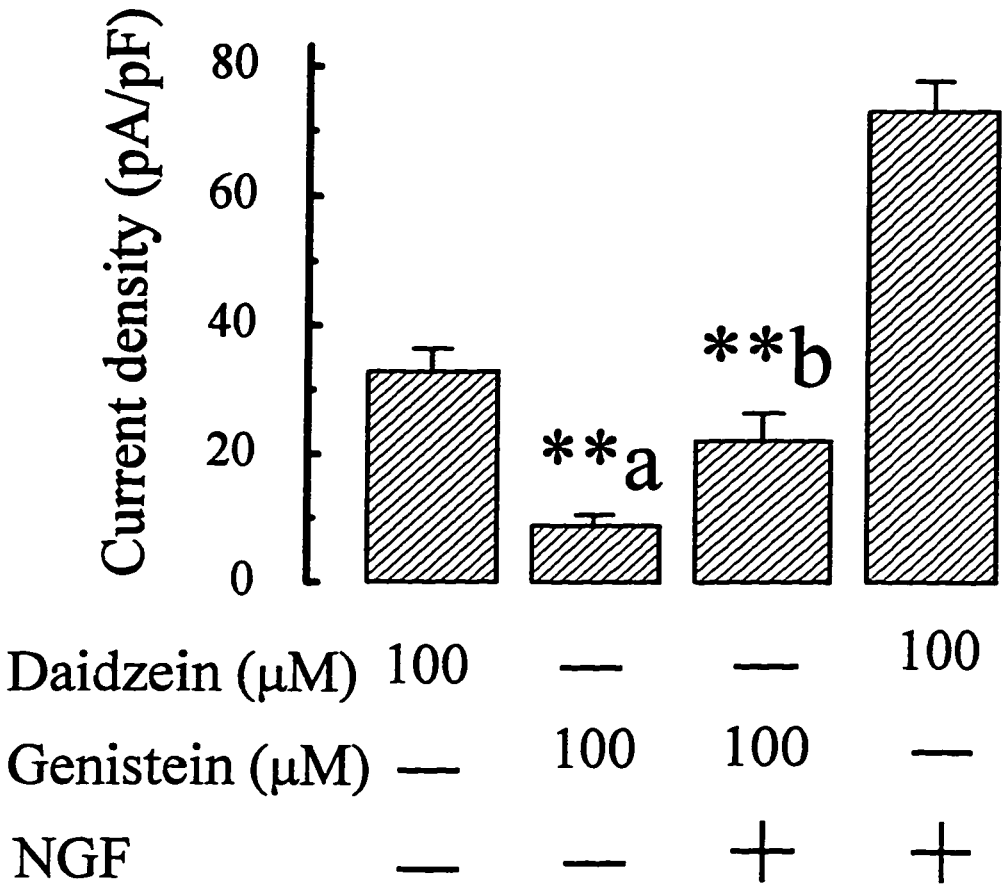


Figure 3-4. Genistein at the concentration of 100 μM inhibited both basal current density and the enhancement of the current density induced by NGF. **a $p < 0.01$ compared with the current density of the cells treated with daidzein alone; **b $p < 0.01$ compared with that of the cells treated with both daidzein and NGF.

Figure 3-5. Lavendustin A, another tyrosine kinase inhibitor, blocked the enhancement of Ca^{2+} channel current density at the concentration of 1 μM and inhibited the basal current density as well at the concentration of 10 μM. Upper panel, *left*, Ca^{2+} current from a cell treated with DMSO alone and a cell treated with DMSO plus NGF; *right*, Ca^{2+} current from a cell treated with 1 μM lavendustin A and a cell treated with 1 μM lavendustin A plus NGF. Lower panel, histogram of the current density. **a, **c, $p<0.01$ compared with the current density of the cells treated with DMSO plus NGF; **b $p<0.01$ compared with the current density of the cells treated with DMSO alone.

(Figure 3-5)

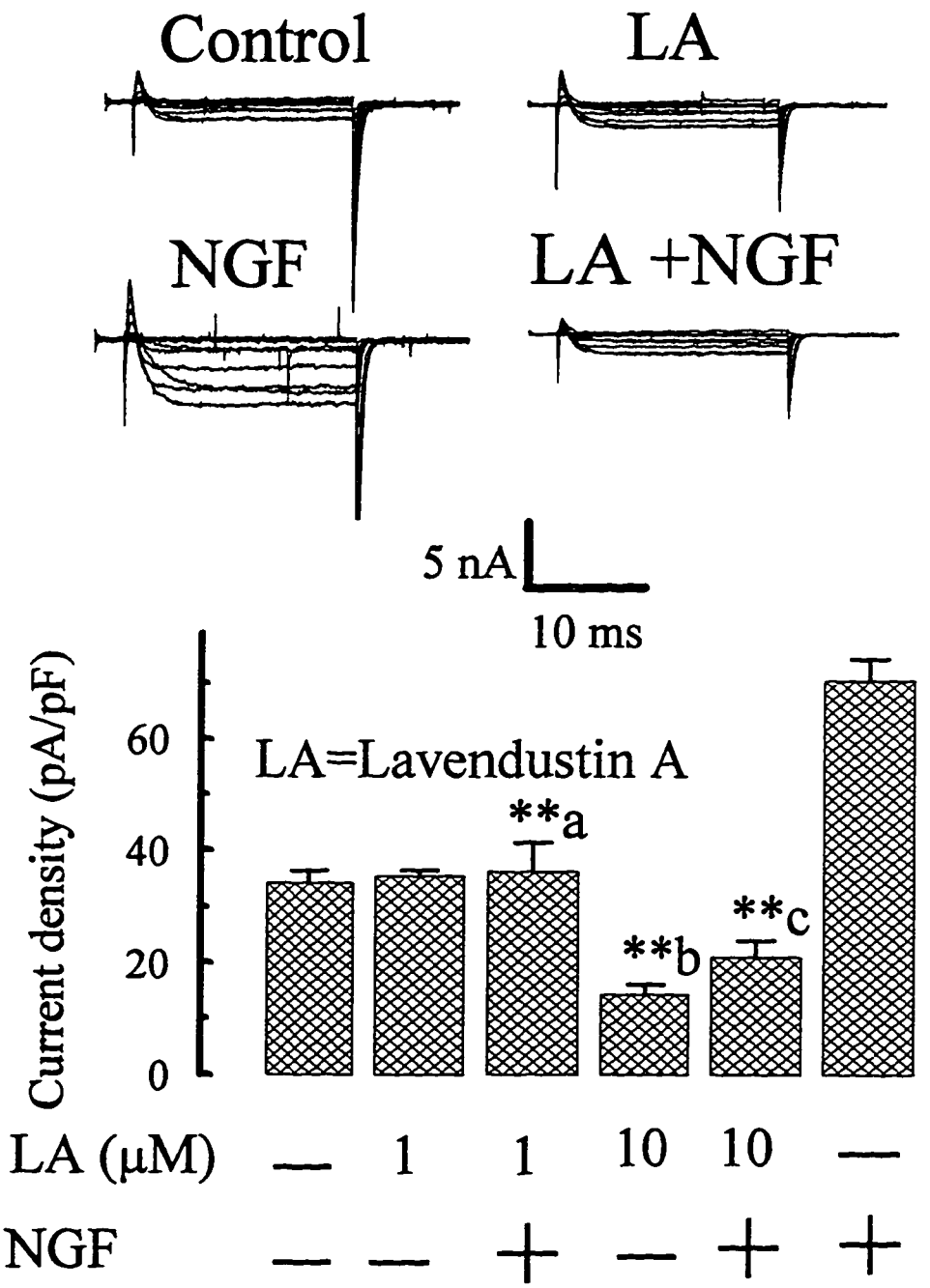

Control
LA
NGF
LA +NGF
5 nA
10 ms
LA=Lavendustin A
Current density (pA/pF)
0
20
40
60
**a
**b
**c
LA (μM) — 1 1 10 10 —
NGF — — + — + +

Table 3-1. Comparison of the capacitance and absolute currents for some cells

	Cin (pF)	Current (nA)	N
DMSO	172.768±8.126	5.119±0.313	112
DMSO+NGF	163.402±5.873	11.346±0.636	107
Cordycepin (20 μM)	107.05±9.429**a	3.227±0.333**a	20
Cordycepin (20 μM) + NGF	109.25±10.094**b	3.845±0.309**b	20
Actinomycin D (0.01 μg/ml)	109.05±10.72**c	4.021±0.391	20
Actinomycin D (0.01 μg/ml) + NGF	125.05±9.548**d	4.381±0.416**d	20
Daidzein (100 μM)	177.857±16.521	5.227±0.542	28
Genistein (100 μM)	115.28±7.325**e	1.121±0.224**e	25
Daidzein (100 μM) + NGF	166.039±11.615	11.613±0.849	26
Genistein (100 μM) + NGF	132.52±8.403*	2.599±0.384**h	25
Lavendustin A (10 μM)	109.95±9.846**f	1.425±0.166**f	20
Lavendustin A (10 μM) + NGF	124.8±11.695**g	2.578±0.403**g	20

**a,c,f $p<0.01$ compared with cells treated with DMSO alone

**b,d,g $p<0.01$ compared with cells treated with DMSO plus NGF

**e $p<0.01$ compared with cells treated with Daizein alone

**h $p<0.01$ compared with cells treated with daidzein plus NGF

* $p<0.05$ compared with cells treated with daidzein plus NGF

Analysis of the capacitance of the cells (Table 3-1) together with microscopic observation indicated that at higher dose, both genistein (100 μM) and lavendustin A (10 μM)

inhibited the outgrowth of neurites. Capacitance was 35% smaller for cells treated with 100 μM genistein compared with cells treated with the same concentration of daidzein (115.28±7.325 pF, n=25 vs 177.857±16.521 pF, n=28; $p<0.01$). In the presence of NGF, genistein reduced capacitance by 20% (132.52±8.403 pF, n=25 vs 166.039±11.615 pF, n=26; $p<0.05$). Capacitance was reduced by 36% in the presence of 10 μM lavendustin A alone (from 172.768±8.126 pF, n=112 to 109.95±9.845 pF, n=20; $p<0.01$) and by 24% in the presence of both lavendustin A and NGF (from 163.402±5.873 pF, n=107 to 124.8±11.695 pF, n=20; $p<0.01$), respectively. These results suggested that tyrosine kinase activity was required for both Ca^{2+} channel induction and neurite outgrowth.

NGF response is related to the activation of Trk A

Since the intracellular, non-receptor tyrosine kinases may be involved in the regulation of Ca^{2+} channels by NGF, the results from the tyrosine kinase inhibitors cannot distinguish between the tyrosine kinase activity from Trk A receptor and that from the intracellular tyrosine kinase or both. Anti-trk antibody which was a rabbit Ig-G raised against mouse Trk extracellular epitopes was therefore used to see whether it could affect the effects of NGF. Ig-G form of the Trk antibody has been reported to mimic the action of NGF on neuronal survival (Clary *et al.*, 1994; Lefcort *et al.*, 1996). To test whether the antibody was effective in our system, explant cultures of BFSG were prepared according to the methods described by Traynor *et al.* (1992). Unlike single, dissociated BFSG neurons in defined medium, addition of NGF to these explants (in serum-rich medium) induces a robust growth response (Kelly *et al.*, 1989). Figure 3-6 shows photomicrographs of control explants treated with 20

Figure 3-6. Photomicrographs of the explant culture. The basic methods used for the culture of BFSG explants were essentially the same as Traynor *et al* (1992). A, Explant cultured in the presence of negative control Ig-G antibody (20 μg/ml) for 15 days. B, Explant cultured in the presence of anti-Trk Ig-G antibody (20 μg/ml) for 15 days. Anti-Trk Ig-G antibody significantly increased the neurite outgrowth.

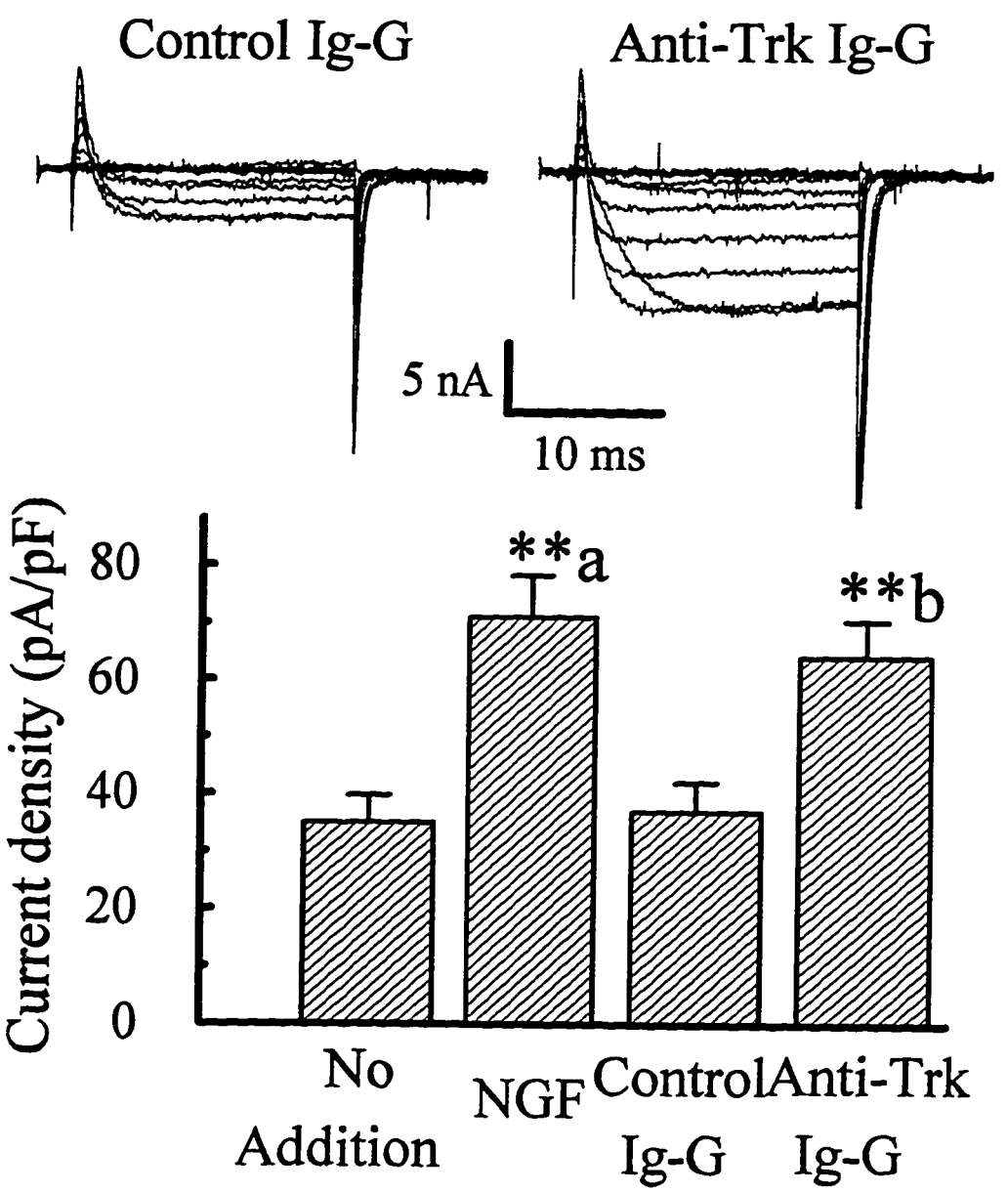
(Figure 3-6)

A. Control Ig-G antibody

B. Anti-Trk Ig-G antibody

Figure 3-7. Anti-Trk Ig-G enhanced Ca^{2+} current and current density. Upper panel, Ca^{2+} current from a cell treated with negative control Ig-G antibody and a cell treated with anti-Trk Ig-G antibody. Lower panel, histogram of the current density. **a, $p<0.01$ compared with the current density of control cells; **b, $p<0.01$ compared with the current density of the cells treated with negative control Ig-G antibody.

(Figure 3-7)


Control Ig-G
Anti-Trk Ig-G
5 nA
10 ms
Current density (pA/pF)
80
60
40
20
0
**a
**b
No Addition
NGF
Control Ig-G
Anti-Trk Ig-G

μg/ml control Ig-G antibody and explants treated with 20 μg/ml Ig-G form of anti-Trk antibody. The antibody enhanced the outgrowth of neurites and therefore mimics the action of NGF. Anti-trk Ig-G (20 μg/ml) also enhanced the Ca^{2+} current density by 72% (from 37.4 ± 4.8 pA/pF, n=20 for cells cultured with the control Ig-G antibody to 64.4 ± 5.9 pA/pF, n=21, for cells cultured with anti-trk Ig-G; $p<0.01$), which was comparable to the effect of NGF (Figure 3-7). The possible explanation for this phenomenon is the double-armed-structure of Ig-G which may cross-link two Trk A monomers together to mediate receptor dimerization. Dimerization of Trk A receptors initiates the activation of the receptors. These results suggest that activation of the Trk receptors can lead to the induction of Ca^{2+} channels.

In line with the results from NGF, anti-trk Ig-G antibody did not influence the capacitance of the cells. There was no significant difference between the capacitance of the cells treated with Trk-Ig-G antibody (177.5 ± 10.3 pF, n=21) and that of the cells treated with the control Ig-G antibody (166.8 ± 12.3 pF, n=20; $p>0.05$). It seems anomalous that NGF or anti-Trk Ig-G antibody increased the neurite outgrowth in explant culture, but they failed to increase neurite outgrowth in dissociated cells judged by cell capacitance. Our explanation for this discrepancy is that capacitance from long neurites cannot be detected by the whole-cell patch-clamp recording because of the space clamp problem. Since only cells which have relatively short processes can be properly voltage-clamped, the capacitances obtained are likely to be restricted to the cell population which has shorter neurites. For the cells which have longer processes, they were discarded because of the space-clamp problem. If those cells represented the typical response of NGF or anti-Trk Ig-G on neurite outgrowth, capacitances might not have reflected the effects of NGF or anti-Trk Ig-G on neurite

outgrowth reliably. We, therefore, simply conclude that anti-Trk Ig-G did not increase the neurite outgrowth judged by the cell capacitance.

Anti-p75 antibody did not block NGF response

A possible role for p75 in NGF-mediated Ca^{2+} channel induction was also examined using p75 monoclonal antibody directed against the extracellular domain of the p75 NGF receptor. The cells were pretreated for half hour with the culture medium containing 20 μg/ml anti-p75 antibody which has been shown to block the binding of NGF to p75 receptors (Cortazzo *et al.*, 1996; Huber and Chao, 1995). The medium was then replaced with medium containing the same concentration of anti-p75 antibody plus NGF and cultured for 6-9 days. In the presence of anti-p75 antibody, NGF was still able to increase current density (Figure 3-8), suggesting that this effect of NGF on Ca^{2+} channel current is independent of p75 receptors.

Treatment of the cells with p75 antibody did not influence cell capacitance either. Capacitance of the cells treated with p75 antibody alone was 170.0 ± 15.0 pF (n=21), which was not significantly different from that of the cells treated with control Ig-G antibody (166.8 ± 12.3 pF, n=20, $p > 0.05$). There was no significant difference between the capacitance of the cells treated with p75 antibody plus NGF (171.4 ± 10.5 pF, n=217) and that of the cells cultured with control Ig-G antibody plus NGF (172.8 ± 10.0 pF, n=21, $p > 0.05$).

PI3K inhibitors did not block NGF response

The role of PI3K in NGF-mediated Ca^{2+} channel regulation was also investigated

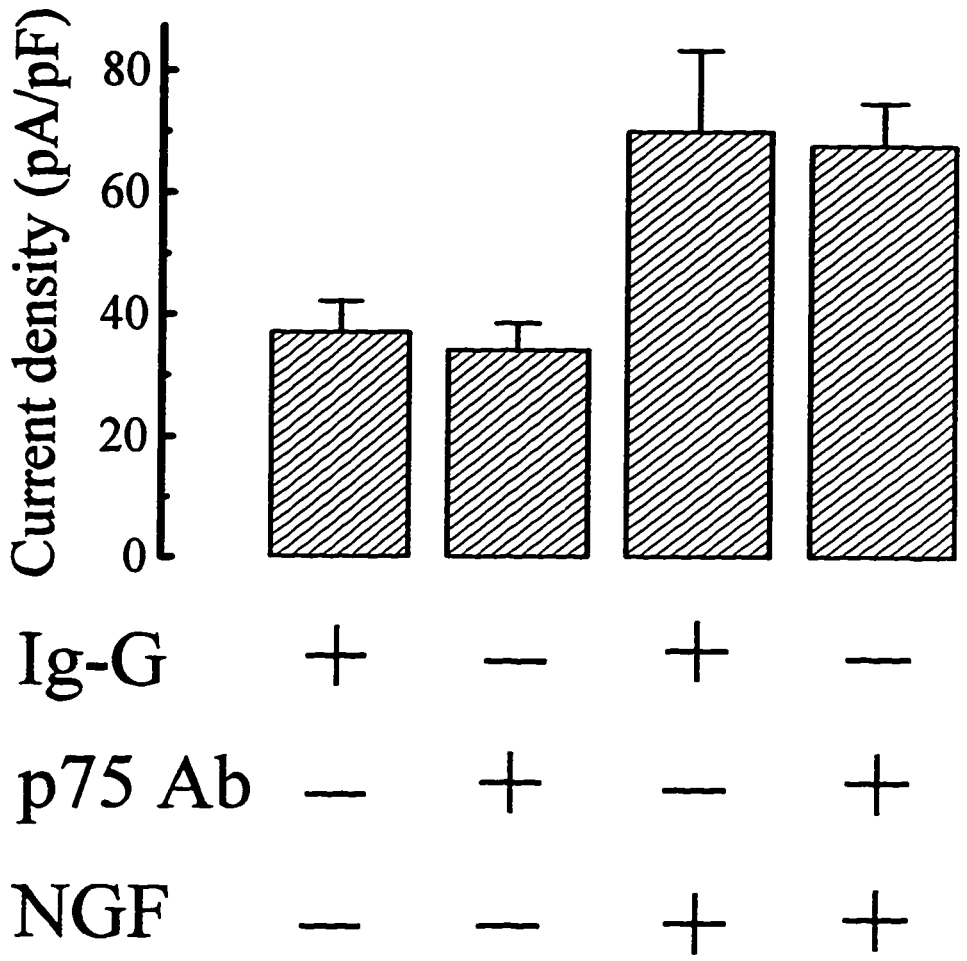


Figure 3-8. Inclusion of anti-p75 antibody (20 μg/ml) in the culture medium did not affect the effect of NGF on Ca^{2+} channel current density. Same concentration of negative control Ig-G antibody (against Aspergillus niger glucose oxidase) was used as a control.

using its two specific inhibitors, wortmannin and LY294002. Wortmannin, a highly potent PI3K inhibitor, acts by binding to the p110 subunit of the enzyme (Yano *et al.*, 1993). LY294002, on the other hand, behaves as a competitive inhibitor for the ATP binding site of PI3K and abolishes PI3K activity both *in vivo* and *in vitro* at low micromolar concentrations but has no inhibitory effect against phosphatidyl-inositol-4-kinase (PI4K) nor a number of intracellular serine/threonine or tyrosine kinases (Vlahos *et al.*, 1994). Both wortmannin (100 nM) and LY294002 (100 μM) were unable to block the effects of NGF on Ca^{2+} current density (Figure 3-9), even though at this concentration both inhibitors inhibited PI3K activity completely (Cheatham *et al.*, 1994; Kimura *et al.*, 1994; Vlahos *et al.*, 1994). These results suggest that activity of PI3K is not necessary for the induction of Ca^{2+} channel currents by NGF.

Input capacitance measurements together with microscopic observation indicated that PI3K inhibitors did not affect neurite outgrowth. This contrasts with the results of Kimura *et al.* (1994) where continuous inhibition of PI3K by wortmannin decreased neurite growth in PC12 cells. Capacitance of the cells treated with 100 nM wortmannin and 100 μM LY294002 was 168.3 ± 18.5 pF (n=26) and 165.0 ± 16.0 pF (n=24), respectively, which was not significantly different from that of the cells cultured with DMSO alone (172.8 ± 8.1 pF, n=112, $p > 0.05$). Inclusion of NGF in the culture medium did not change the capacitance either; the capacitance of the cells cultured with wortmannin plus NGF and LY294002 plus NGF was 162.1 ± 12.8 pF (n=22) and 166.9 ± 10.1 pF (n=30), respectively, which was not significantly different from that of the cells treated with DMSO plus NGF (163.4 ± 5.9 pF, n=107, $p > 0.05$).

Figure 3-9. Lack of effects of PI3K inhibitors, wortmannin and LY294002, on NGF-mediated enhancement of Ca^{2+} channel current density.

(Figure 3-9)

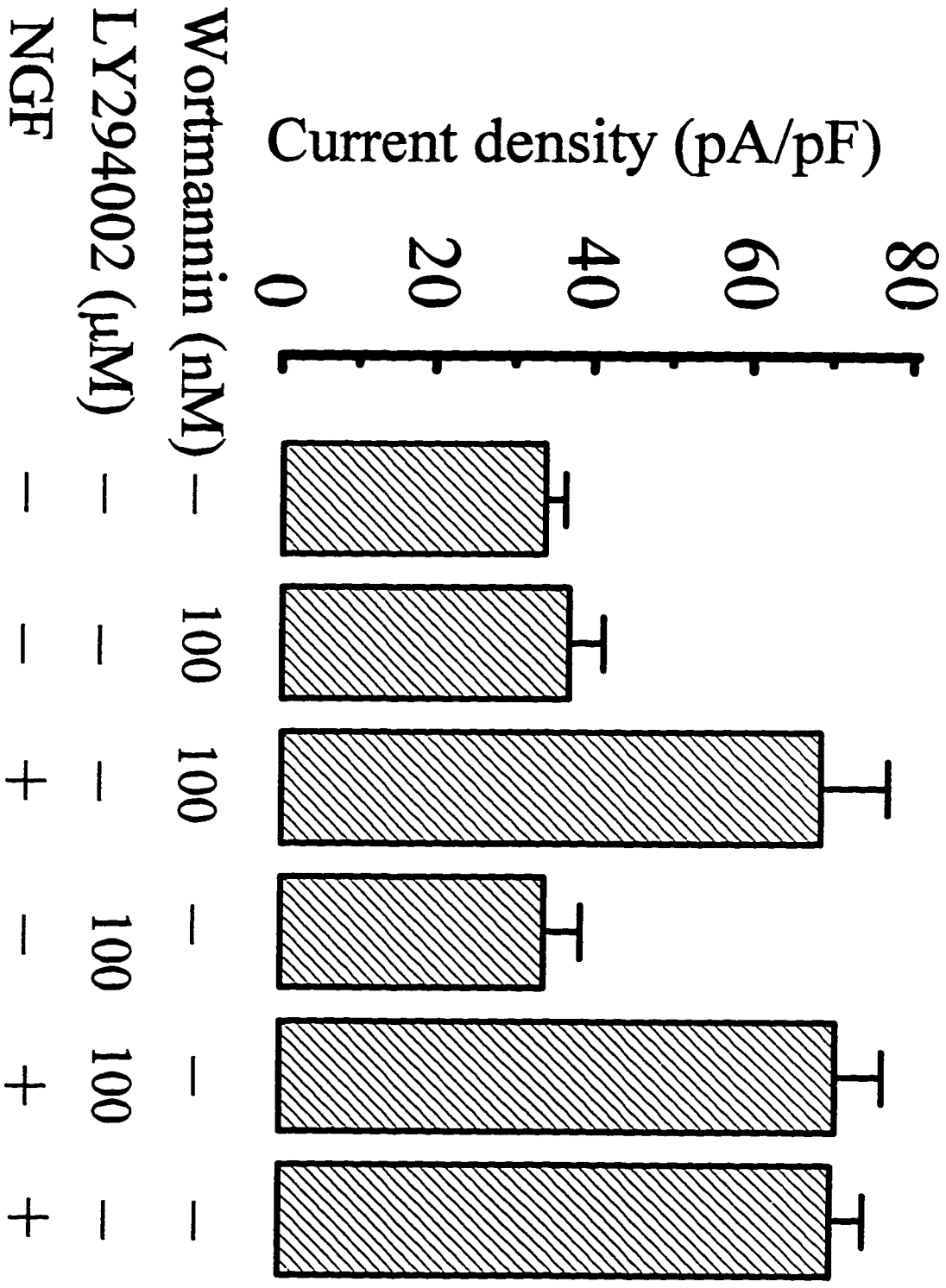
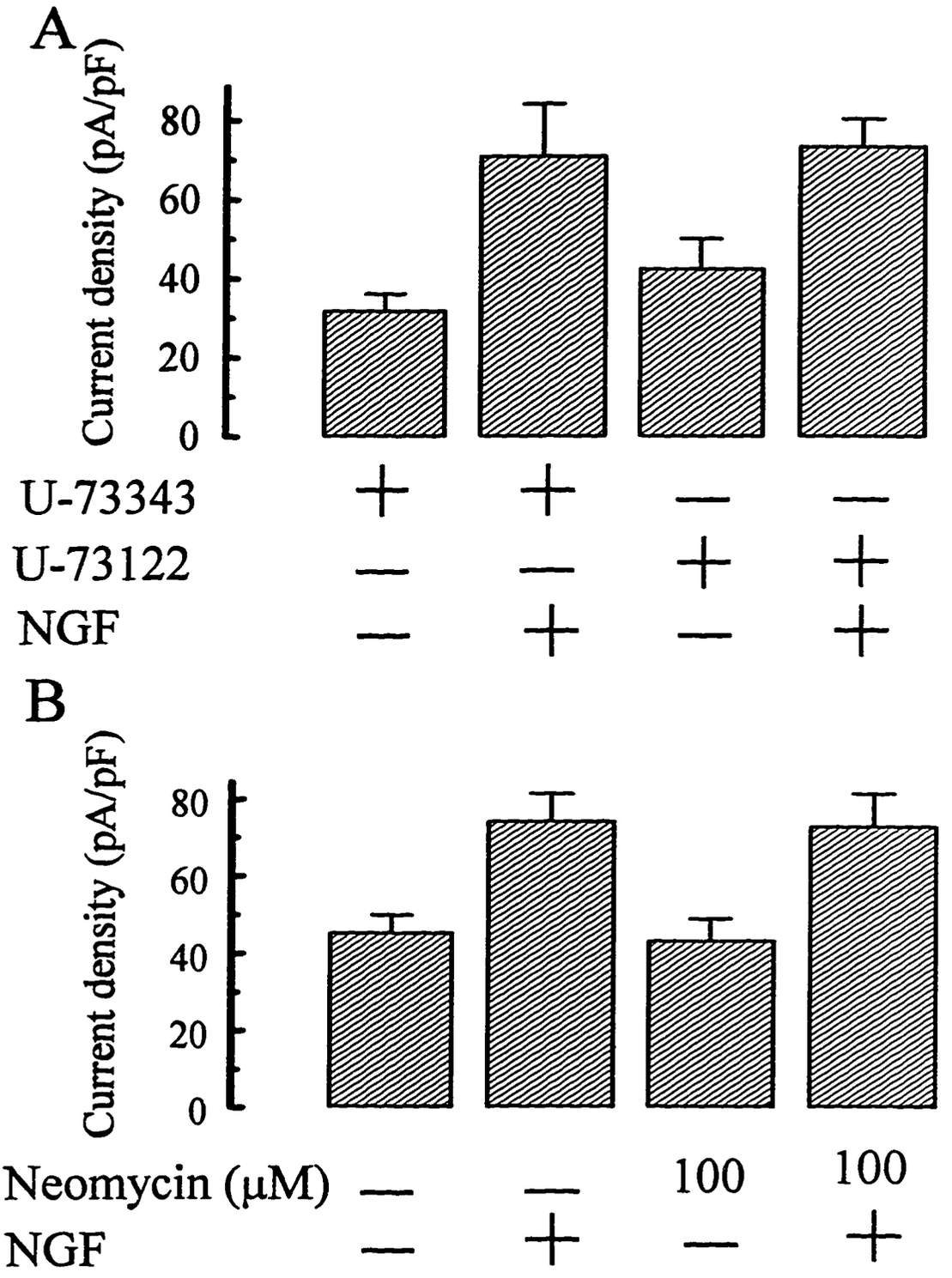

Current density (pA/pF)
0
20
40
60
80
Wortmannin (nM)
LY294002 (μM)
NGF
–
100
100
–
100
–
100
–
–
–
–
–
+
–
–
+
–
+

Figure 3-10. Lack of effects of PLC-γ inhibitor, U-73122 (A) and neomycin (B) on NGF-induced enhancement of Ca^{2+} current density. A, There is no significant difference between the current density of the cells treated with U73122 plus NGF and that of the cells treated with the inactive analogue (U-73343) plus NGF. B, At either 10 or 100 μM, neomycin failed to block the effects of NGF on Ca^{2+} current density.

(Figure 3-10)


A
Current density (pA/pF)
80
60
40
20
0
U-73343
U-73122
NGF
B
Current density (pA/pF)
80
60
40
20
0
Neomycin (μM)
100
100
NGF

PLC-γ inhibitors did not block NGF response

Two PLC-γ inhibitors, U-73122 and neomycin, were used to test whether activity of PLC-γ was involved in the induction of Ca^{2+} channel currents by NGF. U-73122 together with its control inactive enantiomer, U-73343, has been considered to be a useful tool to investigate the involvement of PLC in signal transduction (Smith *et al.*, 1990; Bleassdale *et al.*, 1990; Thompson *et al.*, 1991; Yule and Williams, 1992). At a concentration of 100 μM (the highest concentration tried), U-73122 did not attenuate the effects of NGF (Figure 3-10A). Neomycin, a polycationic aminoglycoside antibiotic, binds strongly to phosphatidyl-inositol-4,5-bisphosphate (PIP_2) (Lodhi *et al.*, 1976; Lang *et al.*, 1977) and prevents its further metabolism (Schacht, 1976; Schibeci and Schacht, 1977; Downes and Michell, 1981). PLC-mediated inositol phospholipid turnover is selectively inhibited by neomycin (Griffin *et al.*, 1980; Carney *et al.*, 1985; Gabev *et al.*, 1989). Inclusion of neomycin in the culture medium (100 μM, the highest concentration tested) failed to affect the NGF-mediated enhancement of Ca^{2+} current density (Figure 3-10B). These results suggested that the effects of NGF on Ca^{2+} channels were not mediated via the activation of PLC-γ.

Inhibitors of posttranslational modification of Ras blocked NGF response

Ras proteins are synthesized as cytosolic precursors and must undergo posttranslational modification at a region of their C-terminal called a CAAX motif (C, cysteine; A, aliphatic amino acid; and X, any amino acid) before they become biologically functional. These modifications include farnesylation at the cysteine residue mediated by farnesyl-protein transferase (FPTase), followed by proteolytic cleavage of the amino acids

AAX, and methyl esterification of the new C-terminal cysteine. These modification are essential for anchoring Ras proteins to the plasma membrane (Willumsen *et al.*, 1984; Hancock *et al.*, 1990) and for a number of biological activities of Ras: malignant transformation of NIH 3T3 cells (Willumsen *et al.*, 1984; Hancock *et al.*, 1990); induction of neuronal differentiation of PC12 cells (Qui *et al.*, 1991) and induction of germinal vesicle breakdown in Xenopus laevis oocytes (Schater *et al.*, 1989) by activated Ras. The activity of H-Ras to activate Raf-1 *in vivo* was also reported to be dependent on the modification (Kikuchi and Williams, 1994).

Farnesylation appears to be the key step in the posttranslational modification of Ras. The substrates used by FPTase are farnesyl diphosphate, the farnesyl moiety of the cholesterol biosynthetic intermediate, and CAAX tetrapeptide of the Ras precursor. This reaction can be hampered by one of the following methods: 1) depletion of the farnesyl diphosphates; 2) inhibition of FPTase by specific inhibitors (Kohl *et al.*, 1993; Gibbs *et al.*, 1994) and 3) modification of the CAAX structure by providing pseudosubstrates for the enzyme (James *et al.*, 1993). In our experiments, we used two substances, α-hydroxyfarnesylphosphonic acid (α-HFA) which is a specific FPTase inhibitor, acting by competition with the farnesyl diphosphate substrate both *in vitro* (Pompliano *et al.*, 1992) and *in vivo* (Gibbs *et al.*, 1993; for a review see Gibbs *et al.*, 1994), and perillic acid (PA) which is thought to interfere with the metabolism of mevalonate pathway (the pathway leading to the production of the farnesyl diphosphate; Crowell *et al.*, 1991) to interfere the posttranslational processes of Ras. PA has been shown to be effective in inhibiting Ras-induced Ca^{2+}-activated K^+ channel expression in balb 3T3 and NIH 3T3 cells (Huang and Rane, 1994).

Inclusion of α-HFA in the culture medium at either 10 or 100 μM did not affect the basic Ca^{2+} channel currents, but it counterbalanced NGF-induced Ca^{2+} channel induction at both concentrations (Figure 3-11), suggesting that function of Ras is required for the induction of Ca^{2+} currents by NGF.

In contrast to the results from transcriptional and tyrosine kinase inhibitors, treatment of the cells with α-HFA failed to influence the neurite outgrowth. This was further confirmed by capacitance analysis. The capacitance was 169.3 ± 16.8 pF (n=25) and 162.9 ± 13.4 pF (n=20) for the cells treated with 10 or 100 μM α-HFA alone and 165.8 ± 14.8 pF (n=22) and 165.8 ± 15.6 pF (n=20) for the cells treated with 10 or 100 μM α-HFA plus NGF, respectively, which were not significantly different from that of the cells treated with DMSO alone (172.8 ± 8.1 pF, n=112; $p>0.05$) or that of the cells treated with DMSO plus NGF (163.4 ± 5.9 pF, n=107; $p>0.05$).

In line with the results of α-HFA, addition of PA at the concentration of either 0.1 mM or 1 mM into the culture medium also blocked the effects of NGF on Ca^{2+} channels (Figure 3-12), but the capacitance was not changed by PA (158.4 ± 16.3 pF, n=20, for cells treated with 0.1 mM PA; 153.2 ± 11.2 pF, n=20, for cells treated with 1 mM PA; vs 172.8 ± 8.2 pF, n=112, for cells treated with DMSO; $p>0.05$) nor PA plus NGF (162.6 ± 19.5 pF, n=20, for cells treated with 0.1 mM PA plus NGF; 148.9 ± 18.0 pF, n=20, for cells treated with 1 mM PA plus NGF; vs 163.4 ± 5.9 pF, n=107, for cells treated with DMSO plus NGF; $p>0.05$).

Figure 3-11. α-Hydroxyfarnesylphosphonic acid (α-HFA) blocked the effects of NGF on Ca^{2+} current density at both 10 and 100 μM. Upper panel, Ca^{2+} currents. α-HFA concentration in the medium is 100 μM. Lower panel, histogram of current density, ** $p < 0.01$ compared with the current density of the cells treated with DMSO plus NGF.

(Figure 3-11)

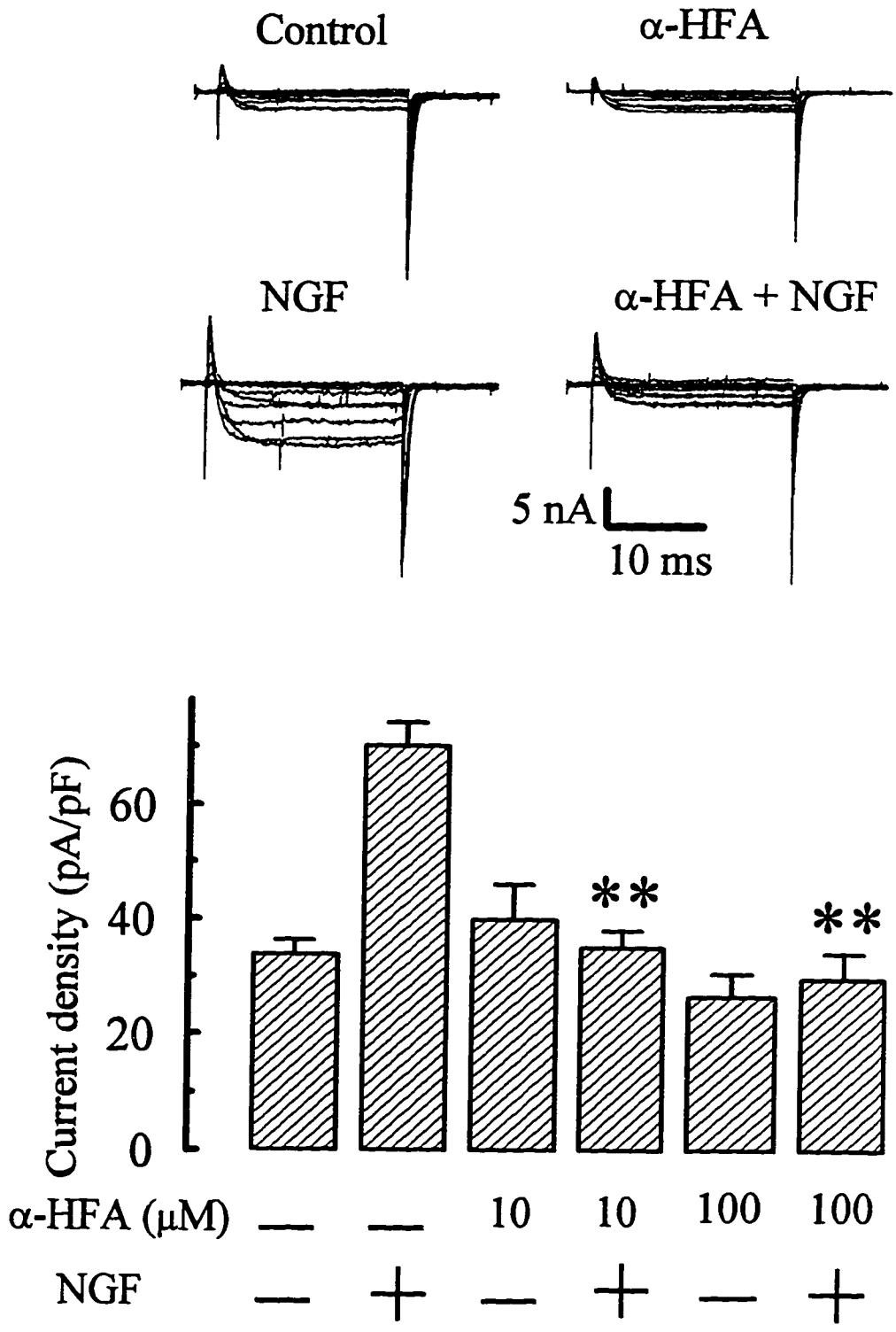

Control
α-HFA
NGF
α-HFA + NGF
5 nA
10 ms
Current density (pA/pF)
0
20
40
60
**
**
α-HFA (μM)
—
—
10
10
100
100
NGF
—
+
—
+
—
+

Figure 3-12. Perrilic acid (PA), another Ras posttranslational inhibitor, blocked the effects of NGF on Ca^{2+} current density at both 0.1 and 1 mM. Upper panel, recordings of Ca^{2+} currents. PA concentration in the culture medium was 1 mM. Lower panel, histogram of the current density. ** $p < 0.01$ compared with the current density of the cells treated with DMSO plus NGF.

(Figure 3-12)

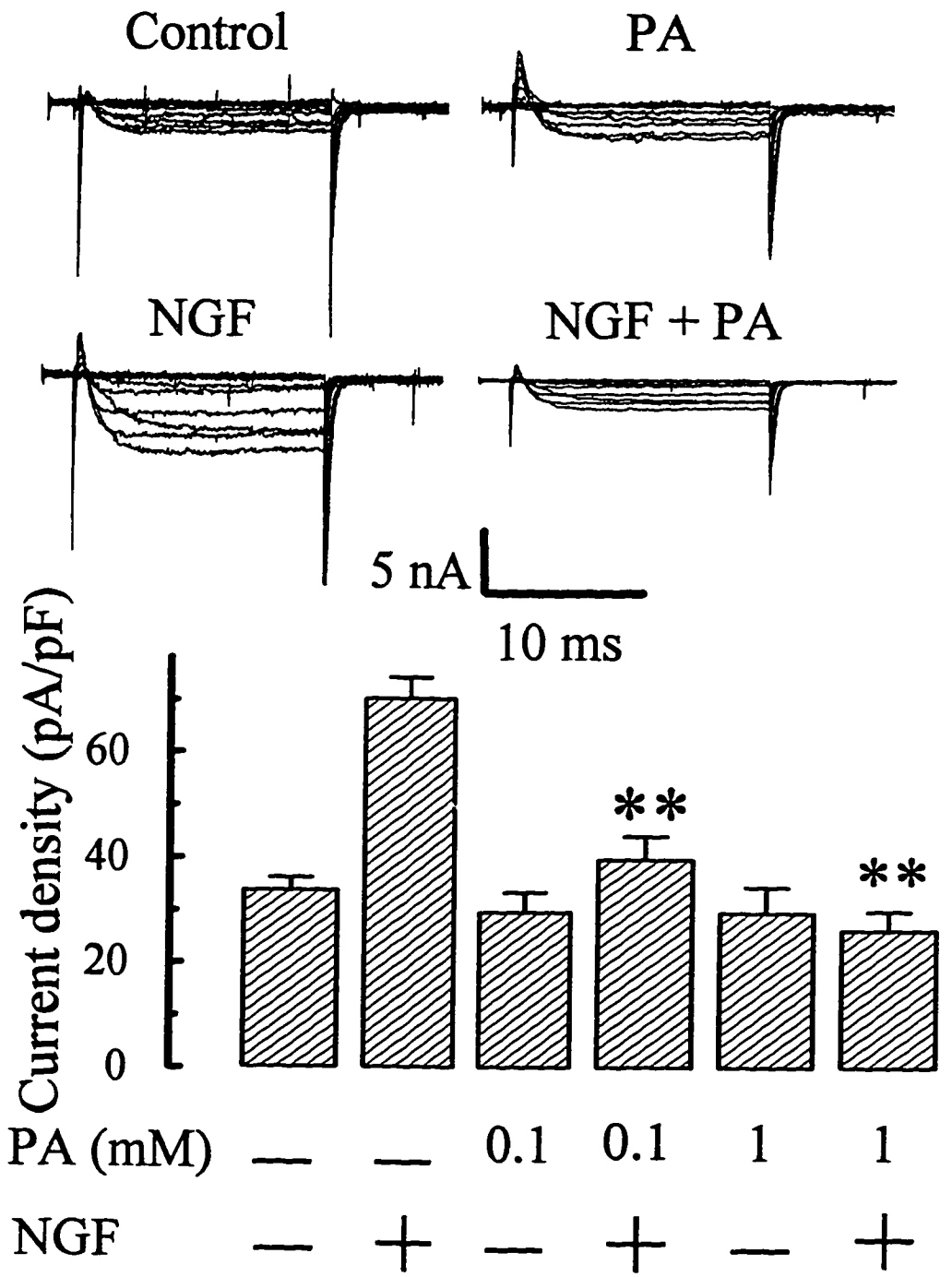
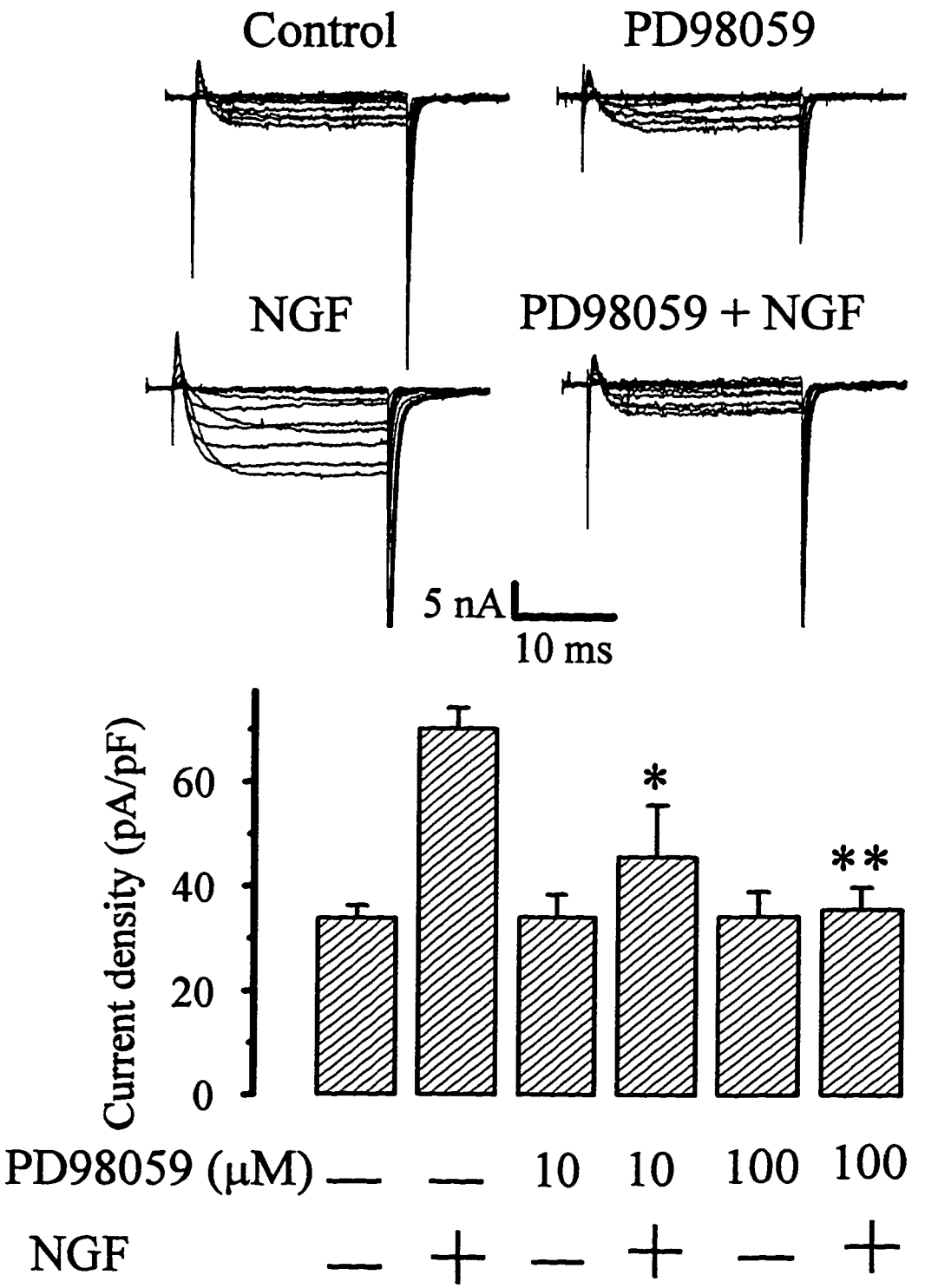

Control
PA
NGF
NGF + PA
5 nA
10 ms
Current density (pA/pF)
0
20
40
60
**
**
PA (mM)
—
—
0.1
0.1
1
1
NGF
—
+
—
+
—
+

Figure 3-13. PD98059, a specific MAPKK inhibitor, blocked the effects of NGF on Ca^{2+} current density at both 10 and 100 μM. Upper panel, recordings of Ca^{2+} currents. PD98059 in the culture medium was 100 μM. Lower panel, histogram of the current density, ** $p < 0.01$ compared with the current density of the cells treated with DMSO plus NGF.

(Figure 3-13)


Control
PD98059
NGF
PD98059 + NGF
5 nA
10 ms
Current density (pA/pF)
0
20
40
60
*
**
PD98059 (μM) — — 10 10 100 100
NGF — + — + — +

MAPKK inhibitor blocked the effect of NGF on Ca^{2+} channels

MAPKK, a down-stream target of Ras was also examined to see whether it is involved in NGF-mediated Ca^{2+} channel induction. PD98059 has been used effectively to block the *in vivo* activation of MAP kinase activity, induced by a variety of agents including platelet-derived growth factor (Dudley *et al.*, 1995), NGF (Pang *et al.*, 1995) and insulin (Lazar *et al.*, 1995). The high degree of specificity of PD98059 *in vitro* and *in vivo* is supported by its failure to inhibit a variety of protein kinases (18 protein Serine/Threonine kinases and 4 protein Tyrosine kinases, Alessi *et al.*, 1995; Dudley *et al.*, 1995). Moreover, it does not prevent the *in vivo* activation of Raf or the activation of other MAPKK and MAP kinase homologous, such as *c-Jun* kinase or p38 (Alessi *et al.*, 1995). Kinetic analysis has indicated that PD98059 inhibits MAPKK activity in a manner which is not competitive with either substrate (MAPK) or ATP binding and has no effect on MAPK itself, suggesting that it most likely inhibits MAPKK through an allosteric mechanism (Dudley *et al.*, 1995). Furthermore, NGF-, EGF- and PDGF-receptor tyrosine autophosphorylation is completely insensitive to PD98059 pretreatment of cells (Lin *et al.*, 1995).

To evaluate its role in NGF-mediated Ca^{2+} channel induction, the cells were treated with 10 or 100 μM PD98059 alone or plus NGF. The increase in the Ca^{2+} channel current density attributed to NGF was reduced in a dose-related manner (Figure 3-13), suggesting that MAPKK was implicated in the regulation of Ca^{2+} channel currents by NGF. However, capacitance was not changed by treatment of PD98059 alone (178.1 ± 19.3 pF, n=20 and 166.5 ± 16.7 pF, n=20, for cells treated with 10 and 100 μM PD98059, respectively; vs 172.8 ± 8.1 pF, n=112, for cells treated with DMSO; $p>0.05$) nor by PD98059 plus NGF (188.5

± 22.6 pF, n=20 and 161.6 ± 9.3 pF, n=20, for cells treated with 10 and 100 μM PD98059 plus NGF, respectively; vs 163.4 ± 5.9 pF, n=107 for cells treated DMSO plus NGF; $p>0.05$).

Discussion

Previously, we have shown that NGF increases both N- and L-type Ca^{2+} currents, and attenuates the inactivation of Ca^{2+} channel currents. In this chapter, we have investigated the mechanisms underlying NGF-mediated increase of the Ca^{2+} channel currents. Because almost all the steady-state inactivation was removed when the cells were held at -80 mV, the present experiments addressed the signal transduction mechanisms underlying the induction of both N- and L-type Ca^{2+} currents by NGF. As our results show that this response was dependent on NGF-induced gene expression, the signal transduction mechanisms underlying NGF-mediated attenuation of inactivation may be different.

Two cell surface receptors have been identified for NGF. Trk A binds NGF with high affinity, whereas p75 binds this peptide with low affinity (Barbacid, 1993). Biological functions of NGF can be ascribed to binding to Trk A or p75 or to a co-operative interaction between the two. The co-operative relationship varies from cell line to cell line and the degree of co-operativity between p75 and Trk A is related to the relative abundance of these two receptors on the cell. Interaction between the two receptors appears to require a 3- to 10-fold excess of p75 (Verdi *et al.*, 1994; Greene and Kaplan, 1995), which is in contrast to the 100-fold excess on SH-SY5Y cells (Azar *et al.*, 1991). This kind of cooperative relationship has not been observed in NGF-mediated Ca^{2+} channel regulation, since treatment

of the cells with anti-Trk Ig-G mimicked the NGF response, but inclusion of the p75 antibody in the culture medium failed to reduce NGF-enhanced Ca^{2+} channel expression.

Our results indicate that Ras/MAPK-mediated signaling appears to be necessary for NGF-induced Ca^{2+} channel enhancement in sympathetic neurons. These results parallel those seen in PC12 cells where constitutive expression of the dominant negative Ras in PC12 cells blocked the growth factor-induced increase in Ca^{2+} channel currents (Pollock and Rane, 1996), but sustained activation of the $p21^{ras}$ signaling pathway failed to increase Ca^{2+} channel current densities, suggesting Ras signaling is necessary but not sufficient on its own to mediate Ca^{2+} channel induction by growth factors. The Ras pathway has also been implicated in the growth factor-mediated induction of potassium channel currents in fibroblast cell lines (Huang and Rane, 1994), but it is not required for growth factor-induced sodium channel expression (Fanger *et al.*, 1993; Pollock and Rane, 1996). This suggests that the expression of different channels is mediated by different regulatory pathways in response to growth factor stimulation.

Our results did not support a role attributed to PI3K, because NGF-mediated induction of Ca^{2+} channel current was not abrogated by two specific PI3K inhibitors, wortmannin and LY294002. The possibility that PI3K participated in the NGF-mediated Ca^{2+} channel regulation through the production of phosphoinositides was thus excluded. However, the catalytic p110 subunit has intrinsic protein serine/threonine kinase activity in addition to phosphoinositide kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). The only good protein substrate for PI3K presently known is the p85 subunit of PI3K, and phosphorylation of this subunit (which results in the inhibition of the lipid kinase activity) is orders of

magnitude slower than phosphorylation of phosphoinositides (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). Furthermore, the p85/p110 type PI3K has been shown to bind to a wide variety of molecules *in vivo* (e.g. protein-tyrosine kinases, Grb2, ras, tubulin) and it would potentially act as an adaptor protein to assemble signaling complexes that do not require the PI3K activity (Carpenter and Cantley, 1996). If this is the case, PI3K may simply be a helper in Ras-mediated NGF-induced Ca^{2+} channel expression or a down-stream target of Ras (Rodriguez-Viciana *et al.*, 1996).

Our results precluded a role for PLC-γ in NGF-mediated Ca^{2+} induction. This pathway has been implicated in signaling modulation of a voltage-activated potassium channel by peptide growth factor receptors expressed in *Xenopus* oocytes (Timpe and Fantl, 1994). More recently, NGF or NT-3 has been shown to activate Ca^{2+}-dependent potassium channels (BK channels) acutely in rat cortical neurons via PLC-γ activity (Holm *et al.*, 1997).

Effects of SNT pathway in NGF-mediated Ca^{2+} channel induction have not been probed in our experiments because no specific inhibitor for this pathway has been found yet. If this pathway is parallel to Ras, our experimental results indicate that this pathway is not necessary for NGF-mediated Ca^{2+} channel induction. More recently, SNT has been proposed to serve as a co-pathway to Ras in NGF-promoted neuritogenesis, since deletion of a conserved juxtamembrane sequence in the Trk receptor resulted in impaired neurite outgrowth and the mutated receptor mediated unimpaired tyrosine phosphorylation of the signaling proteins other than SNT (Peng *et al.*, 1995). If this is the case, the role of SNT in NGF-mediated Ca^{2+} channel induction still needs to be investigated.

All the evidence from our results indicate that Trk A is the receptor by which NGF

exerts its effects on Ca^{2+} channel induction. The downstream targets of Trk A involve Ras and MAPKK. The nucleus must be involved because gene transcription is required for NGF-mediated increase in Ca^{2+} channel currents. The interaction of MAPK with the nucleus leads to an increase in the synthesis of Ca^{2+} channel proteins. The whole-cell Ca^{2+} channel current is, therefore, increased by treatment with NGF.

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Chapter 4

Regulation of Sodium and Potassium Channel Currents by Nerve Growth Factor in Adult Bullfrog Sympathetic B-cells

Introduction

One of the features that distinguish neurons from the inexcitable types of cells is their capacity to create action potentials (a.p.) which are dependent on the properties of voltage-dependent ion channels, such as calcium (Ca^{2+}), sodium (Na^+) and various potassium (K^+) channels in the membrane. Generation, conduction of the a.p. and their patterns of frequency ultimately leads to the coding and transfer of information in the nervous system.

Nerve growth factor (NGF), a protypical member of neurotrophin family, induces differentiation of pheochromocytoma (PC12) cell line into sympathetic-like neurons accompanied by the appearance of excitability due to the insertion of Na^+ channels on the plasma membrane (Dichter *et al.*, 1977; Rudy *et al.*, 1982; 1987; Mandel *et al.*, 1988; Pollock *et al.*, 1990). In PC12 cells, NGF selectively induces the expression of brain type II Na^+ channels (Mandel *et al.*, 1988; Ginty *et al.*, 1992; Fanger *et al.*, 1993; 1995; D'Arcangelo *et al.*, 1993) via a Ras-independent pathway (Fanger *et al.*, 1993). Cyclic AMP has been proposed to be involved in the NGF-mediated Na^+ channel expression (Kalman *et al.*, 1990) by activating cAMP-dependent protein kinase at a posttranslational level (Ginty *et al.*, 1992). Recently, platelet-derived growth factor (PDGF)-mediated induction of Na^+ channels has been shown to involve multiple intracellular pathways including phosphatidyl-inositol-3-kinase (PI3K), phospholipase C-γ, GAP (for GTPase activating protein) and Syp (a tyrosine phosphatase) in PC12 cells (Fanger *et al.*, 1997). These experiments investigated the role of Na^+ channels induced by NGF in cell differentiation. Whether or how NGF regulates Na^+ channel properties in differentiated neurons is unknown.

Voltage-gated K^+ channels are important in the repolarization of a.p. and regulation

of firing frequency and excitability. Inconsistent results have been reported with regard to the effects of growth factors on K^+ channels. In PC12 cells, NGF failed to change K^+ channel currents measured by whole-cell patch-clamp recording (Garber *et al.*, 1989). However, a 4-fold increase in the level of kv2.1 (a delayed rectifier K^+ channel) polypeptide has been detected in NGF-treated PC12 cells, but the level of kv2.1 mRNA was not changed with NGF treatment (Sharma *et al.*, 1993). In addition, the expression of Ca^{2+}-activated K^+ channels increased in NGF-treated NIH3T3 and C3H10T1/2 cell lines through NGF-mediated activation of $p21^{ras}$ and its immediate downstream target, the Raf kinase (Huang and Rane, 1994). On the other hand, platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) acutely decreased the kv1.5 (a K^+ channel) currents in *Xenopus oocytes* transfected with kv1.5 gene together with PDGF or FGF receptors (Timple and Fantl, 1994). These disparate results suggest that the effects of growth factors on K^+ channels depend on cell type or growth factors regulate different K^+ channel subtypes in distinctive manners. Therefore, the functions of growth factors on K^+ channels in differentiated neurons must be examined.

Previously, we have shown that NGF increases the N-type and induces the expression of L-type Ca^{2+} channel currents and attenuates the inactivation of the Ca^{2+} currents in adult bullfrog sympathetic ganglion (BFSG) B-cells and the NGF response is mediated via Ras/MAP kinase pathway (see Chapter 2 and 3). It is unknown whether NGF influences Na^+ channels or various K^+ channels such as the large Ca^{2+}-activated K^+ channels (I_C), delayed rectifier K^+ channels (I_K), voltage- and time-dependent muscarine-sensitive K^+ channels (I_M) in BFSG B-cells. Therefore, in this chapter, we have examined the effects of NGF on Na^+ and various K^+ channels in adult BFSG B-cells.

Methods

Tissue culture

Tissue culture method was the same as described in "Methods" in Chapter 2. Neuron-enriched culture was used in all the experiments. The dissociated ganglion cells were preplated into two or three 35 mm culture dishes. After 1-2 h, most of the non-neuronal cells were adhered to the bottom of the dishes and the nonadherent cells which were primarily neurons were harvested, redistributed to 30 dishes and cultured in 3 ml/dish of culture medium containing 73% L-15 medium supplemented with 10 mM glucose, 1 mM $CaCl_2$, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μM cytosine arabinoside. For the culture groups with NGF, NGF was added to the culture medium to make a final concentration of 200 ng/ml. The dishes were placed in a humidified glass chamber and cultured at room temperature (22°C) for up to 15 days. The culture medium was changed daily.

Electrophysiology

Voltage-gated Na^+ and various K^+ channel currents were recorded by whole-cell patch-clamp recording using an Axoclamp 2A amplifier. To record Na^+ currents, the solution in the bath (external) contained (mM): NaCl, 97.5; TEA-Br, 20; $MnCl_2$, 4; Tris-Cl, 2.5 (pH7.2) and the solution inside the pipette (internal) contained (mM): CsCl, 103; NaCl, 9; TEA-Br, 5; Cs-Hepes, 2.5; Cs-EGTA, 1 (pH7.2; Jones, 1987). To study the Ca^{2+}-activated, voltage-dependent potassium current (I_c), the internal solution consisted of (mM): KCl, 110; NaCl, 10; $MgCl_2$, 2; $CaCl_2$, 0.4; EGTA, 4.4; Hepes, 5; D-glucose, 10; cyclic AMP, 0.125;

leupeptin, 0.1 (pH 7.2). Total outward current was initially recorded in an external solution containing (mM): KCl, 40; $CaCl_2$, 2; NMG-Cl, 40; Tris-Cl, 2.5; sucrose, 204; D-glucose, 10. After replacing the above external solution with a solution containing (mM): $CdCl_2$, 0.1; KCl, 40; $MgCl_2$, 2; NMG-Cl, 40; Tris-Cl, 2.5; sucrose, 203.9; D-glucose, 10, the current was recorded again. The difference between the two currents recorded in these two different solutions was the I_C activated by the influx of extracellular Ca^{2+} (Jassar *et al.*, 1994). The delayed rectifier potassium current (I_t) was recorded using the same internal solution, but the external solution contained 0.1 mM $CdCl_2$ which was used to block I_C. The voltage- and time-dependent muscarine-sensitive potassium current (I_M) was studied using an external solution containing (mM): NaCl, 117; KCl, 2; $MgCl_2$, 2; $CaCl_2$, 2; HEPES/NaOH (pH 7.2), 5; D-glucose, 10 and an internal solution consisting of (mM): KCl, 110; NaCl, 10; $MgCl_2$, 2; $CaCl_2$, 0.4; EGTA, 4.4; HEPES/KOH (pH 7.2), D-glucose, 10 (Selyanko *et al.*, 1990). Generally, external solutions were 250 mOsmol/kg and internal solutions were 240 mOsmol/kg. The Petri dishes were superfused with external solution at a flow rate of 2 ml/min , which allowed exchange of solutions within about 2 min. The holding potential was -80 mV unless otherwise stated. Capacitance was calculated by integrating the capacitative transient which accompanied a 10 mV depolarizing command from -80 mV. For I_c and I_t, leak subtraction was done by applying 1/4 amplitude hyperpolarizing pulses, multiplying the responses by four and addition. For I_M, the leak currents were calculated by linear regression of the currents measured from -110 mV to -80 mV, because this range of voltage was beyond the voltage range for the activation of I_M and only leak current existed (Adams *et al.*, 1982). The 'pure' I_Ms at the voltage between -70 mV and -30 mV were acquired by subtracting the leak current

(Jassar *et al.*, 1994). All data are presented as means ± S.E.M and Student's two-tailed t-test or analysis of variance has been used to assess statistical significance ($p<0.05$). In graphs where no error bars are visible, the error bars are smaller than the symbols used to designate the data points.

Results

Effects of NGF on Na^+ currents

Figure 4-1A, B and C illustrate typical recordings of Na^+ currents from an acutely-dissociated cell, a cell maintained in defined medium without NGF for 15 days and a cell maintained in the medium in the presence of NGF for 15 days, respectively. The absolute Na^+ current increased after culture with NGF for 15 days. Currents were elicited by a series of 12 ms depolarizing voltage commands from a holding potential of -80 mV in 10 mV steps. The Na^+ currents activated quickly and almost completely inactivated at the end of the depolarization. The macroscopic Na^+ currents began to activate near -20 mV and the peak currents generally occured at +10 mV for acutely-dissociated cells and for cells cultured with or without NGF. Figure 4-1D shows the current-voltage relationship from 23 acutely-dissociated cells, 20 cultured cells in the absence of NGF for 15 days and 20 cultured cells in the presence of NGF for 15 days. Treatment of the cells with NGF for 15 days significantly increased the total Na^+ ($p<0.01$) compared with the current of the cells cultured without NGF for 15 days. However, judged by the current-voltage relationship, the voltage dependence of the macroscopic Na^+ channel currents did not change significantly as a result

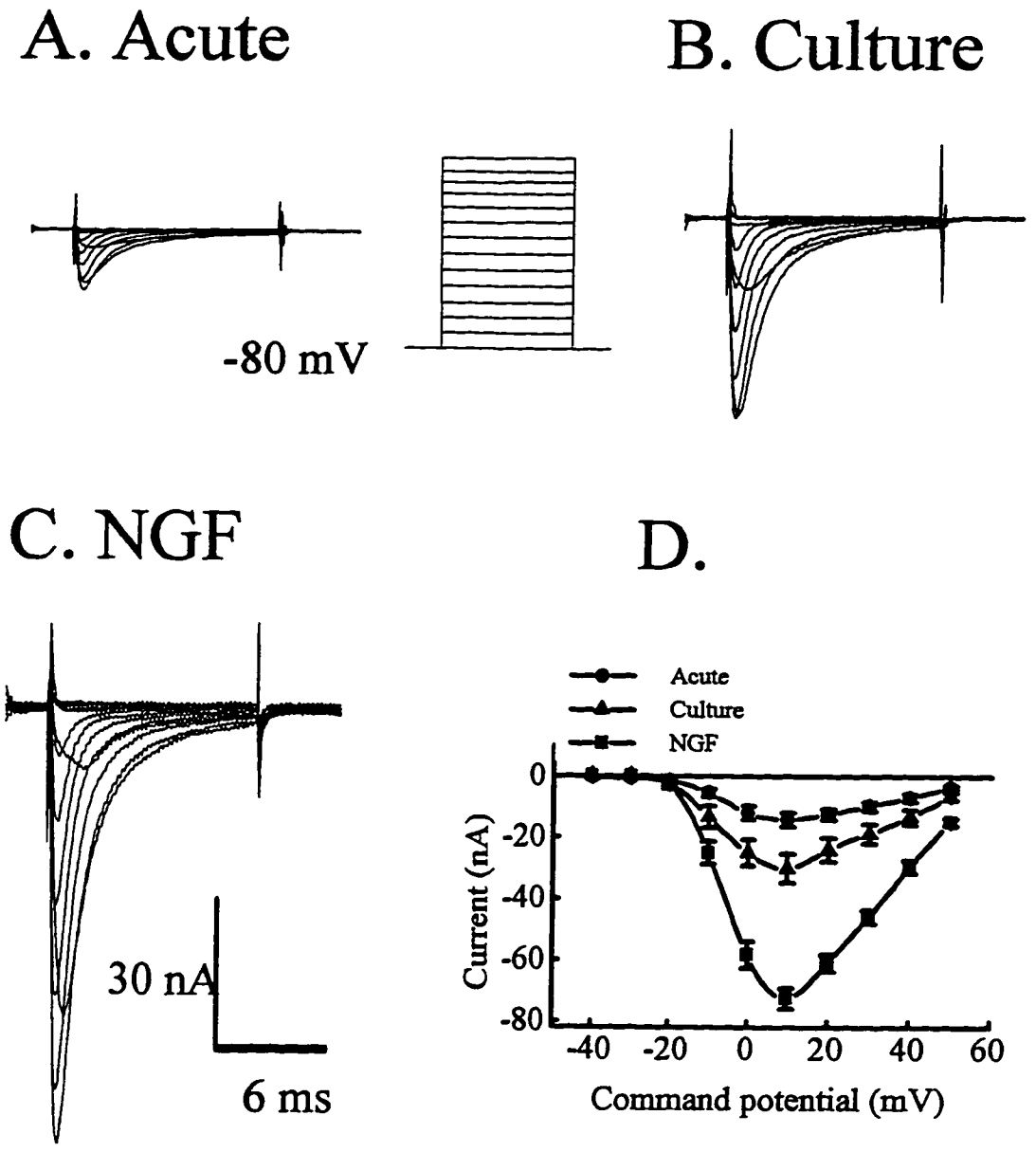


Figure 4-1. NGF increases the total Na^+ current. A, Na^+ current recorded from an acutely dissociated cell. B, Na^+ current from a cultured cell without NGF for 15 d. C. Na^+ current from a cultured cell with NGF for 15 d. D, Current-voltage relationship. Protocol and scale bar are applicable to all the currents.

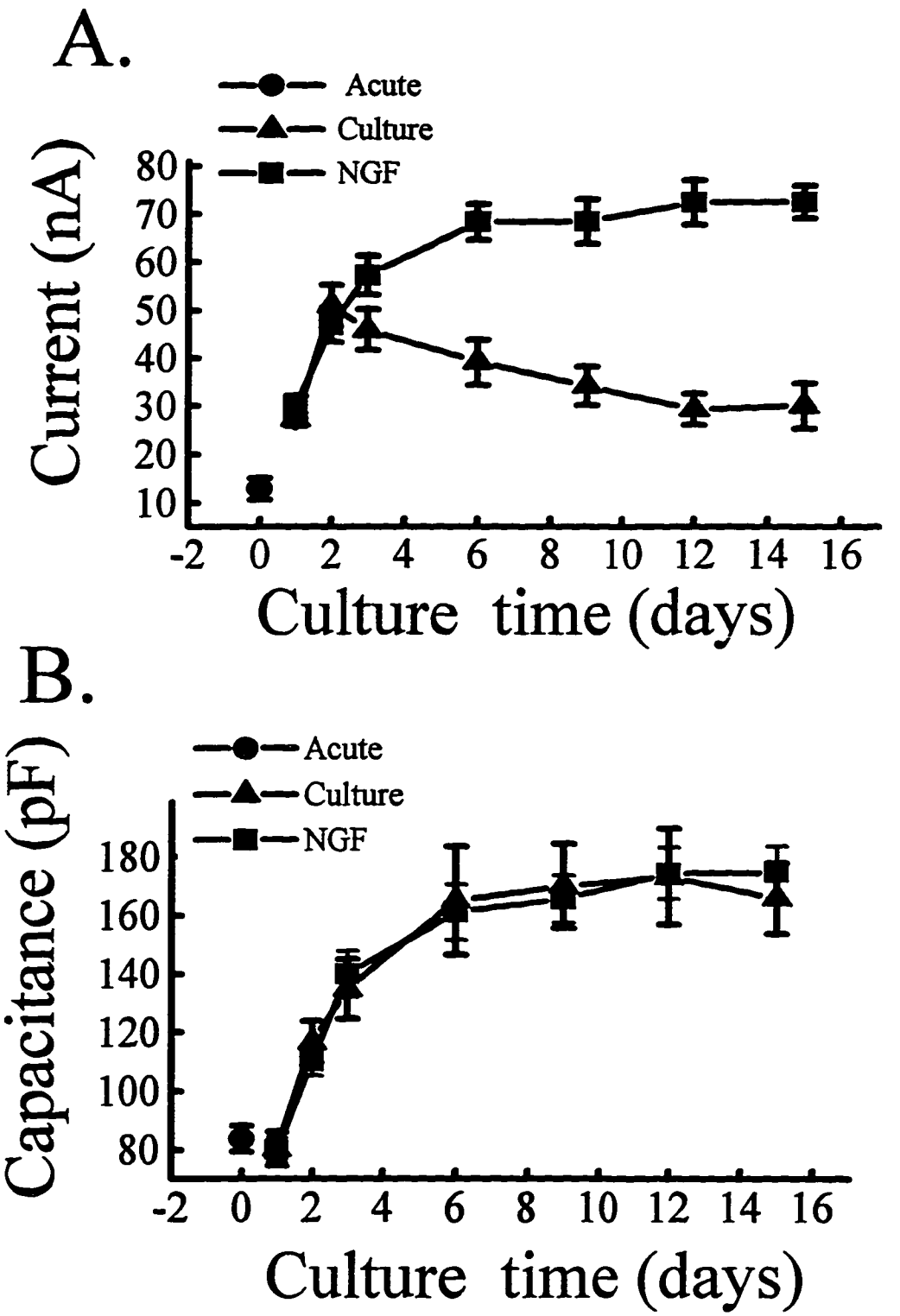
of culture with or without NGF.

Because the cell sizes changed significantly during the culture period, absolute current, capacitance and current density (absolute current normalized to capacitance) were carefully analysed. Figure 4-2A shows the time course of the development of the absolute Na^+ currents. Compared with that of the acutely-dissociated cells, Na^+ current increased 2.6-fold within the first 2d of culture (from 14.20 ± 2.18 nA, n=23 to 50.95 ± 4.30 nA, n=20, $p<0.01$). Afterwards, the current declined gradually, but it was still maintained at a level higher than that of the acutely-dissociated cells even on the 15th day of culture (29.99 ± 4.72 nA, n=20 vs 14.20 ± 2.18 nA, n=23, $p<0.01$). These results show that the viability of the cells cultured in the defined medium was still maintained with frequent exchange of the culture medium. Inclusion of NGF in the culture medium failed to influence the current significantly until 6th day of culture. In the presence of NGF, the current was maintained at a higher level in contrast to that of the cultured cells. On the 15th day of culture, the current of the cultured cells with NGF was still 1.4-fold larger than that of the cells cultured in the absence of NGF (72.40 ± 3.41 nA, n=20 vs 29.99 ± 4.72 nA, n=20, $p<0.01$).

To account for the influence of cell size on the macroscopic currents, cell size was analysed by measuring the capacitance. Figure 4-2B shows the change of capacitance of acutely-dissociated cells, cultured cells with and without NGF. Within the first day of culture, the cell capacitances remained the same as those of the acutely-dissociated cells (80.25 ± 5.78 pF, n=20 vs 83.70 ± 4.40 pF, n=20, $p>0.05$), which was in contrast to the 92% increase of the currents on the first day (Figure 4-2A). On the second day of culture, the capacitances increased by only 39% in contrast to 2.6-fold enhancement of the current (Figure 4-2A).

Figure 4-2. NGF increases the total Na^+ current, but does not influence capacitances of the cells. A, Time course of the development of the peak Na^+ currents at +10 mV. B, The corresponding changes in cell capacitances.

(Figure 4-2)


A.
Acute
Culture
NGF
Current (nA)
80
70
60
50
40
30
20
10
-2
0
2
4
6
8
10
12
14
16
Culture time (days)
B.
Acute
Culture
NGF
Capacitance (pF)
180
160
140
120
100
80
-2
0
2
4
6
8
10
12
14
16
Culture time (days)

These results suggested increase in the Na^+ current occured prior to the the enhancement of the capacitance, which may reflect that different cellular mechanisms controlled the regulation of ion channels and neurite growth. Comparison of the capacitances of the cultured cells in the presence of NGF with those of the cells cultured in the absence of NGF shows that NGF failed to influence capacitance (Figure 4-2B). Because the values of the capacitances were just obtained from those cells in which the voltages were adequently controlled and those cells generally have short processes, at this stage, we can only say NGF has not been found to increase the neurite outgrowth in adult bullfrog sympathetic ganglion cells judged by the capacitance of the cells. A similar conclusion has also been obtained in adult dorsal root ganglion neurons (Lindsay, 1988) and basal forebrain neurons (Levine *et al.*, 1995).

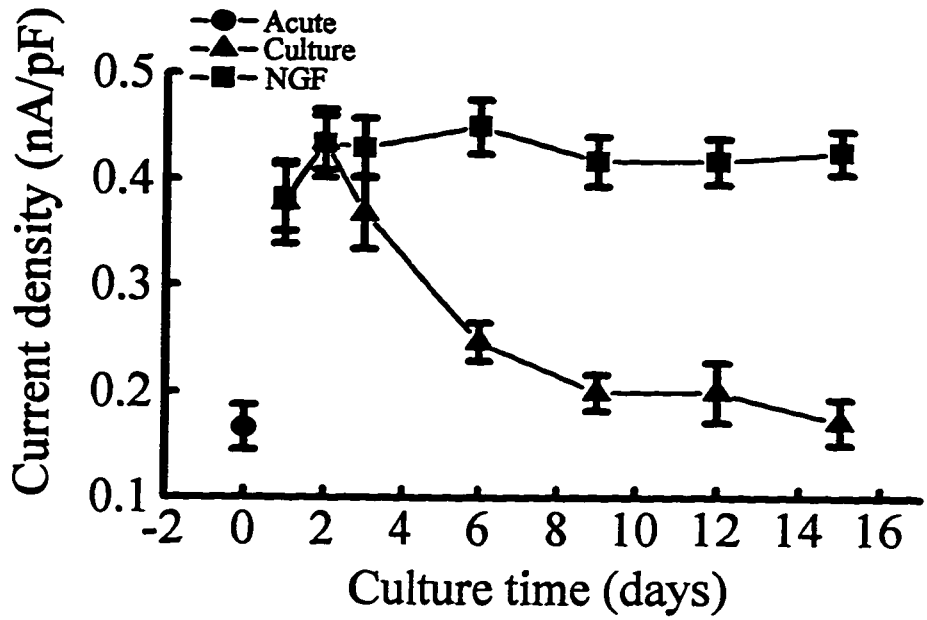
The effects of NGF on Na^+ channels were further studied by analysis of the current density (Figure 4-3A). The initial peak seen in the absence of NGF was more conspicuous when current density was used as an indicator. On the second day of culture, the current density reached maximum. The current density of the cultured cells remained at a higher level until the 6th day and the value on day 1, day 2 and day 3 was 1.3-, 1.6- and 1.2-fold larger than that of the acutely-dissociated cells, respectively ($p<0.01$ for all the time points). On the 6th day, the current density was still 48% larger than that of the acutely-dissociated cells ($p<0.05$). Afterwards, there was no statistical difference between the current density of the cultured cells and that of the acutely-dissociated cells ($p>0.05$). Addition of NGF in the culture medium did not change the initial enhancement of the current density, but it prevented the current density from declining with time after the initial peak (Figure 4-3A).

Our attention was drawn to the initial peak on the time course curves of both absolute

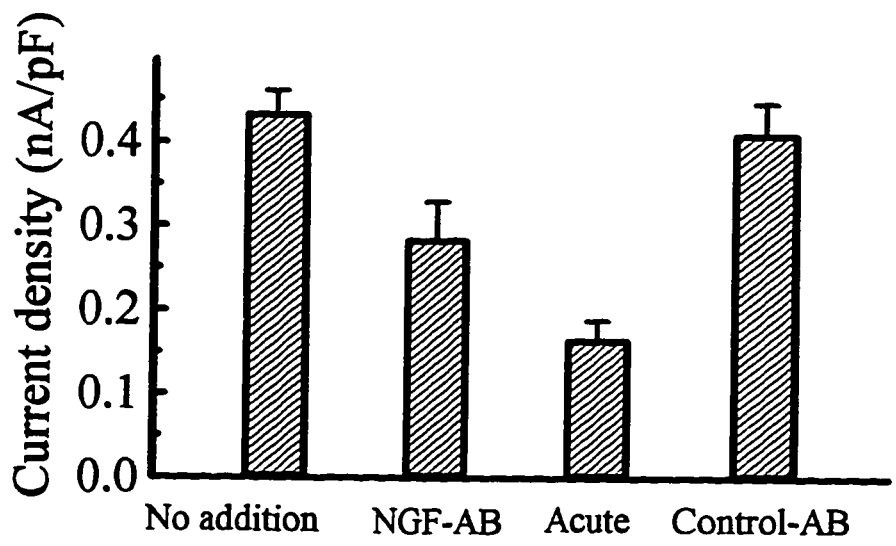
Figure 4-3. A, Time course of the peak Na^+ current density at +10 mV. NGF can prevent the decline of the Na^+ current density. B, Effects of NGF antibody on Na^+ current density of the cultured cells for 2 d in the absence of NGF. NGF antibody only partially inhibited the increase in current density.

(Figure 4-3)

A.


Acute
Culture
NGF
Current density (nA/pF)
0.5
0.4
0.3
0.2
0.1
-2
0
2
4
6
8
10
12
14
16
Culture time (days)

B.


Current density (nA/pF)
0.4
0.3
0.2
0.1
0.0
No addition
NGF-AB
Acute
Control-AB

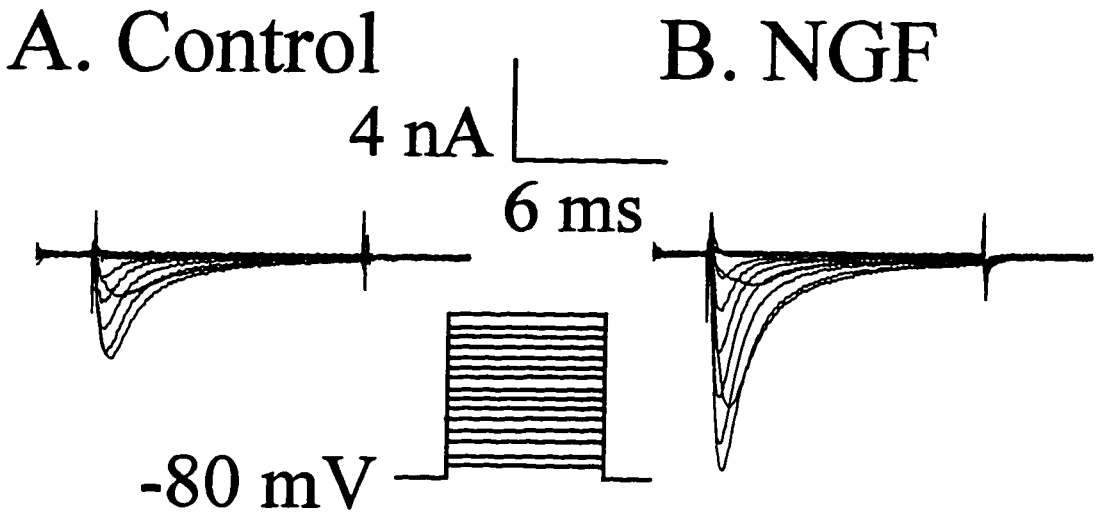
current and current density. We hypothesized that some neurotrophic factor(s) might be generated in the system, which was responsible for the initial increase in the Na^+ current or current density because neurotrophic factors can be produced from both neuronal and non-neuronal cells (Korsching, 1993). To test whether NGF was the proposed neurotrophic factor in the system, serum containing NGF antibody was included in the culture medium. The control group was treated with serum without NGF antibody. Anti-NGF (0.5 μg/ml) inhibited only 34% of the enhanced current density on the second day of culture (from 0.43 ± 0.04 nA/pF, n=20 for cells cultured for 2 days with control serum to 0.29 ± 0.04 nA/pF, n=20 for cells cultured with anti-NGF serum for 2 days, $p < 0.05$; Figure 4-3B). But the current density of the cells cultured with anti-NGF for 2 days was still 71% higher than that of the acutely-dissociated cells (0.29 ± 0.04 nA/pF, n=20 vs 0.17 ± 0.02 nA/pF, n=23, $p < 0.05$). These results suggested that NGF could only be partially responsible for the initial increase in Na^+ current. It is also possible that some other trophic factor which has similar immunological properties to NGF is responsible for the initial peak.

NGF increases both TTX-sensitive and TTX-resistant components of Na^+ currents

NGF has been reported to induce TTX-resistant Na^+ channels in PC12 cells (Rudy *et al.*, 1987). In BFSG neurons, the total Na^+ current consists of a larger TTX-sensitive component and a smaller TTX-resistant component (Jones, 1987; Jassar *et al.*, 1993). To assess the effects of NGF on these two components, the total Na^+ currents were recorded first in the normal external solution, and then TTX-resistant currents were recorded after replacing

Figure 4-4. NGF increases TTX-resistant Na^+ current. A, TTX-resistant Na^+ current from a cultured cell without NGF for 15 d. B, TTX-resistant Na^+ current from a cell cultured with NGF for 15 d. C, Current density and voltage relationship for cells cultured with or without NGF for 15 days. NGF significantly increases TTX-resistant current density.

(Figure 4-4)


A. Control
B. NGF
4 nA
6 ms
-80 mV

C.

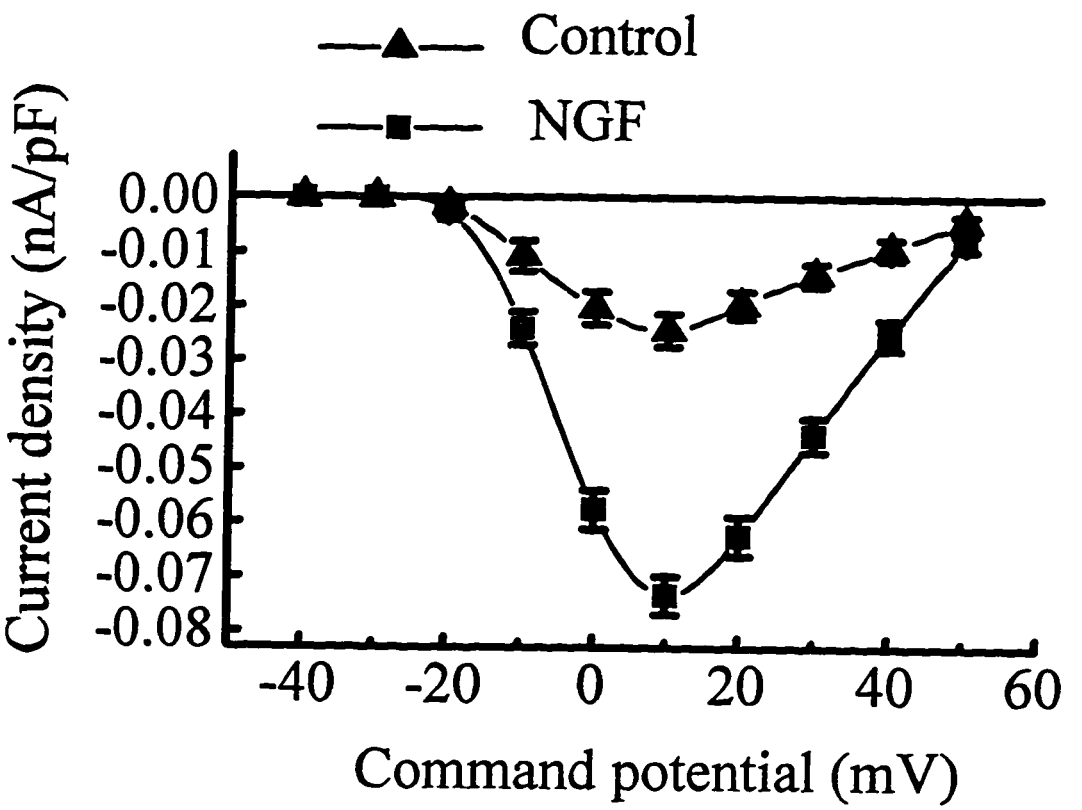
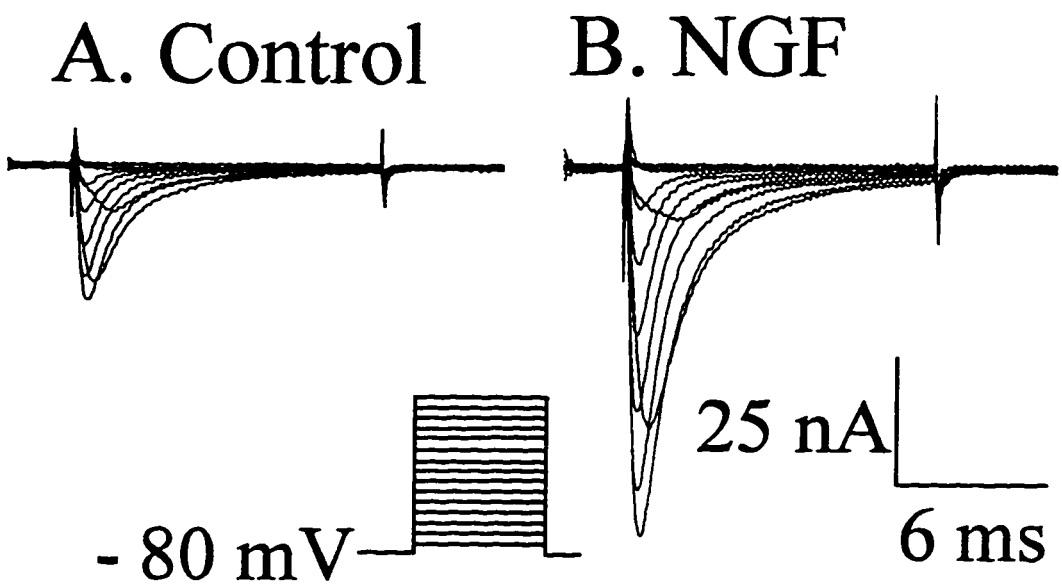

Control
NGF
Current density (nA/pF)
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
-0.07
-0.08
-40
-20
0
20
40
60
Command potential (mV)

Figure 4-5. NGF increases TTX-sensitive Na^+ current. A, TTX-sensitive Na^+ current from a cell cultured without NGF for 15 d. B, TTX-sensitive Na^+ current from a cell treated with NGF for 15 d. C, TTX-sensitive current density and voltage relationship for those cells. NGF significantly increases TTX-sensitive Na^+ current density.

(Figure 4-5)


A. Control
B. NGF
25 nA
6 ms
- 80 mV

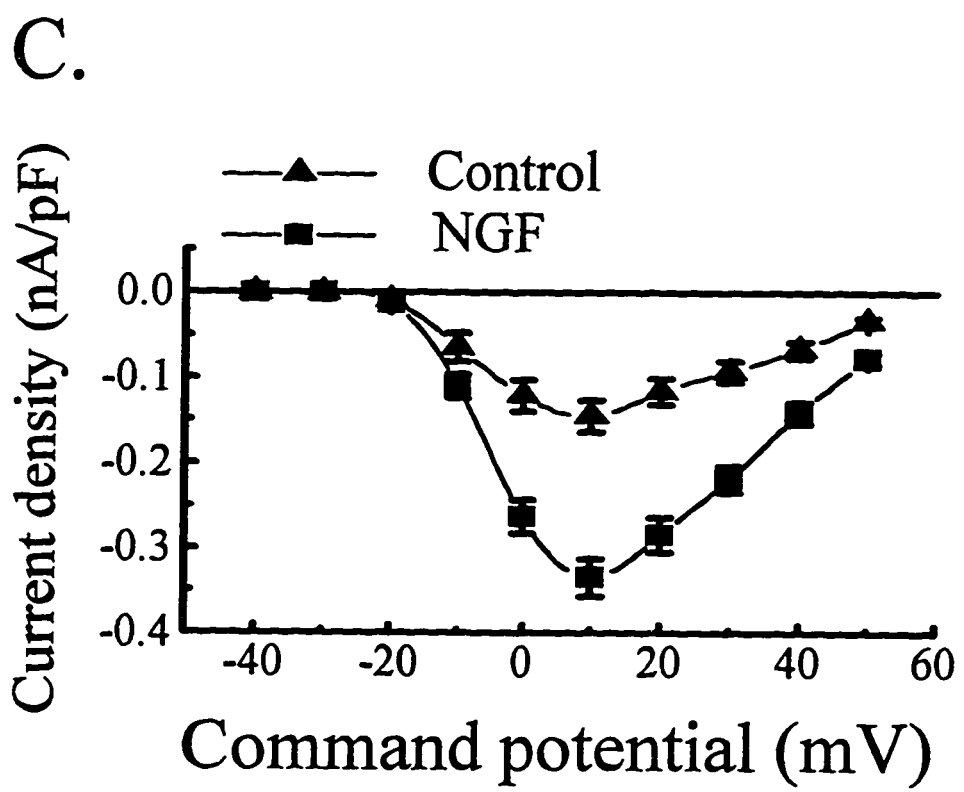

C.
Control
NGF
Current density (nA/pF)
0.0
-0.1
-0.2
-0.3
-0.4
-40
-20
0
20
40
60
Command potential (mV)

Table 4-1. Percentage of the TTX-resistant and TTX-sensitive currents in the total Na^+ currents

	Control	NGF
TTX-resistant	15.8 ± 2.3 %	21.8 ± 2.9 %
TTX-sensitive	84.2 ± 2.3 %	78.2 ± 2.9 %

with the external solution containing 1 μM TTX. TTX-sensitive current was the difference between the total current and the TTX-resistant current. Figure 4-4A and B show typical recordings of TTX-resistant Na^+ currents. NGF increased the peak TTX-resistant current density by 2.4-fold (from 27.0 ± 5.0 pA/pF, n=20 for the cells cultured without NGF for 15 days to 91.2 ± 12.6 pA/pF, n=20 for the cells cultured with NGF for 15 days, $p<0.01$; Figure 4-4C). TTX-sensitive currents were obtained by subtracting the TTX-resistant currents from the total currents. Digitally subtracted current traces from a cell cultured without NGF for 15 days and a cell cultured with NGF for 15 days are shown in Figure 4-5A and B, respectively. In the presence of NGF, the peak TTX-sensitive current density increased by 1.3 fold (from 144.5 ± 18.8 pA/pF, n=20 for cells cultured without NGF for 15 days to 334.2 ± 21.9 pA/pF, n=20 for the cells cultured with NGF for 15 days; $p < 0.01$).

The TTX-resistant current has been reported to inactivate more slowly than the TTX-sensitive component (Jones, 1987). One possibility was that the increase in the slowly-inactivating TTX-resistant current induced by NGF might contribute to the enhancement of the total Na^+ current. To see whether this was possible, the relative proportion of the TTX-sensitive and TTX-resistant currents in the total currents was calculated (Table 4-1). Even though NGF increased the absolute current densities of both components, their relative ratios

in the total currents were not significantly changed by NGF ($p > 0.05$ for two groups).

NGF did not change the activation kinetics

Although NGF failed to change the voltage-dependence of activation as judged from the current-voltage relationship, the activation and inactivation processes still needed to be further studied by comparing the parameters which can detect more subtle changes of the kinetics. To study the activation kinetics, the absolute current recorded from each acutely-dissociated cell, cell cultured with NGF for 15 days and cell cultured without NGF for 15 days was converted to conductance by the equation $g_{Na}=I_{Na}/(V_m-E_{Na})$, where g_{Na} is the conductance of Na^+ channel, I_{Na} is the Na^+ current, V_m is the command potential, E_{Na} is the Na^+ equilibrium potential which was computed to be +60 mV for $[Na^+]_o$=97.5 mM and $[Na^+]_i$=9 mM in the recording system. This potential is very close to the observed reversal potential for the Na^+ channel current of each cell. Once obtained, the conductance values were normalized and fitted to a Boltzmann relation $g/g_{max}=\{1+\exp[-(V-Va_{1/2})/k_a]\}^{-1}$, where g is the conductance; g_{max}, the maximal conductance; V, the command potential; $Va_{1/2}$, voltage at which half the activation achieved; k_a, slope factor of activation curve. For each cell, $Va_{1/2}$ and k_a can be obtained by Boltzmann fitting. Table 4-2 summarizes the the results fitted from 23 acutely-dissociated cells, 20 cells cultured without NGF for 15 days and 20 cells cultured with NGF for 15 days. There were no stastically significant differences among the values of $Va_{1/2}$ for the acutely-dissociated cells, cells cultured with and without NGF for 15 days, suggesting the voltage-dependence of activation for those cells was not changed. Although the slope factor (k_a) for acutely-dissociated cells was statistically larger than that of the cells

cultured without NGF for 15 days ($0.01<p<0.05$) and that of the cells cultured with NGF for 15 days ($p<0.01$), there was no significant difference between the k_a value of the NGF-treated cells and that of the cells cultured alone ($p>0.05$). This was further confirmed by Boltzmann fitting the means of the pooled data (Figure 4-6).

Table 4-2. Activation and inactivation parameters of Na^+ channels

	$Va_{1/2}$	k_a	$Vi_{1/2}$	k_i
Acute	0.82 ± 1.29	7.29 ± 0.53	-19.32 ± 2.42	7.70 ± 0.37
Culture	-2.14 ± 0.66	5.44 ± 0.48*	-17.46 ± 2.59	7.74 ± 0.40
NGF	-2.40 ± 0.93	5.31 ± 0.21**	-21.39 ± 2.19	7.22 ± 0.29

* $0.05>p>0.01$ compared with acutely-dissociated cells

** $p<0.01$ compared with acutely-dissociated cells

NGF did not change the inactivation kinetics

The effects of NGF on the rate of onset of inactivation and the steady-state inactivation were examined. First, the inactivation time constants during the 12 ms depolarizing pulses were obtained by single-exponential fitting to the decay of the current. The reciprocals of the time constants were defined as the inactivation rate. The results were shown in Figure 4-7. Between -10 and +40 mV, there was no significant difference for the inactivation rate among the acutely-dissociated cells, cultured cells without NGF for 15 days and cultured cells with NGF for 15 days ($p>0.05$).

Second, the voltage-dependence of steady-state inactivation (h_∞ plot) has been further studied by clamping the cells to a series of relatively positive holding potentials (from -70 to +10 mV) for 20 ms to allow inactivation to develop prior to the application of a 12 ms test pulse to +10 mV. Figure 4-8A and B show some typical recordings from a cultured cell without NGF for 15 days and a cultured cell with NGF for 15 days by this protocol, respectively. The amplitude of Na^+ current generated during this test pulse provided a measurement of the amount of inactivation which developed at various holding potentials. The currents were normalized to the maximal current and then fitted by Boltzmann equation $I/I_{max}=\{1+\exp[(V-Vi_{1/2})/k_i]\}^{-1}$ (I, current; I_{max}, maximal current; V, prepulse potential; $Vi_{1/2}$, voltage at which half inactivation reached; k_i, slope factor of inactivation curve) for each cell. Values of $Vi_{1/2}$ and k_i for each cell can be obtained from the fitting. The results are summarized in Table 4-2. There were no significant differences for the values of $Vi_{1/2}$ and k_i among the acutely-dissociated cells, cultured cells with and without NGF for 15 days ($p>0.05$ for all the groups), suggesting that the voltage-dependence of inactivation has not been changed by culture with or without NGF (Figure 4-8C).

NGF response is transcription-dependent

NGF-mediated enhancement of the Na^+ currents was unlikely due to NGF-induced functional change of Na^+ channels because NGF did not alter the activation and inactivation kinetics and did not change the relative proportion of TTX-resistant component which inactivated more slowly in the total currents. The possibility that NGF increased the Na^+ channel gene induction which led to the augmentation of channel protein synthesis was

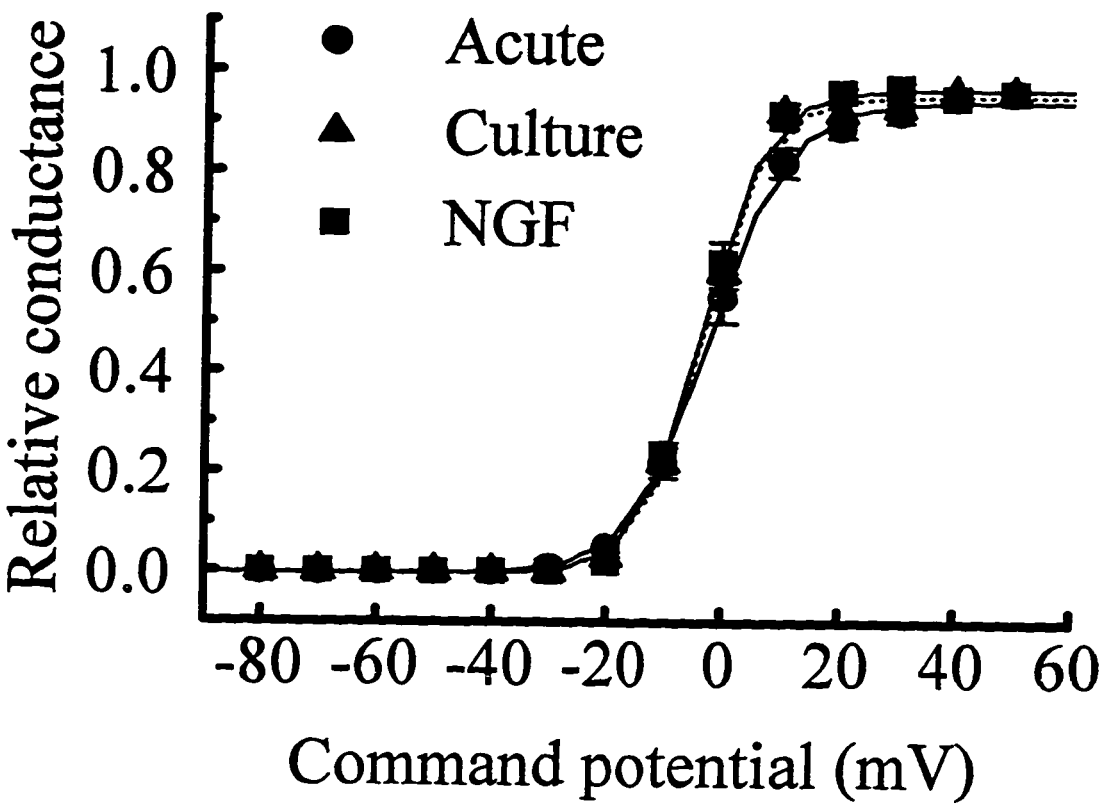


Figure 4-6. NGF did not change the activation kinetics of cultured cells. The current was converted to conductance first, and Boltzmann fitting was conducted on the means. The activation of acutely-dissociated cells is slower than that of the cultured cells with or without NGF for 15 days. The is no significant difference between the cells cultured with and without NGF for 15 days.

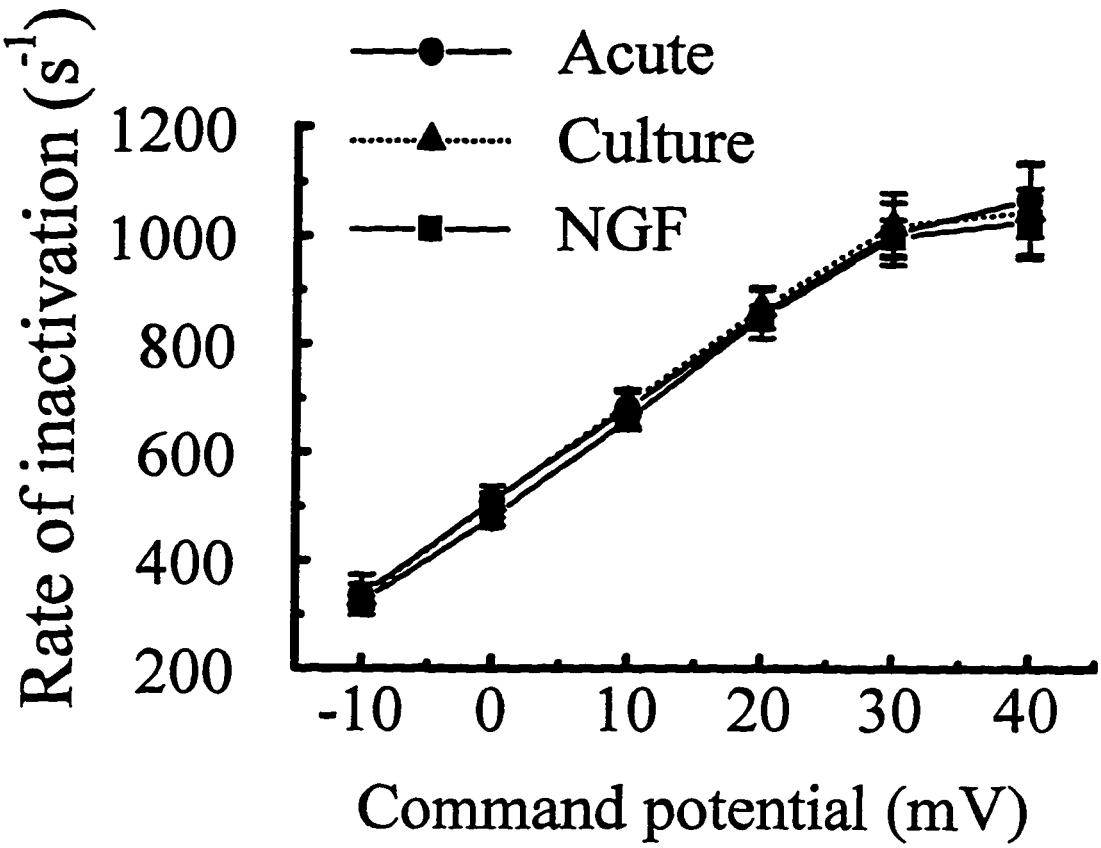
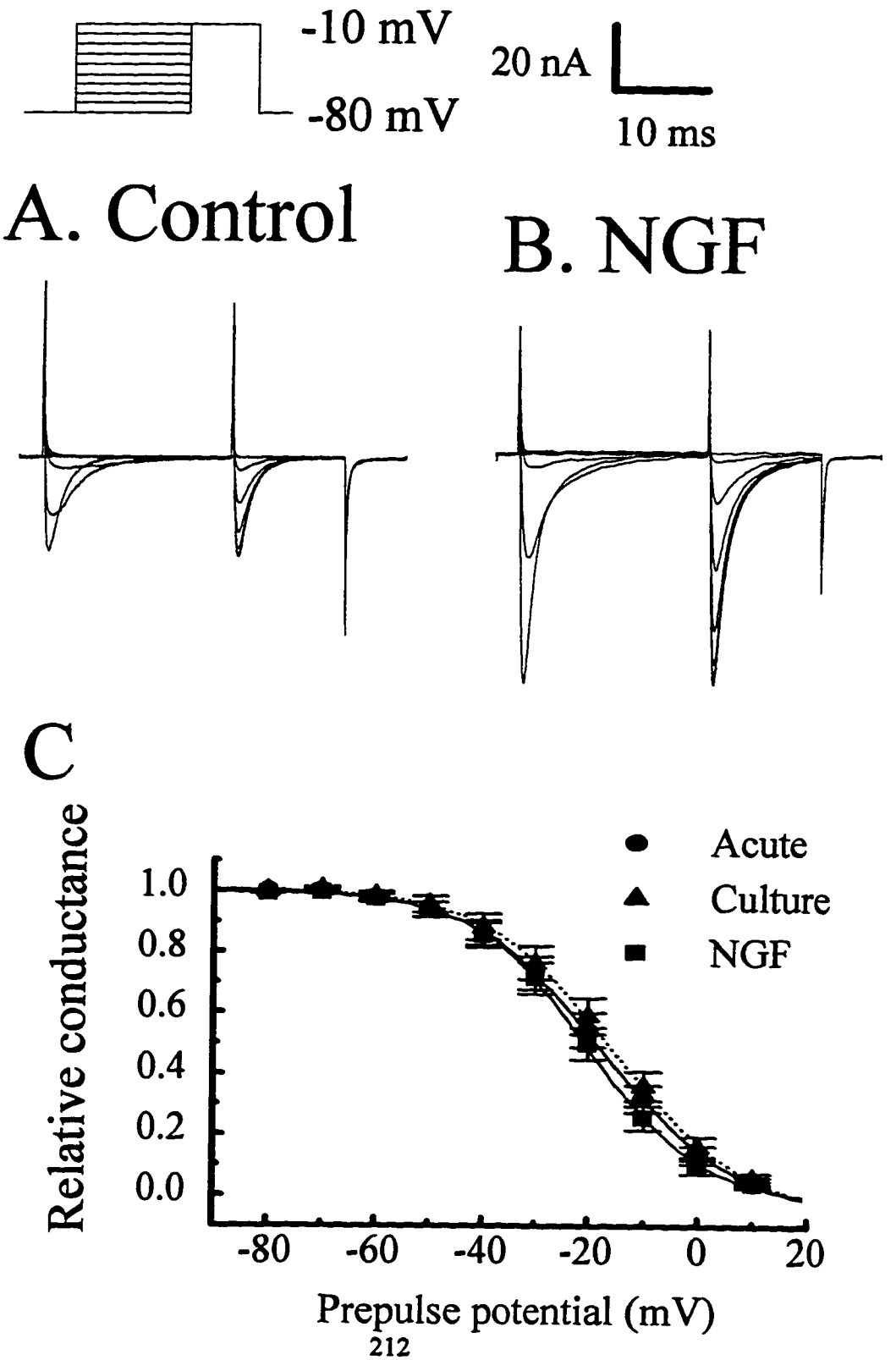


Figure 4-7. NGF did not change the inactivation rate. Time constants of inactivation were obtained by single-exponential fitting of the decay of the total current. The reciprocals of the time constants were defined as the inactivation rate. There is no significant difference among the inactivation rate of the acutely-dissociated cells, cultured cells with or without NGF for 15 days.

Figure 4-8. NGF did not change the voltage-dependence of steady-state inactivation. A, B, Currents recorded by a two pulse protocol with a series of 20 ms prepulses at different potentials to induce Na^+ channel inactivation and a 12 ms test pulse to +10 mV to assess the inactivation of the Na^+ channels from a cultured cell without NGF for 15 days and a cell cultured with NGF for 15 days, respectively. C, Boltzmann fitting plot of the inactivation conductance from 30 acutely dissociated cells, 26 cultured cells without NGF for 15 days and 33 cells treated with NGF for 15 days. There is no significant difference among the inactivation curve of the acutely-dissociated cells, cultured cells with and without NGF for 15 days.

(Figure 4-8)


-10 mV
-80 mV
20 nA
10 ms
A. Control
B. NGF
C
Relative conductance
1.0
0.8
0.6
0.4
0.2
0.0
-80
-60
-40
-20
0
20
Prepulse potential (mV)
Acute
Culture
NGF

therefore examined. Two transcriptional inhibitors, actinomycin D and cordycepin were used in the culture medium. These two inhibitors were dissolved in dimethyl sulphoxide (DMSO)

Table 4-3. Effects of transcriptional inhibitors on Na^+ currents and cell capacitances

	Current (nA)	Capacitance (pF)	n
DMSO	28.65 ± 3.43 **a	166.60 ± 16.91	20
DMSO + NGF	70.44 ± 5.07	169.91 ± 9.99	23
Cordycepin	20.19 ± 3.42	114.25 ± 9.15 **b	20
Cordycepin + NGF	26.62 ± 4.51 **a	126.70 ± 10.31 **a	20
Actinomycin D	21.59 ± 2.79	116.55 ± 7.05 **b	20
Actinomycin D + NGF	22.46 ± 2.07 **a	128.80 ± 9.39 **a	20

**a, $p < 0.01$ compared with DMSO+NGF

**b, $p<0.01$ compared with DMSO

and their final concentrations in the culture medium were 0.01 μg/ml and 20 μM, respectively. The final concentration of the DMSO was controlled to be 0.1% and two control groups, DMSO alone and DMSO plus NGF, were set. At this concentration, DMSO did not influence either Na^+ channel currents or NGF response.

The effects of the inhibitors on absolute current and capacitance are shown in Table 4-3. Inclusion of cordycepin (20 μM) or actinomycin D (0.01 μg/ml) in the culture medium for 12 to 15 days did not significantly change the basal currents, but they completely inhibited

the increase of the current attributed to NGF. However, they reduced the cell capacitance by 30% from the value seen after 15 days culture without NGF. In the presence of the inhibitors, NGF failed to increase the cell capacitance significantly (Table 4-3).

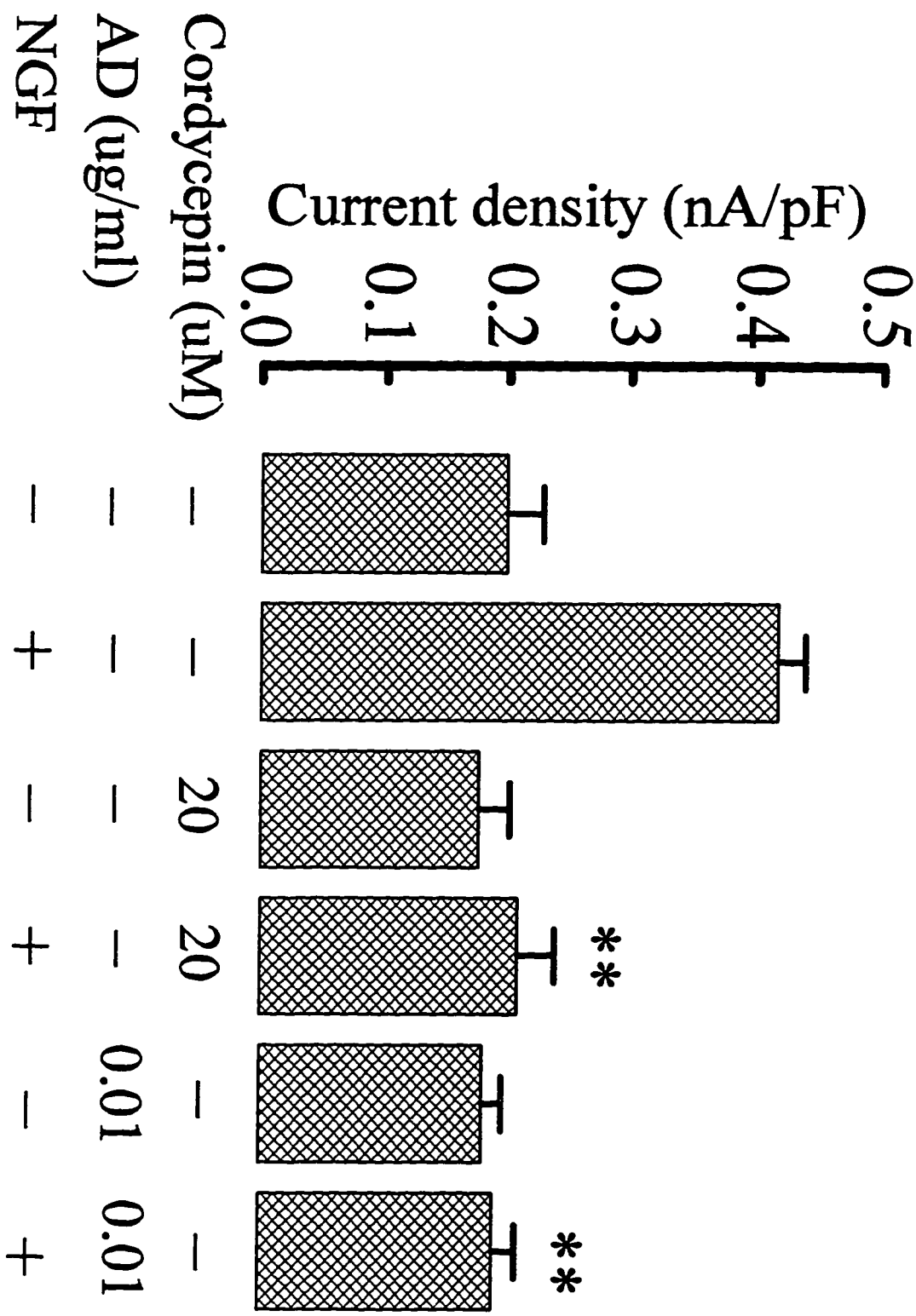
Effects of the inhibitors on Na^+ channels were further analysed by calculation of the current density. The results were shown in Figure 4-9. Both of the inhibitors did not significantly alter the basal current density, but they completely blocked the augmentation of current density induced by NGF. These results suggested that the effects of NGF on Na^+ channels were dependent on gene induction and possibly the increase in Na^+ channel protein synthesis.

Effects of NGF on Ca^{2+}-activated potassium channel currents (I_C)

One of the major functions of I_C in BFSG B-cells is to generate the fast repolarization of the action potential (Adams *et al.*, 1982; Lancaster and Pennefather, 1987). The activation of this current requires influx of Ca^{2+} via voltage-gated Ca^{2+} channels. To estimate whether NGF affects the properties of I_C, the current was elicited by 50 ms pulses from a holding potential of -40 mV which minimized contamination by I_K because at this voltage I_K is largely inactivated (Adams *et al.*, 1982; Xu and Adams, 1992). While this holding potential caused pronounced inactivation of N-type Ca^{2+} channels which limited the influx of Ca^{2+}, the large conductance of I_C made the recording still practical. In fact, initially, we used an external solution containing 4 mM $[Ca^{2+}]_o$ and 2 mM $[K^+]_o$ to record I_C, but the currents were too large to allow reliable control of voltage. To obtain better voltage control, experiments were conducted by lowering the $[Ca^{2+}]_o$ to 2 mM and raising $[K^+]_o$ to 40 mM to limit the influx of

Figure 4-9. Effects of NGF on Na^+ channels were blocked by transcriptional inhibitors, cordycepin and actinomycin D (AD). **$p<0.01$, compared with the cells treated with NGF plus DMSO.

(Figure 4-9)


Current density (nA/pF)
0.0
0.1
0.2
0.3
0.4
0.5
Cordycepin (uM)
AD (ug/ml)
NGF
-
-
-
+
-
-
-
-
20
+
-
20
-
-
0.01
+
-
0.01
**
**

$[Ca^{2+}]_o$ and decrease the K^+ gradient. Under these recording conditions, the amplitude of I_C was adequent for proper voltage-clamp.

Figure 4-10A and B illustrate pure I_C traces obtained by digitally subtracting the remaining Cd^{2+}-resistant current from the total current for a cell cultured without NGF for 15 days and a cell cultured with NGF for 15 days, respectively. Figure 4-8C shows the current-voltage relationship of I_C from those cells. I_C began to activate when the command potential was -30 mV and reached a maximum at +30 mV. At more positive voltages, the current declined gradually because of the decreased influx of Ca^{2+} via Ca^{2+} channels (Jassar *et al.*, 1994). I_C of the cells cultured with NGF was significantly greater than that of the cells cultured without NGF for 15 days (the differences between the values of the cultured cells without NGF and those of the cells cultured with NGF were statistically significant for all voltages positive to -20 mV, $p<0.01$). Figure 4-10D shows the time course of I_C change. The total I_C remained relatively constant throughout the 15d experimental period and inclusion of NGF in the culture medium more than doubled the current in the absence of NGF (Figure 4-10D).

Effects of NGF on I_C have been further examined by analyzing the change in capacitance and the current density of the cells. After 6d of culture, capacitance was almost double that of the first day, but NGF failed to affect the cell capacitance (Figure 4-11A). However, the current density decreased significantly after 3d of culture because the capacitance increased at this time but the absolute current remained almost the same (Figure 4-10D). Inclusion of NGF prevented the decrease of the current density (Figure 4-11B).

Previously, we have shown that NGF increases N-type and L-type Ca^{2+} currents and

Figure 4-10. Effects of NGF on I_C. Currents were evoked from -40 mV by a series of 50 ms depolarizing pulses. I_C was obtained after subtracting the Cd^{2+}-resistant current from the total current. A, I_C from a cell cultured in the absence of NGF for 15 days. B, I_C from a cell cultured with NGF for 15 days. The protocol and scale bar are applicable to both cells. NGF can increase I_C current. C, Current and voltage relationship from 22 cultured cells without NGF and 20 cultured cells with NGF for 15 days. NGF significantly increased I_C current. D, Time course of I_C for cells cultured with or without NGF.

(Figure 4-10)

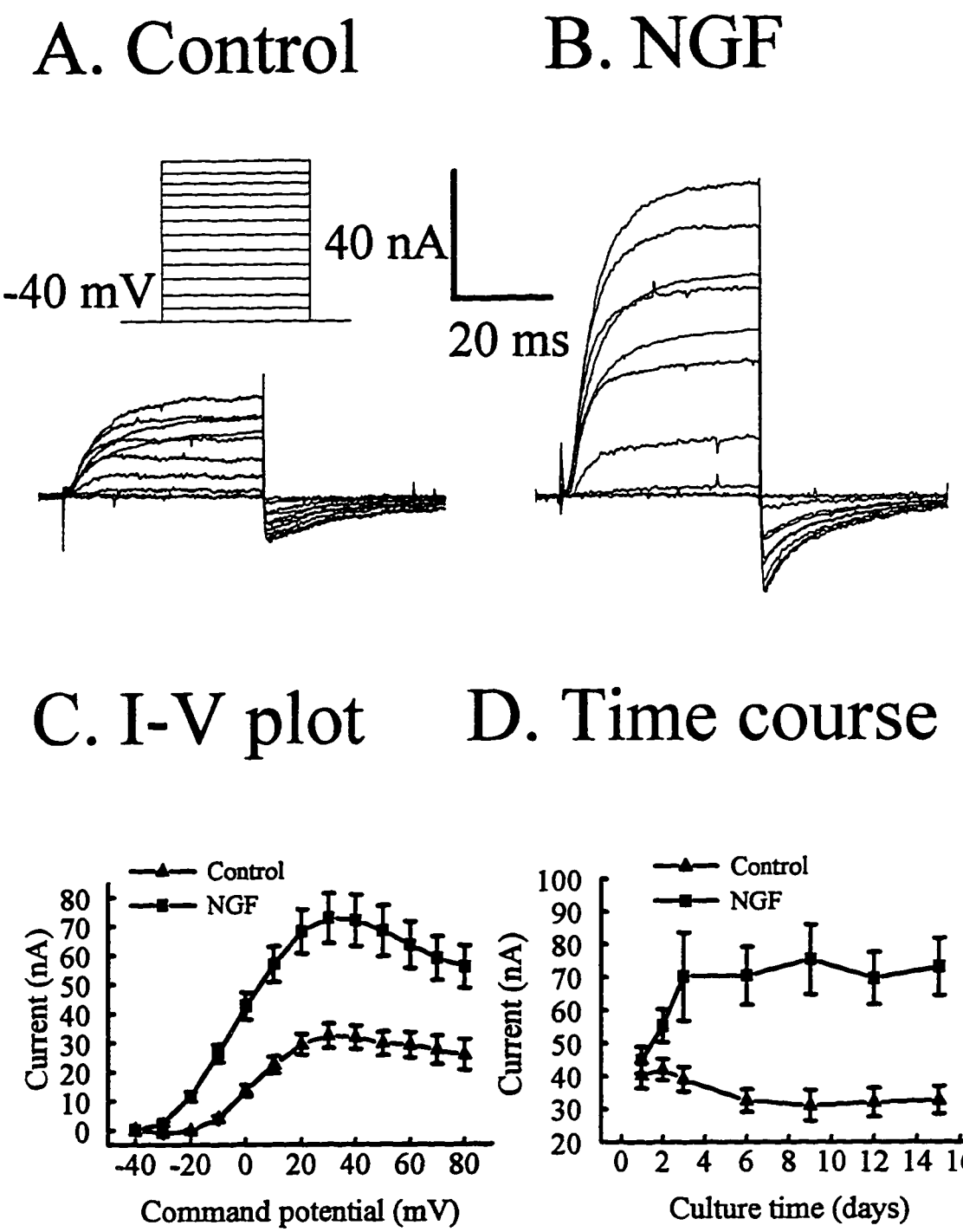
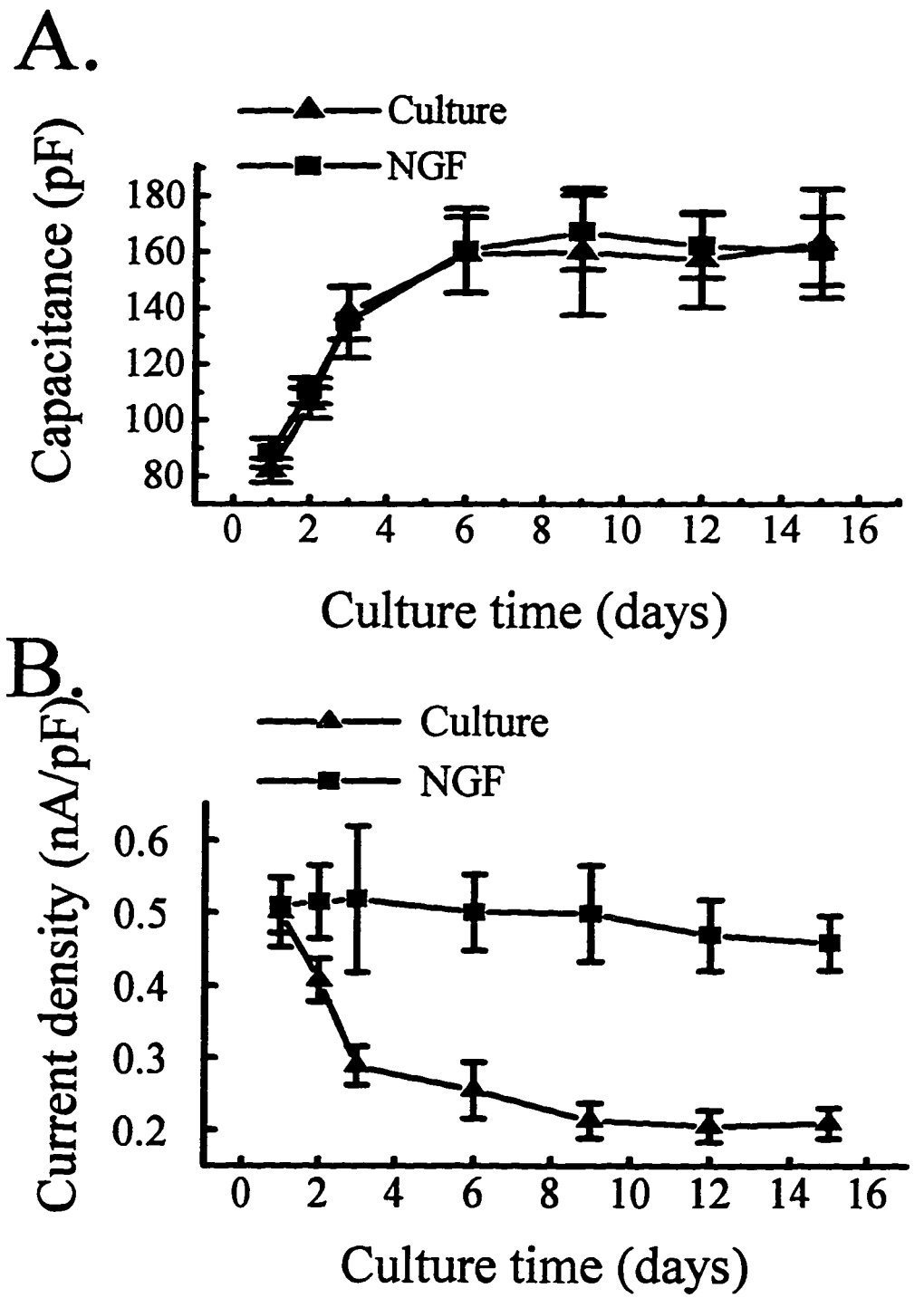

A. Control
B. NGF
-40 mV
40 nA
20 ms
C. I-V plot
D. Time course
Control
NGF
Current (nA)
Command potential (mV)
Culture time (days)

Figure 4-11. Effects of NGF on the capacitance (A) and I_C current density (B). A, Capacitances of the cells has not been changed by treatment with NGF. B, In the absence of NGF, I_C current density declined gradually. NGF prevented the decrease of the I_C current density of the cultured cells.

(Figure 4-11)


A.
Culture
NGF
Capacitance (pF)
180
160
140
120
100
80
0
2
4
6
8
10
12
14
16
Culture time (days)
B.
Culture
NGF
Current density (nA/pF)
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
12
14
16
Culture time (days)

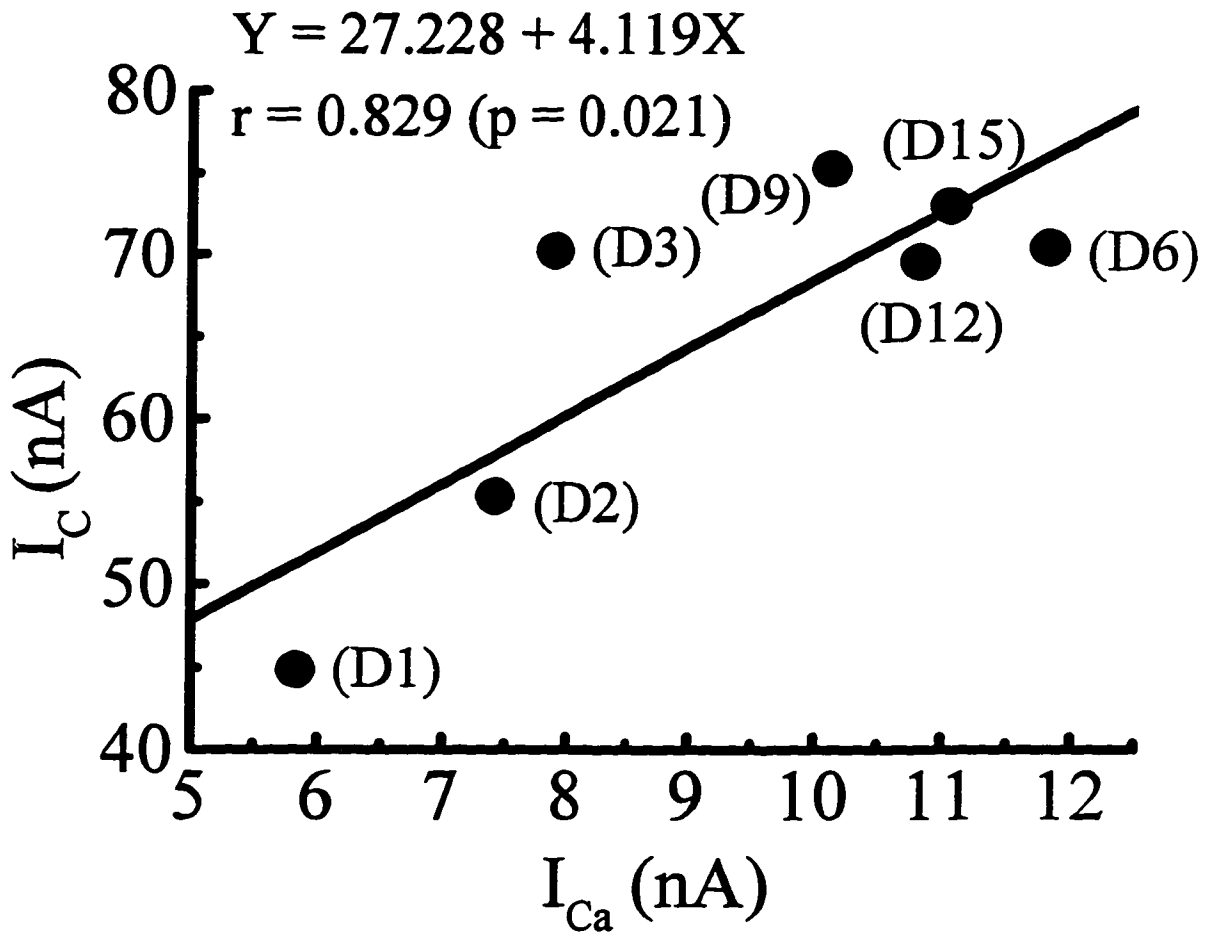


Figure 4-12. Correlation between Ca^{2+} channel current (I_{Ca}) and Ca^{2+}-dependent K^+ channel currents (I_C) in NGF-treated cells. I_{Ca} data were obtained from Chapter 2. The relationship between I_C and I_{Ca} can be described by equation Y=27.228+4.119X, Y is the I_C amplitude, X is the I_{Ca} amplitude. The correlation coefficient is 0.829 which is statistically significant (p=0.021)

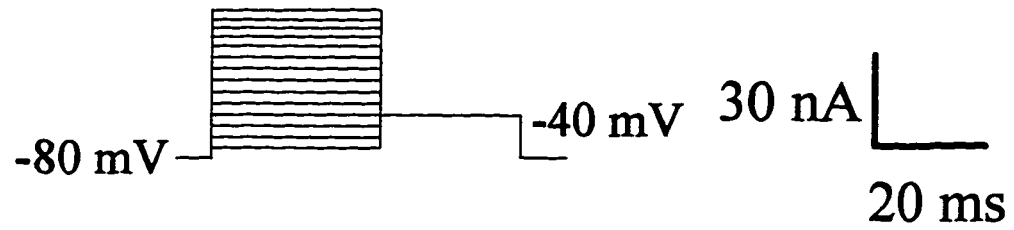
attenuates the inactivation of the Ca^{2+} channels. Since the activation of I_C channels requires influx of Ca^{2+} via voltage-gated Ca^{2+} channels, it is possible that effects of NGF on I_C reflect its action on Ca^{2+} channels rather than on I_C channels *per se*. We made an initial attempt to see whether this is a possibility by linear regression of the means of the Ca^{2+} current from the cultured cells with NGF for 1 to 15 days (from Chapter 2) with the I_C amplitude of the cells treated with NGF for the same period of time. I_C amplitudes are proportional to their corresponding Ca^{2+} current amplitudes (Figure 4-12, $p<0.01$ for the correlation coefficient). These results suggested that I_C was dependent on Ca^{2+} channel currents for NGF-treated cells and the effects of NGF on I_C might be secondary to its effects on Ca^{2+} channels.

Delayed rectifier K^+ current (I_K) was not affected by NGF

I_K was recorded from a holding potential of -80 mV when the solution was switched to Cd^{2+}-containing external solution to block Ca^{2+} influx via Ca^{2+} channels and hence I_C. Figure 4-13A and B illustrate the current responses from a cultured cell without NGF and a cultured cell with NGF for 15 days, respectively. I_K started to activate at -30 mV and increased with increasing depolarization. During the 50 ms depolarization, the current did not inactivate. The current-voltage relationship was not changed by NGF (Figure 4-13C). Figure 4-13D shows the time course of the I_K at +80 mV. I_K progressively decreased with time in the absence of NGF, but NGF failed to prevent the decrement of I_K.

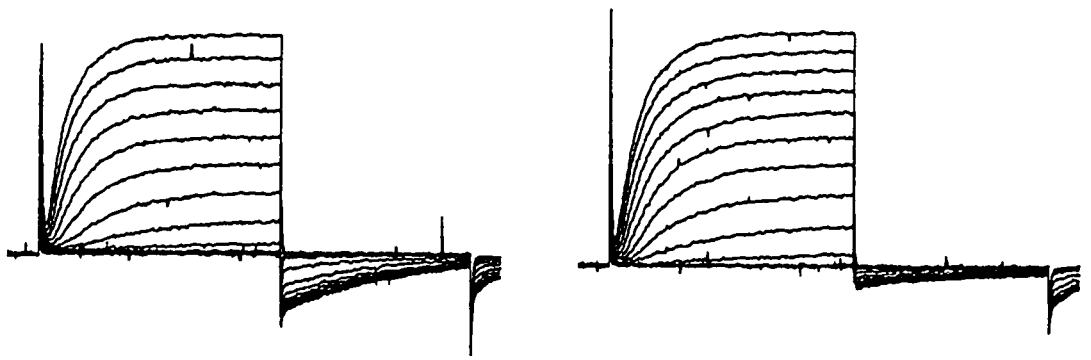
Figure 4-13. Lack of effects of NGF on I_K. A, B, I_K evoked by a series of 50 ms depolarizing pulses from -80 mV for a cell cultured without NGF for 15 days and a cell treated with NGF for 15 days, respectively. Protocol and scale bar are applicable to both cells. C, Current density and voltage relationship of 22 cultured cells in the absence of NGF for 15 days and 20 cells treated with NGF for 15 days. D, Time course of the current density. NGF did not influence I_K.

(Figure 4-13)



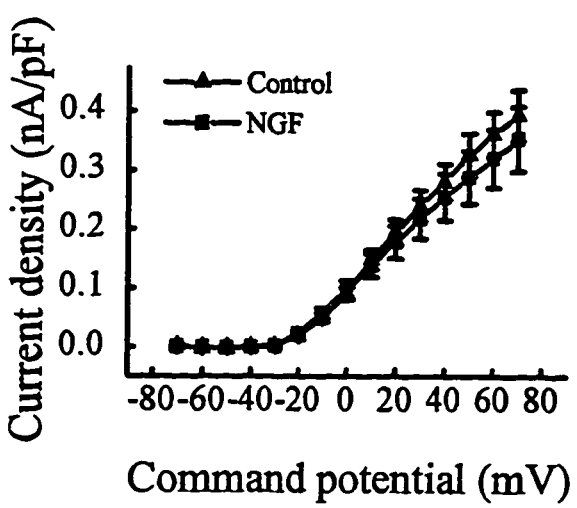
A. Control

B. NGF



C. I-V plot

D. Time course



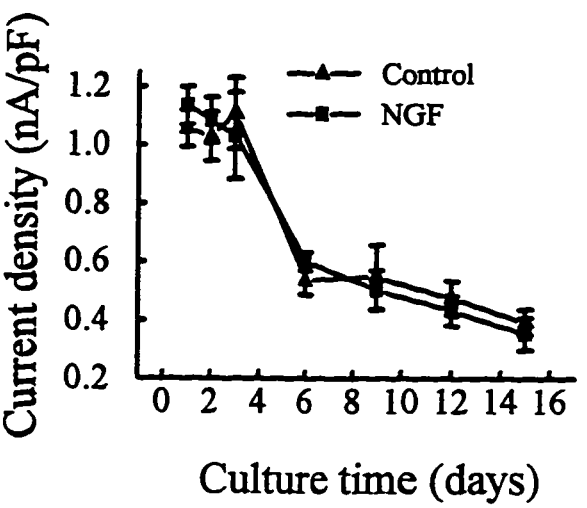
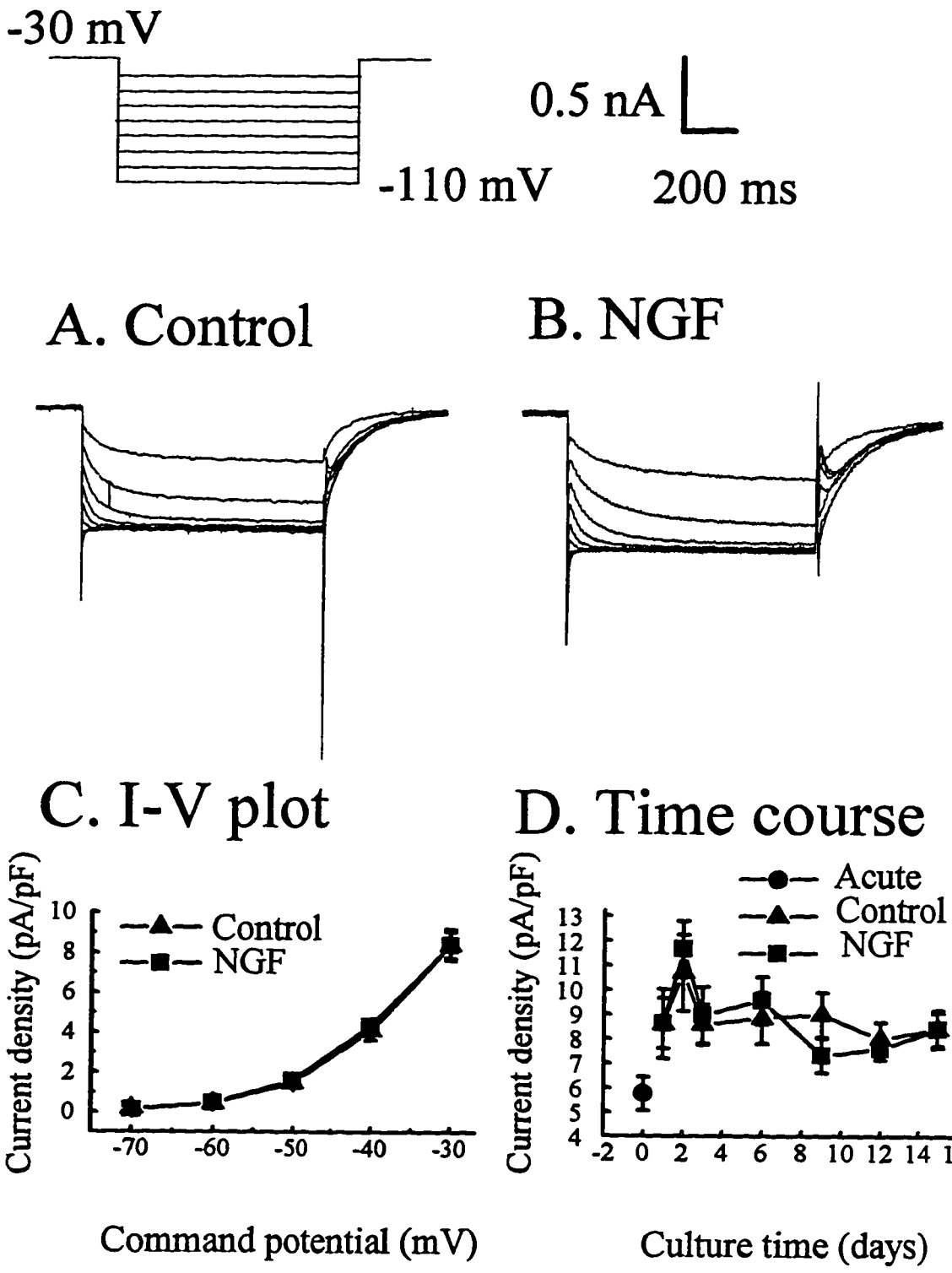


Figure 4-14. Lack of effects of NGF on I_M. I_M deactivation was evoked by a series of hyperpolarizing pulses from a holding potential of -30 mV for a cultured with NGF for 15 days (A) and a cell treated with NGF for 15 days (B). Protocol and scale bar are applicable to both cells. C, Current density and voltage relationship of I_M for 24 cultured cells without NGF for 15 days and 22 cultured cells with NGF for 15 days. D, Time course of I_M. NGF had no effects on I_M.

(Figure 4-14)


-30 mV
0.5 nA
-110 mV
200 ms
A. Control
B. NGF
C. I-V plot
D. Time course
Current density (pA/pF)
Control
NGF
Command potential (mV)
Acute
Control
NGF
Culture time (days)

I_M was not affected by NGF

I_M is a muscarine-sensitive, slowly activating and non-inactivating K^+ current (Brown and Adams, 1980; Adams *et al.*, 1982; 1986; Marrion *et al.*, 1992). Hyperpolarizing command pulses from a holding potential of -30 mV promote deactivation of I_M. The current then reactivates upon repolarization to -30 mV (Adams *et al.*, 1982). Figure 4-14A and B illustrate representive current responses evoked from a cultured cell without NGF for 15 days and a cultured cell with NGF for 15 days. There were no obvious differences of amplitude and kinetics between these two groups. Figure 4-14 C shows the current-voltage relationship of the I_M from the cultured cells with or without NGF for 15 days. Treatment of the cells with NGF did not change the current-voltage relationship of I_M. Figure 4-14D shows the time course for changes in I_M at -30 mV for cells cultured with or without NGF. There is an initial transient increase followed by a sustained increase in I_M over the 15 day experimental period. Inclusion NGF did not change the time course of the I_M.

Discussion

Effects of NGF on Na^+ channels

Our results show for the first time that voltage-gated Na^+ channels are subject to regulation by NGF in adult sympathetic neurons. The major effect of NGF on voltage-gated Na^+ channels was to prevent the gradual decline of Na^+ currents and current density (Figure 4-3A). NGF maintained both TTX-sensitive and TTX-resistant currents on the 15th day of culture, but NGF failed to affect the relative ratio of TTX-sensitive and TTX-resistant

components in the total currents. The activation or inactivation kinetics was not been changed significantly by NGF. The effects of NGF on Na^+ channel currents involved NGF-induced gene expression and possibly channel protein synthesis.

Comparison of the potency of NGF on Na^+ channel in BFSG neurons with its effect in PC12 cells shows that NGF response in PC12 cells is stronger than that in BFSG neurons. The reported increases in Na^+ channel density in PC12 cells after up to 21 days of NGF treatment range from 3- to 13-fold (D'Arcangelo *et al.*, 1993; Ginty *et al.*, 1992; Ifune and Steinbach 1990; Mandel *et al.*, 1988; Pollok *et al.*, 1990). A five-fold increase in sodium current can be detected as early as 24 h after NGF treatment (D'Arcangelo *et al.*, 1993). In BFSG neurons, there was an initial peak during the first 3 day of culture. Effects of NGF were not observed until the 6th day of culture by which time the Na^+ current had begun to decline. Furthermore, effects of NGF on adult BFSG neurons were not as potent as those on PC12 cells since from 6th to 15th day of culture, the current density was only about 1.5-fold higher than that of the corresponding cells cultured without NGF. The lower potency of NGF on Na^+ channel expression in BFSG neurons in comparison to PC12 cells may reflect different phenotypical states of these two types of cells. PC12 cells had never been in contact with NGF before experimental exposure to NGF and the effects of NGF are to differentiate the cells into sympathetic-like neurons and to generate excitability by inserting Na^+ channels into the plasma membrane. The sensitivity to NGF of these cells is likely to be higher than that of the adult BFSG neurons which receive a supply of neurotrophic factor from their targets *in vivo* (Kuffler and Megwinoff, 1994; Dobretsov *et al.*, 1994). Exogenous application of NGF in the system may just mimic the function of target to help maintenance of the neuronal

functions.

The effects of NGF on Na^+ channels are unlikely to be secondary to its beneficial effects on cell viability. First, NGF was not required for the survival of BFSG neurons in the culture system and NGF did not promote the increase in cell sizes or neurite outgrowth as judged by the cell capacitances. Second, the changes of the Na^+ channel currents deviated from those of the cell sizes (capacitances) in the absence of NGF. On the first day of culture, current increased by 92%, but the capacitance remained the same. On the second day of culture, the current increased 2.6-fold with only 39% increase in capacitance. On the sixth day of culture, the current decreased from the initial peak, but the capacitance reached maximal level (Figure 4-2B). These results suggest that two different signaling pathways controlled the expression of Na^+ channels and neurite generation and NGF has just influenced the signals controlling Na^+ channels in adult BFSG neurons.

Our results suggest that induction of gene expression in response to NGF was involved in the regulation of Na^+ channels because transcriptional inhibitors completely blocked the increase in Na^+ channel currents attributed to NGF. At this stage, we do not know whether the induced gene was the one encoding Na^+ channel proteins or other gene encoding some subsidiary proteins required for the function of Na^+ channels. In PC12 cells, NGF has been shown to selectively induce brain type II Na^+ channel mRNA (Mandel *et al.*, 1988; Fanger *et al.*, 1993; 1995; D'Arcangelo *et al.*, 1993). The type of Na^+ channel gene that was induced by NGF in adult BFSG neurons still remains to be determined.

In adult BFSG neurons, NGF did not change the activation and inactivation kinetics of the Na^+ channels and both TTX-sensitive and TTX-resistant components have been

induced by NGF. This conclusion was in accord with the results from PC12 cells in which NGF increases the functional Na^+ channels and induces TTX-resistant currents (Rudy *et al.*, 1987) and cultured rat chromaffin cells in which Na^+ channel current density resistant to 12 nM TTX has been increased by NGF treatment (Islas-Suárez *et al.*, 1994). The activation and inactivation kinetics of Na^+ channels from these cells were also changed by NGF.

Effects of NGF on K^+ channels

Of all the K^+ channels examined in BFSG neurons, only Ca^{2+}-activated K^+ channels (I_C) appeared to be affected by NGF. The significance of the regulation of I_C by NGF may reflect the role of NGF in the regulation of a.p. shape in cultured BFSG explants (Traynor *et al.*, 1992). NGF increased the amplitude of the afterhyperpolarization of the a. p., which may be due to NGF-induced increase in I_C.

Several mechanisms can be proposed to explain NGF-mediated regulation of I_C. First, the effects of NGF on I_C were a consequence of its effects on voltage-gated Ca^{2+} channel expression. This is possible because the I_C amplitude correlated well with that of the Ca^{2+} channel currents in NGF-treated cells (Figure 4-12). Second, activation of Trk A receptors by NGF stimulates phospholipase C-γ (PLC-γ) activity which in turn hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to produce two second messengers: diacylglycerol (DAG) which activates protein kinase C and inositol trisphosphate (IP3) which mobilizes intracellular Ca^{2+} (Kaplan and Stephens, 1994). More recently, NGF has been reported to evoke about a 3-fold increase in cytosolic free Ca^{2+} concentration via the activity of Trk A in C6-2B glioma cells (De Bernardi *et al.*, 1996). Even though, influx of

extracellular Ca^{2+} was supposed to be the major trigger for the activation of I_C, some possible subsidiary roles of intracellular Ca^{2+} release in the activation of I_C can not be fully excluded. NGF might increase I_C current by enhancing intracellular Ca^{2+} release. Third, NGF may directly increase gene expression and I_C channel protein synthesis or facilitate the function of dormant I_C channels. Further work needs to be done to distinguish these possibilities.

More recently, neurotrophin-3 (NT-3) and NGF have been demonstrated to activate calcium-dependent potassium channels (BK channels) in rat cortical neurons within several minutes after application of NT-3 or NGF (Holm *et al.*, 1997). The acute response was blocked by inhibitors of protein kinases, phospholipase C, and serine/threonine protein phosphatase 1 and 2a. Omission of Ca^{2+} from the extracellular medium prevented the NT-3 effects. These authors did not examine whether NGF or NT-3 acutely increases Ca^{2+} channel currents in these neurons. It is unknown whether the effects of NGF or NT-3 on BK channels are secondary to their effects on Ca^{2+} channels because NGF can also acutely increase Ca^{2+} channel currents in molluscan neurons (Wildering *et al.*, 1995).

In BFSG neurons, I_K was not affected by NGF, which is in contrast to the delayed rectifier K^+ channels in PC12 cells (Sharma *et al.*, 1993). In PC12 cells, NGF increases a delayed rectifier K^+ channel (kv2.1) polypeptide by 4-fold without changing kv2.1 mRNA (Sharma *et al.*, 1993). This increase in polypeptide level could be seen within 12 h and elevated levels were maintained for at least 6 days of continuous NGF treatment. However, RNase protection assays indicate that steady-state levels of kv2.1 mRNA following NGF treatment have not increased following NGF treatment, suggesting that mechanisms other than regulation of gene expression can affect ion channel expression in PC12 cells. By

contrast, treatment of PC12 cells with NGF failed to alter the total K^+ channel currents measured by whole-cell patch-clamp recording (Garber *et al.*, 1989). It is unkown whether the same situation existed for I_K in BFSG neurons.

NGF did not influence I_M in BFSG neurons. This is surprising because I_M can be regulated by numerous neurotransmitters or neuropeptides in BFSG neurons (for a review, see Smith, 1994). Lack of effects of NGF on I_M suggests that NGF selectively regulates those channels wich are important in the generation of a.p.

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Chapter 5

General Discussion

Introduction

In this thesis, the regulation of voltage-dependent ion channels (Ca^{2+}, Na^+ and K^+) in adult sympathetic neurons by NGF has been characterized. Figure 5-1 shows the overall time course for normalized Ca^{2+}(I_{Ca}), Na^+ (I_{Na}) and Ca^{2+}-dependent potassium (I_C) channel current density. The Ca^{2+} channel was used as an example to investigate the signal transduction mechanisms whereby NGF might exert long-term control of an ion channel. In this chapter, the results from different chapters will be connected and discussed in order to provide a comprehensive overview of the regulation of ion channels by NGF in an adult neuron.

The problem we sought to solve is whether or how NGF regulates voltage-dependent ion channels in adult neurons for which NGF is not required for neuronal survival. This question arose from some reports that NGF was able to regulate the a.p. shape in peripheral neurons (Chalazonitis *et al.*, 1987; Ritter and Mendell, 1992; Traynor *et al.*, 1991). These results suggested that the underlying ion channels were changed by NGF. Furthermore, treatment of PC12 cells with NGF causes them to differentiate into sympathetic-like neurons (Greene and Tishler, 1976) accompanied with a distinguishing neuronal trait, the appearance of an a.p. (Ditchter *et al.*, 1977; Rudy *et al.*, 1982). NGF also increases expression of Ca^{2+} (Streit and Lux, 1987; Garber *et al.*, 1989; Usowicz *et al.*, 1990; Lewis *et al.*, 1993; Cavalié *et al.*, 1994), Na^+ (Rudy *et al.*, 1982; 1987; Pollock *et al.*, 1990; Kalman *et al.*, 1990; Ginty *et al.*, 1992; D'Arcangelo *et al.*, 1993; Fanger *et al.*, 1993; Toledo-Aral *et al.*, 1995) and K^+ (Sharma *et al.*, 1993) channels in PC12 cells. These results suggest that during the differentiation of PC12 cells, one role for NGF is to induce voltage-dependent ion channels and furnish the cells with excitability. However, the function of NGF in adult, differentiated

neurons is likely to be different from its role on PC12 cells. With this in mind, we began to explore the effects of NGF on voltage-dependent ion channels in adult neurons.

Significance of NGF-mediated Ca^{2+} channel regulation

To examine the effects of NGF on Ca^{2+} channels *in vitro*, I developed a serum-free, defined medium culture system. NGF exerted three separate effects; it increased ω-conotoxin-GVIA-sensitive N-type currents; it increased nifedipine-sensitive L-type currents and decreased inactivation of the total Ca^{2+} channel conductance. NGF-mediated increase in the total Ca^{2+} current enables the current density to keep pace with the neurite outgrowth. The physiological relevance of *in vitro* effects of NGF on Ca^{2+} channel function was demonstrated in experiments that included injection of NGF or NGF antiserum *in vivo*. These data unambiguously show that NGF plays a role in the maintenance of Ca^{2+} channel currents in adult amphibian sympathetic neurons.

Previous data from mammalian dorsal root ganglion (DRG) had produced ambiguous results in that the *in vitro* effects of NGF on spike width (Chalazonitis *et al.*, 1987) were the converse of those seen *in vivo* (Ritter and Mendell, 1992). Furthermore, although an action of NGF on Ca^{2+} channels was implicated in its effects on DRG neurons *in vitro* (Chalazonitis *et al.*, 1987), this effect was not explicitly demonstrated. While it is clear that neurotransmitters (Dunlap and Fischbach, 1981; Wanke *et al.*, 1987; Tsien *et al.*, 1988; Lipscombe *et al.*, 1989) and second messengers (DeRiemer *et al.*, 1985; Rane and Dunlap, 1986; Gross and MacDonald, 1989) can rapidly and reversibly inhibit or enhance neuronal Ca^{2+} currents, very little is known about conditions that cause long-lasting changes in Ca^{2+}

channel function. As discussed in Chapter 2, this may be especially relevant to understanding the response of neurons to injury. It may also help to explain why different electrophysiological characteristics can be seen in different individual cells within a population of neurons. For example, the diversity in a.p. shape seen in sensory neurons (Rose *et al.*, 1986) may arise from differential activities of trophic factors on different subtypes of DRG neurons. Some aspects of the phenotypic expression, however, are probably pre-programmed at early developmental stages (Hall *et al.*, 1997). Our results indicate that NGF may be one of those factors that underlie the long-term regulation of Ca^{2+} channels. Furthermore, Ca^{2+} channels are important in the transfer of charge, the modulation of gene expression, the activation of enzymes or ionic channels, and the regulation of exocytosis and synaptic plasticity (Tsien *et al.*, 1988; Sheng and Greenberg, 1990). The capacity of NGF to regulate Ca^{2+} channels implies that NGF may also play a role in the regulation of the above neuronal functions.

The functions ascribed to the activation of Ca^{2+} channels occur either in the somata or in the axon terminals. Nerve terminals need enough Ca^{2+} channels to fulfill their primary function of neurotransmitter release. This requires localized influx of extracellular Ca^{2+} via voltage-dependent Ca^{2+} channels. The supply of an appropriate amount of Ca^{2+} to the transmitter release sites within fractions of a millisecond needs a high density of Ca^{2+} channel. Maintenance of a constant relationship between cell size and current density in the presence of NGF may therefore permit a better performance of the functions attributed to Ca^{2+} channels in the terminals. In addition, it has been proposed that Ca^{2+} plays a critical role in the survival of many neuronal populations (Johnson *et al.*, 1992; Murrel and Tolkovsky,

1993) and is involved in the effects of NGF on neurite outgrowth (Rogers and Hendry, 1990). However, we believe that NGF-mediated regulation of Ca^{2+} channels is irrelevant to the neuronal survival and the production of neurites in this system, because BFSG neurons survived well and generated neurites in the absence of NGF and extracellular Ca^{2+}(see Chapter 2 and 3).

It has been suggested that Ca^{2+} can activate distinct intracellular signalling pathways, depending on its mode of entry into neurons (Bading *et al.*, 1993). Activation of voltage-dependent Ca^{2+} channels stimulates the activity of growth factor receptor signals, Ras and MAPK (Rosen *et al.*, 1994; 1996). It is unknown whether NGF-induced increase in the expression of N-type and L-type Ca^{2+} channels aims at facilitating NGF-mediated activation of Ras/MAPK pathway.

Inactivation of Ca^{2+} channels in the axon terminals of magnocellular neurosecretory cells has been found to be slower than that in the somata (Fisher and Bourque, 1996). In BFSG neurons, NGF can attenuate the inactivation of Ca^{2+} channel currents and the cultured BFSG neurons grow processes. One possibility is that NGF-induced Ca^{2+} channels which are less inactivated are inserted into the growing neurites. Therefore, NGF-mediated induction of Ca^{2+} channels may be important for the facilitation of neuronal functions in the nerve terminals.

Inactivation of Ca^{2+} channel currents in BFSG neurons is modulated by phosphorylation by intracellular second messengers (Werz *et al.*, 1993). NGF can attenuate the inactivation of Ca^{2+} channel currents. NGF, thus, provides an example for exogenous ligand-mediated modification of Ca^{2+} channel inactivation. Furthermore, activation and

inactivation of Ca^{2+} channels has been shown to be related to the stoichiometry of expression of different subunits in the channel macromolecule. Heterologous expression of different subunits of L-type Ca^{2+} channels from skeletal muscle induces Ca^{2+} channels with different kinetic properties. Expression of α-subunit alone results in the Ca^{2+} channels with slow activation and inactivation kinetics whereas co-expression of α and β subunits induces channels with fast kinetics (Varadi *et al.*, 1991). It seems unlikely that NGF-mediated alteration of inactivation of Ca^{2+} channel currents in the present experiments reflects changes in the expression of different subunits of Ca^{2+} channels, because only inactivation, not activation kinetics were changed (see Chapter 2).

Signal transduction mechanisms for NGF-mediated Ca^{2+} channel regulation

The next question we have addressed is the mechanisms underlying NGF-mediated Ca^{2+} channel regulation. Chapter 3 presents the results. NGF can interact with any of the following processes to increase Ca^{2+} channel currents: (1) reduced inactivation of channels, (2) increased synthesis of channels, (3) decreased degradation of channels, or (4) unmasking of covert Ca^{2+} channels by phosphorylation mechanisms (Knox *et al.*, 1992; Conn *et al.*, 1989). Although NGF can attenuate the inactivation of Ca^{2+} channel currents, this effect cannot fully explain NGF-mediated enhancement of Ca^{2+} channel current because the level of Ca^{2+} current seen in the presence of NGF cannot be reached, no matter how negative a holding potential is used to remove inactivation (Figure 2-4E). Because the effects of NGF on the total Ca^{2+} channels can be completely blocked by transcriptional inhibitors (Figure 3-1), NGF-mediated enhancement of gene expression is the major reason for the increase in the

total Ca^{2+} channel currents induced by NGF. However, we cannot exclude the third possibility because altered gene expression may impair the mechanism of channel degradation. It is unlikely that the mechanism involves unmasking of a covert Ca^{2+} channels as described when *Aplysia* bag cells are treated with phorbol esters to activate protein kinase C (Knox *et al.*, 1992; Conn *et al.*, 1989) because this effect occurred within minutes, whereas the changes we saw took several days and therefore probably involved changes in protein synthesis.

The first step to dissect the signal transduction pathway is to discern which NGF receptor (Trk A and p75) is involved in NGF-mediated regulation of Ca^{2+} channel currents. Even in PC12 cells, this question has not been systematically addressed. Activation of tyrosine kinase ($pp60^{v\text{-}src}$) can mimic the effects of NGF on Ca^{2+} channels in PC12 cells (Lewis *et al.*, 1993). The tyrosine kinase inhibitor, genistein, can block the effects of FGF on Ca^{2+} channels (Rane and Pollock, 1994). Those results cannot indubitably prove that Trk A receptor is required for the induction of Ca^{2+} channel currents by NGF because it is possible that intracellular tyrosine kinase is required for NGF-mediated expression of Ca^{2+} channels. In our experiments, tyrosine kinase inhibitors and antibodies against Trk or p75 receptors have been used . The effects of NGF on Ca^{2+} currents in BFSG B-cells can be completely inhibited by tyrosine kinase inhibitors, genistein and lavendustin A, suggesting tyrosine kinase activity is required for NGF-mediated Ca^{2+} channel induction. Furthermore, the effects of NGF on Ca^{2+} channels can be mimicked by a bivalent antibody that activates mammalian TrkA, TrkB and TrkC receptors. However, p75 antibody has no effects on NGF-mediated Ca^{2+} channel regulation. These results suggest Trk A, not p75 is required for NGF-mediated induction of Ca^{2+} channel in adult neurons.

The downstream pathways of Trk A have been further dissected by specific inhibitors to individual signal proteins. The major finding from these experiments is that Ras/MAPK pathway is necessary for NGF-mediated Ca^{2+} channel induction. This pathway can interact with nucleus to affect gene expression. Our results indicate that NGF-mediated increase in Ca^{2+} channel currents depends on NGF-mediated induction of gene expression. This suggests that the effects of NGF on Ca^{2+} channel must be mediated via the nucleus.

At this stage, we do not know how MAPK interacts with the nucleus to increase Ca^{2+} channel current. However, several lines of evidence suggest that *c-fos* and *c-jun* are the possible targets for MAPK in the signal transduction pathway from receptor to membrane Ca^{2+} channels. First, treatment of the PC12 cells with NGF increases the levels of *c-fos* and *c-jun* (Cavalié *et al.*, 1994). Second, transfection of *c-fos* and *c-jun* can increase the Ca^{2+} channel currents in a time frame of days (Cavalié *et al.*, 1994). Third, recent progress in understanding MAPK-related signal transduction systems reveals that *c-fos* and *c-jun* are common targets of these systems (for a review, see Seger and Krebs, 1995).

Effects of NGF on Na^+ channels

The major function for Na^+ channels is to generate and propagate a.p. in the nervous system. In BFSG neurons, NGF can increase the Na^+ channel current and current density. That means the effect of NGF on Na^+ channels in BFSG neurons is to bestow the neurons with better ability to produce excitability since NGF also increases the functional activity of Ca^{2+} channels but not K^+ channels (Chapter 4), its effects seem to be primarily directed at augmenting the basic neuronal function of conduction of impulses and release of

neurotransmitters. In BFSG neurons, Na^+ channel is responsible for the upshoot of the a.p. NGF can increase the amplitude of the a.p. in cultured BFSG explants (Traynor *et al.*, 1992). This may reflect NGF-mediated increase in the Na^+ channel density.

The effects of NGF on Na^+ channel involved NGF-mediated gene transcription because the effects of NGF on Na^+ channel were completely blocked by transcriptional-inhibitors. At this stage, we do not know what kind of signal transduction pathways are involved in the NGF-mediated Na^+ channel induction. Activation of a variety of receptor tyrosine kinases increased Na^+ channel expression in PC12 cells (Fanger *et al.*, 1995). This result suggests that NGF-mediated increase in Na^+ channel expression is related to Trk A. However, the role of p75 in NGF-mediated Na^+ channel expression cannot be completely excluded because p75 can increase the production of cAMP (Dobrowsky *et al.*, 1994) and cAMP-dependent protein kinase is required (Kalman *et al.*, 1990) at a posttranslational level (Ginty *et al.*, 1992) for NGF-mediated increase in the number of Na^+ channels in PC12 cells. The downstream target of Trk A in NGF-mediated increase in Na^+ channel expression is unknown. Ras has been excluded (Fanger *et al.*, 1993). Recently, redundant intracellular pathways including PI-3-K, PLC-γ, GTPase activating protein (GAP) and Syp (a tyrosine phosphatase) have been proposed to be related to platelet-derived growth factor (PDGF)-mediated induction of Na^+ channels in PC12 cells (Fanger *et al.*, 1997). This raises the interesting possibility that different signaling pathways may mediate induction of Ca^{2+} and Na^+ channels by NGF in adult sympathetic neurons. Because of the differences of the phenotypic states between PC12 cells and differentiated, adult neurons, the signal transduction mechanisms for NGF-mediated induction of Na^+ channels in adult neurons need to be

investigated further. There also seems to be some mechanism whereby functional Na^+ channel expression increases as neurons grow in culture.

Effects of NGF on K^+ channels

NGF can increase Ca^{2+}-dependent K^+ current (I_C). This effect is possibly the consequence of its action on Ca^{2+} channels (Figure 4-12). In BFSG neurons, I_C contributes to the repolarization of the action potential and the generation of the early part of the afterhyperpolarization (Adams *et al.*, 1982; Pennefather *et al.*, 1985; Lancaster and Pennefather, 1987; Yamanda *et al.*, 1989). In cultured BFSG explants, NGF increased the amplitude of afterhyperpolarization (Traynor *et al.*, 1992). This may reflect the effects of NGF on I_C channels.

In cultured BFSG explants, NGF increased the a.p. duration (Traynor *et al.*, 1992). Changes in several channel properties can lead to the increase in a.p. width, including a decrease in the inactivation of Na^+ channels or a decrease in I_C channel currents. NGF did not influence the inactivation of Na^+ channels, yet increased rather than decreased I_C in dissociated BFSG neurons. Therefore, these two hypotheses must be rejected leaving us to search for an alternative explanation. N-type Ca^{2+} channels are proposed to be co-localized with I_C channels so that Ca^{2+} influx via them immediately activates I_C channels (Sah, 1995; Jassar *et al.*, 1994). The width of the a.p. produced in this way is likely to be narrow. However, L-type Ca^{2+} channels induced by NGF may be located at a distance from I_C channels. If there is a significant distance between L-type Ca^{2+} channels and I_C channels,

diffusion of the Ca^{2+} via L-type Ca^{2+} channels would take time to reach I_C channels. This may be the reason that the a.p. width is increased by treatment of NGF.

NGF failed to influence delayed-rectifier K^+ current (I_K) and muscarine-sensitive, non-inactivating K^+ current (I_M). In BFSG neurons, voltage-insensitive Ca^{2+}-activated K^+ current (I_{AHP}) also regulates the duration of the afterhyperpolarization. Because I_{AHP} cannot be recorded by whole-cell patch-clamp recording in dissociated cells, (Jassar *et al.*, 1994), we do not know whether NGF influences this channel.

Significance of NGF-mediated regulation of ion channels in adult neurons

Whilst it is well-known that NGF is a trophic molecule essential for the survival of peripheral and some central neurons during development, the role of NGF in the maintenance or regulation of differentiated phenotypes of mature peripheral neurons is much less clear. Several lines of evidence suggest that NGF is not required for the survival of adult peripheral neurons *in vitro* (Lindsay, 1988; Kuffler and Megwinoff, 1994; Dobretsov *et al.*, 1994). Sequestration of endogenous NGF by NGF antibodies appears to have no effect upon neuronal survival in the adult dorsal root ganglia (DRG) (Gorin and Johnson, 1980; Rich *et al.*, 1984). These results are in accordance with our results that the survival of adult BFSG neurons is independent of exogenous NGF. However, we cannot exclude the possibility that neurons can generate endogenous neurotrophic factors which can be secreted in to the medium (autocrine) (Wilkinson *et al.*, 1989; Elde *et al.*, 1991; Schnürch and Risau, 1991; Ernfors *et al.*, 1990; Wetmore *et al.*, 1991; Acheson *et al.*, 1995; for a review, see Korsching, 1993). Furthermore, the neurotrophic factors synthesised by a neuron may be kept inside the

neuron and used by itself (intracrine) (Rifkin and Moscatelli, 1989; Vlodavsky *et al.*, 1991). We do not know whether this is the case for BFSG neurons.

Two very general aspects of the results should be addressed here. The first is the rather vague notion that the inclusion of NGF in culture simply makes the neurons more 'healthy' and this improved 'health' of the cultures is reflected in increased channel activity. We think this is unlikely for two reasons, 1) NGF only affects Na^+ and Ca^{2+} channels, but K^+ channels are unaffected; 2) treatment of neurons *in vivo* with NGF also increases Ca^{2+} current, i. e. NGF increases Ca^{2+} current in neurons *in vivo* that are presumably already quite 'healthy'.

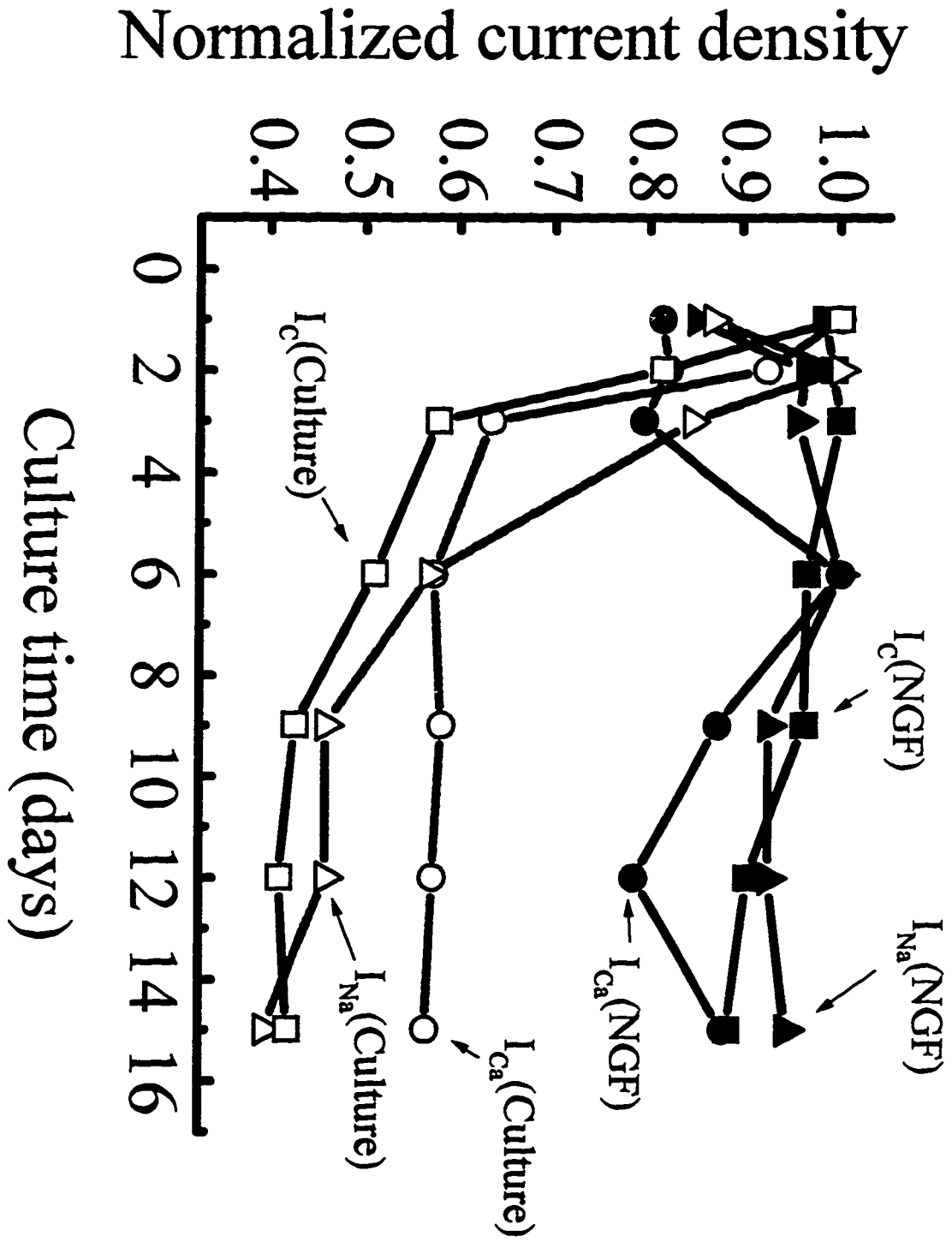
The second idea is that effects of NGF on ion channels reflect its primary action and that other maintenance or growth-inducing effects occur downstream of this action. It has for example been suggested that actions of ras/raf on a K^+ channel are required for mitogenic signaling in murine fibroblasts (Huang and Rane, 1994). K^+ channels do not however seem to be involved in the action of NGF in BFSG as they are essentially unaffected.

NGF still plays a continuous dynamic role in the regulation of normal physiological homeostasis in adulthood. Adult peripheral neurons still express the high-affinity NGF receptor (Yamamoto *et al.*, 1993; Vega *et al.*, 1994) and continue to retrogradely transport NGF (Stoeckel *et al.*, 1975). NGF administration can prevent neuronal death and atrophy following nerve injury (Rich *et al.*, 1987). Furthermore, variation of NGF level leads to the changes in neuropeptide expression in adult sensory neurons (Lindsay and Harmar, 1989; Lindsay *et al.*, 1989; Schwartz *et al.*, 1982; Kessler and Black, 1980; Otten *et al.*, 1980) and pelvic ganglion neurons (Tuttle and Steers, 1992). NGF was also shown to prevent neurotoxic effects of some anticancer drugs on adult peripheral neurons (Hayaka *et al.*, 1994;

Apfel *et al.*, 1991). In this thesis, we further enlarged the scope of NGF-mediated regulation of physiological functions in adult neurons. NGF also regulates the properties of voltage-dependent ion channels in adult neurons. NGF-mediated regulation of voltage-dependent ion channels may underline the physiological functions attributed to NGF.

Figure 5-1. Time course for normalized Ca^{2+}(I_{Ca}), Na^{+} (I_{Na}) and Ca^{2+}-dependent potassium (I_C) channel current density in the presence (filled symbols) and absence (emty symbols) of NGF. The means of the individual current density were normalized to the maximal value to show the relative relationship of the three channels.

Figure 5-1


Normalized current density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0 2 4 6 8 10 12 14 16
Culture time (days)
I_C(Culture)
I_{Na}(Culture)
I_{Ca}(Culture)
I_C(NGF)
I_{Ca}(NGF)
I_{Na}(NGF)

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