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THE UNIVERSITY OF ALBERTA

PHARMACOLOGICAL MODULATION OF TRANSMITTER RELEASE IN MOTOR NEURONS

BY

HSINYO CHEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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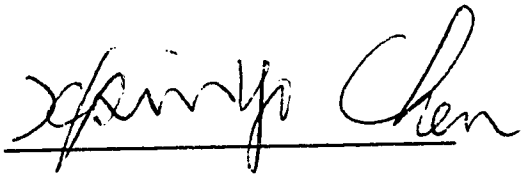

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ABSTRACT

Some presynaptic heteroreceptors on the mouse phrenic nerve-diaphragm were identified by using conventional microelectrode methods. Mepp frequency was monitored as the parameter of quantal transmitter release from the motor nerve terminal. The signal transduction mechanisms underlying presynaptic receptor activity were also investigated.

Adenosine analogues (L-PIA, NECA, 2-ClA, and CV-1674) inhibited transmitter release in a concentration-dependent manner and could be antagonized by theophylline, thus confirming the presence of a presynaptic adenosine receptor. The concentration-effect relationships of the four drugs formed two parallel pairs with a potency order of L-PIA > NECA and 2-ClA $\geq$ CV-1674. The effect of 1 μM 2-chloroadenosine was abolished by preincubation with pertussis toxin and also by H-7, suggesting the involvement of a G_i protein. The evidence was consistent with an A_1 receptor acting through G_i, to inhibit adenylate cyclase.

Adrenocorticotropic hormone induced a sustained increase in mepp frequency already accelerated by exposure to 15 mM K^+. This stimulant effect was not affected by lithium, but potentiated by the pretreatment with Pertussis toxin and blocked by the prior treatment with H-7 and A-3. This was taken to indicate the involvement of G_s protein and protein

kinase A in the transduction sequence.

Epinephrine, norepinephrine and phenylephrine caused an increase in accelerated transmitter release in a concentration -dependent manner. Furthermore, this excitatory effect of epinephrine was blocked by prazosin but not by yohimbine and nadolol, suggesting the presence of an alpha-1 adrenoceptor on the motor nerve terminal. The enhanced release of quanta caused by norepinephrine was blocked by nonspecific protein kinase inhibitors (e.g. clomiphene, polymyxin B, auranofin) but not by H-7 and Pertussis toxin. The effect was inhibited by W-7, but further increased in the presence of lithium. The suggested transduction sequence involves a G_p protein, which in turn triggers the production of inositol triphosphate and mobilizes calcium from an intracellular pool to activate a calmodulin dependent process.

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CHAPTER I

INTRODUCTION

1. THE MOTOR NERVE TERMINAL

The neuromuscular junction in vertebrate skeletal muscle can be divided into two major components: the presynaptic terminal and the postjunctional endplate. Each neuronal axon branches repeatedly within the target muscle and so each motor neuron has several nerve terminals. Each axon is covered with a myelin sheath, which extends to the terminal branches lying between the muscle fibers, and ends immediately before the nerve terminal (Ellisman et al., 1976). The bulblike nerve terminal expansions are a highly specialized region of the neuron, and are characterized by the presence of synaptic vesicles containing transmitter, which are concentrated near the presynaptic membrane (Heuser and Reese, 1977). In addition, the nerve terminals also contain microtubules (Gray, 1975), neurofilaments, large numbers of mitochondria to meet the metabolic demands of synthesis and release of acetylcholine (ACh), and endoplasmic reticulum which stores calcium and may have a role in the replenishment of axonal membrane and, because of its geometric association with vesicles (De Robertis and Bennett, 1955), function in the formation of synaptic vesicles (Heuser and Reese, 1973; Droz et al., 1975).

Synaptic vesicles are also formed in the cell bodies and transported to the terminal by fast axonal flow (Dahlstrom,

1967). Vesicles are seen associated with microtubules (Gray, 1975) which supports the suggestion that vesicles are transported down the axon and to the active zones via microtubules (Smith et al., 1970; Gray, 1975).

The synaptic vesicle is spherical in shape, and 20 to 40 nm in diameter with a less dense center and dense periphery. Most are without a discernible internal structure, though some are granular in appearance (De Robertis and Bennett, 1955). At mouse and rat neuromuscular junctions, the vesicle is about 40-50 nm in diameter, and the density is about 1600-2750 vesicles/μm^3 (Andersson-Cedergren, 1959; Hubbard, 1970; Jones and Kwanbunbumpen, 1970). In rat phrenic nerve terminals, the average number of vesicles was estimated to be 270,000 $\pm$ 70,000 (Elmqvist and Quastel, 1965; Hubbard et al., 1969).

In the Torpedo electric organ, a vesicle is a highly hydrated core with approximately 0.5 M (or 40,000 molecules) ACh solution, 0.2 M ATP and Ca^{2+}, a pH of about 5.1-5.7 and enveloped with lipoprotein membrane consisting of at least five specific protein constituents including actin, an ADP/ATP exchange carrier similar to the ATP carrier in mitochondria, a Ca^{2+},Mg^{2+}-activated ATPase, an ACh transporter, glucosaminoglycan (vesiculin) which has a role in binding Ca^{2+} and ACh by electrostatic attraction to its negative charges, and synapsin I, a calmodulin-associated vesicular protein

(Whittaker, 1971; 1982; Fuldner and Stadler, 1982; Nestler and Greengard, 1983; 1985).

Both within the living terminal and when terminals are disrupted either experimentally or naturally, vesicles play a role in protecting ACh from destruction by acetylcholinesterase (E.C.3.1.1.7; AChE), whereas the soluble ACh is accessible to AChE and is hydrolyzed (Whittaker et al., 1963; Marchbanks and Israel, 1971).

The presynaptic membrane has a series of dense bars attached to its inner surface. These have been termed "active zones" (Couteaux and Pecot-Dechavassine, 1970) and are areas of dense osmium staining lying opposite postjunctional folds (Andersson-Cedergren, 1959; Birks et al., 1960; Hubbard, 1970). Synaptic vesicles cluster along these dense bars and it is suggested that these represent sites of quantal release of neurotransmitter.

2. PHYSIOLOGY OF NEUROMUSCULAR TRANSMISSION

2.1. SYNTHESIS OF ACH

The content of ACh in the Torpedo electroplax and rat phrenic nerve terminals is quite constant whether the terminal is at rest, incubated with choline or during nerve stimulation. This is due to the efficiency of choline uptake

and hence synthesis of ACh (Potter, 1970).

At nerve terminals, ACh is synthesized by the cytoplasmic enzyme, choline acetyltransferase (E.C. 2.3.1.6; CAT), which combines choline with an acetyl group from acetyl coenzyme A (acetyl CoA) and hydrolyzed by AChE which is distributed around the junction.

Acetyl CoA is derived from a metabolic pathway, based in the mitochondria but depending on a putative carrier mechanism to transport suitable intermediates from the mitochondrion into the cytoplasm for reconstitution as acetyl CoA (Bowman, 1990; Cooper et al., 1986). Choline, an endogenous compound, is believed to be made primarily in the liver and transported to other organs through the blood and spinal fluid, and is presumably also the breakdown product of phospholipids (e.g. phosphatidylcholine, sphingomyelin, and plasmalogens) which are present in the membranes of all cells including nerve terminal membrane (Diamond, 1971; Yamamura and Snyder, 1972; Blusztajn and Wurtman, 1983). However, the majority of choline comes from the liberated ACh which is hydrolyzed by AChE (Perry, 1953; Collier and MacIntosh, 1969), though some choline was believed to be taken up by muscle fibers and metabolized to phosphatidylcholine or phosphorylcholine and incorporated into membrane (Collier and Lang, 1969; Wessler and Sandmann, 1987). About 50 to 60% of liberated choline

from cat superior cervical ganglion enters the nerve terminal (Collier and Katz, 1974) by either a sodium co-transport (sodium-dependent), high affinity (K_m=1-5 μM) active (Na^+,K^+-ATPase-required) uptake system or low affinity (40-80 μM) passive diffusion transport, the proportional contribution of each depending on the choline concentration (Yamamura and Snyder, 1972; Blusztajn and Wurtman, 1983). Both the high and low affinity choline transport mechanisms were demonstrated to be inhibited by hemicholinium-3 (HC-3), a choline analogue, with potent (K_m=0.05-1 μM) and weak (K_m=10-120 μM) inhibitory actions respectively (MacIntosh, 1961; Potter, 1970).

The level of ACh can be regulated by at least three factors:

(1) The availability of choline and acetyl CoA (Jope and Jenden, 1977; Kuhar and Murrin, 1978).

(2) The availability of CAT (Kaita and Goldberg, 1969).

(3) Neuronal activity and drugs (e.g. HC-3).

Stimulation of the nerve terminal increases choline uptake and thus accelerates ACh synthesis and release (Collier and MacIntosh, 1969; Hubbard, 1971). The ACh formed in the neuron is believed to exert a feedback mechanism on choline uptake, i.e., high affinity uptake of choline is inhibited by excess of ACh (Kuhar and Murrin, 1978) and on CAT that synthesizes ACh (Kaita and Goldberg, 1969).

2.2. UPTAKE OF ACH INTO VESICLES

In Torpedo electric organ, synthesized ACh exists initially in solution in the cytosol (46% of total ACh) and is transferred to synaptic vesicles (54% of total ACh). It was demonstrated that a sodium, potassium, chloride-independent mitochondrial-like ATPase is present in the isolated Torpedo vesicle membrane (Breer et al., 1977; Batteiger and Parsons, 1986). Because this is the only Ca^{2+},Mg^{2+}-activated ATPase in Torpedo cholinergic synaptic vesicles, Breer et al., (1977) suggested that this cytoplasmically oriented ouabain-insensitive ATPase is an ATP-dependent proton pump which is responsible for the ACh transport.

Harlos et al., (1984) confirmed the presence of a proton-translocating ATPase and proposed that it is probably located on the outer surface of vesicles. The ATPase pumps protons into the vesicle to produce an internal proton gradient (Anderson et al., 1982; Fuldner and Stadler, 1982; Harlos et al., 1984) and a separate ACh transporter is hypothesized to draw on the positively charged proton gradient to drive ACh uptake via the exchange of a proton for an ACh molecule (Marshall and Parsons, 1987). These two activities certainly are attributable to two different molecules (Anderson et al., 1982), for example, a mercurial compound blocks the active ACh transport without affecting ATPase activity (Parsons and

Koenigsberger, 1980). In the absence of Mg^{2+} and ATP, ACh uptake is nonspecifically and electrophoretically carried out by a passive transport process (Carpenter et al., 1980). The transporter is known to be inhibited with a good degree of specificity by three compounds, 2-(4-phenylpiperidino) cyclohexanol (AH5183, vesamicol) which is a tertiary amine, quinacrine, and tetraphenylboron (Anderson et al., 1983). None of the above inhibitors, however, affected ATPase activity despite their inhibition of the ACh transport system. Vesamicol interferes with the ACh transport system by blocking intravesicular ACh uptake. Vesamicol was ineffective in causing any acute change in miniature endplate potential (mepp) amplitude at resting neuromuscular junctions indicating its lack of postsynaptic effect. In contrast, when the nerve was stimulated rapidly over a prolonged time interval, it caused a reduction in mepp amplitude is due to a reduction in quantal size, leading to the conclusion that the effect is presynaptic in nature (Van der Kloot, 1986; Whitton et al., 1986). However, it was unclear whether vesamicol might interfere with either the ACh transport system by binding to the allosteric site of the ACh transporter or act on an unknown mechanism that is related to the ACh transporter (Marshall and Parsons, 1987). Recent evidence suggests that the transporter molecule is a proteoglycan rather than the more conventional glycoprotein (Parsons, 1990).

2.3. STORAGE OF ACH

The synthesized ACh seems to be stored in a heterogenous pool (Dowdall and Zimmermann, 1974; Zimmermann and Whittaker, 1977). At least two populations of vesicular stores of ACh were isolated by using the sucrose density zonal gradient technique (Zimmermann and Denston, 1977a). Vesicles are either readily available for transmitter release (VP_2, 20%) or consist of reserves (VP_1, 80%) which are mobilized slowly at rest (Birks and MacIntosh, 1961; Whittaker, 1968; Israel et al., 1979; Weiler et al., 1982).

Vesicles close to the membrane are believed to contain the immediatedly available store (IAS) of ACh which is enriched with newly synthesized ACh, as the newly formed (labelled) ACh is preferentially released in Torpedo electric organ, cat superior cervical ganglia and rat phrenic nerve after repetitive nerve stimulation (Collier and MacIntosh, 1969; Hubbard, 1970; Barker et al., 1972). When the rate of nerve stimulation was increased to a higher frequency (20 Hz), the release was significantly decreased, implying the incomplete mobilization of ACh from the reserve store to the IAS (Collier, 1969; Potter, 1970; Zimmermann and Whittaker, 1974). Recycled vesicles (VP_o), which are empty and in the course of migration inwards from the presynaptic membrane, are more likely to acquire newly synthesized ACh and hence, by

virtue of their location, are released even though they may be incompletely filled and osmotically shrunken in size (Hubbard, 1970; Barker et al., 1972; Zimmermann and Whittaker, 1977; Suszkiw et al., 1978; Whittaker, 1986). On the other hand, vesicles at a distance from the presynaptic membrane are less able to approach the membrane and release their contents than adjacent ones.

Del Castillo and Katz (1954b) proposed that the number of quanta released by nerve stimulation (m-the quantal content of the end plate potential or epp) represented only a proportion of a store of quanta present in the terminal. Elmqvist and Quastel (1965) estimated the total store in phrenic nerves to be comprised of 2.7×10^5 quanta, and the number of quanta released by a nerve impulse after a period of rest is presently estimated to about 40 (Glavinovic, 1979b). Thus the loss from the terminal caused by a single episode of evoked release is a very small fraction of the total content of transmitter, and yet significant depression of transmitter release during tetanic stimulation has long been recognised (Liley and North, 1953; Liley, 1956b; Brooks and Thies, 1962; Hubbard, 1963; Hubbard and Wilson, 1973; Fletcher and Forrester, 1975; Wilson, 1979; Glavinovic and Narahashi, 1988). The larger the quantal content of an initial epp, the more the IAS is depleted and the greater the effect on subsequent epp's (Glavinovic and Narahashi, 1988). Experiments

using pulse pairs revealed that maximum depression was observed with between-pulse intervals of 200-400 msec. Shorter intervals produced a lesser depression, while at longer intervals the depression apparently recovered (Thies, 1965). The responses at shorter intervals were thought to be elevated due to residual Ca^{2+} in the cytosol left over from the initial conditioning pulse, while the recovery in transmitter release seen at longer interpulse intervals was attributed to mobilization from deeper stores.

An additional impediment to the free migration of vesicles within the terminal may be the physical attachment of many if not most vesicles to the cytoskeleton. The evolution of this hypothesis rests on studies on the phosphoprotein, synapsin I, by Greengard and his coworkers over the past 18 years. Synapsin was associated with synaptic vesicles from its discovery and binds to the cytoplasmic surface of the vesicle with high affinity (K_d = 10 nM). Synapsin also binds to actin, spectrin, neurofilaments and microtubules, all constituents of the cytoskeleton of most cells including neurons (Baines and Bennett, 1985, 1986; Goldenring et al., 1986; Bahler and Greengard, 1987). Synapsin is in addition, a substrate for a number of kinases. Protein kinase A (cyclic AMP dependent) and Ca^{2+}/calmodulin dependent kinase I phosphorylate at a site located in the globular head of the molecule, while Ca^{2+}/calmodulin dependent kinase II acts at a

site on the collagen-like tail (Ueda and Greengard, 1977; Huttner et al., 1981, 1983). Phosphorylation of synapsin I cleaved the molecule from both its binding site on the synaptic vesicle and from actin and this phosphorylation is observed under conditions in which depolarization and Ca^{2+} entry occur (Greengard and Browning, 1988; Sihra et al., 1989). Initially it was suggested that phosphorylation might be associated with release of transmitter itself (Browning et al., 1985), but release occurs faster than a process requiring phosphorylation of proteins (Almers, 1990). Greengard later suggested that phosphorylation was but a priming step that prepared vesicles for release (Greengard and Browning, 1988). Evidence in support of this supposition came from experiments in which organelle movement in axoplasm was decreased by injecting dephosphosynapsin I but not phosphorylated synapsin I (McGuiness et al., 1989). In other experiments, the quantal content of epsp's resulting from transmitter release from the goldfish Mauthner axon was reduced following injection of dephosphosynapsin I in a manner that was consistent with the removal of quanta from the immediatedly available store (Hackett et al., 1987).

Thus the evidence for two stores of transmitter may have an identifiable basis in vesicles immobilized to the cytoskeleton by dephophosynapsin I forming the immobile or reserve store, and vesicles liberated after the

phosphorylation of synapsin I, which are capable of movement within the nerve terminal and form the immediately available store.

2.4. CO-TRANSMITTERS

Silinsky and Hubbard (1973) reported that after high frequency stimulation of rat phrenic nerve, ATP was released concomitantly with ACh from nerve terminals. This was the first evidence demonstrating the co-storage of transmitters in motor nerve terminals. In addition to rat phrenic nerve terminals, cholinergic synaptic vesicles isolated from the Torpedo electric organ also contain ATP (Dowdall et al., 1974). Whether ACh coexists with ATP in the same vesicle is not known.

By using immunocytochemical methods, Matteoli et al. (1988) demonstrated that two types of vesicles, different in size, co-exist in the frog motor nerve terminals. The small synaptic vesicles (SSV) which are 40-60 nm in diameter contain classical neurotransmitter ACh, whereas the large dense-core vesicles (LDCV), which are 80-120 nm in diameter, contain the calcitonin gene-related peptide (CGRP). However, LDCV's represent only about 1% of the total vesicle population.

CGRP, which is a 37 amino acid polypeptide, is the

product of a distinct messenger RNA (mRNA) found in neural tissue, in contrast to the 32 amino acid polypeptide, calcitonin secreted by the thyroid gland (Amara et al., 1982; Rosenfeld et al., 1983).

CGRP enhanced the contraction produced by indirect or direct stimulation at the mouse or rat diaphragm, and it was supposed that CGRP might act on both pre- and post-synaptic junction (Takami et al., 1985; Ohhashi and Jacobowitz, 1988).

The distribution of large number of CGRP-containing neurons and binding sites throughout the central nervous system (CNS) (Rosenfeld et al., 1983; Tschopp et al., 1985) and co-release of the peptide with ACh from the frog motor neurons (Matteoli et al., 1988) suggested that CGRP acts as a peptide neurotransmitter or neuromodulator and may have a role in neuromuscular transmission (Ohhashi and Jacobowitz, 1988).

2.5. RELEASE OF ACH

2.5.1. Measurement of release

The interaction of released ACh with postsynaptic nicotinic receptors on the endplate which are concentrated at the postjunctional region of muscle induces receptor channel openings, each of which provides a measurable, small,

transient net inward current of sodium that causes a depolarization of the membrane. Collectively these single channel events form the conventional quantal phenomena termed miniature endplate potentials (mepp) and currents (mepc) (Fatt and Katz, 1950, 1952; del Castillo and Katz, 1955; Boyd and Martin, 1956b; Katz and Miledi, 1970; 1971). Evoked release of transmitter following nerve stimulation can be measured at the postsynaptic site as an endplate current (epc) or potential (epp).

2.5.2. Quantal release of ACh

The quantal theory of synaptic transmission was proposed by Del Castillo and Katz in 1954 to explain the phenomena that had been observed. Simply stated, the quantum is the basic unit of transmitter release and is recognized as the mepp. The epp was composed of the additive response to simultaneous release of numerous quanta (Del Castillo and Katz, 1954b; Boyd and Martin, 1956a, Liley, 1956b). The arrival of an action potential caused the synchronous release of quanta of ACh, causing a transient potential change which is detectable as the epp. The number of quanta which are released in this way at any one time may range from 4 in a small terminal to as much as 400 in a large terminal (Del castillo and Katz, 1954b; Boyd and Martin, 1956a, Kuno et al., 1971).

(a) The vesicular hypothesis. Del Castillo and Katz (1957) unified their quantal theory with the electron microscopic observation of vesicles (De Robertis and Bennett, 1955, Whittaker, 1971) and propounded the "vesicle theory of transmitter storage and release"; i.e., a vesicle not only contains the neurotransmitter but represents the morphological counterpart of a single quantum. A quantum of ACh was calculated to consist of approximately $1x10^3$-10^4 molecules (Whittaker and Sheridan, 1965; Whittaker, 1971; Whittaker et al., 1972; Kuffler and Yoshikami, 1975).

By using the quick-freezing technique, Heuser (1978) showed that, at frog nerve terminals, depletion of synaptic vesicles by rapid nerve stimulation was associated with the appearance of vesicles apparently fused with the terminal membrane. The contents of vesicles were assumed to be released by exocytosis.

Evidence for the recycling of the empty vesicle after liberation of the contents came from the use of horseradish peroxidase, an extracellular marker, which was located within some vesicles after periods of nerve stimulation (Heuser and Reese, 1973; 1981; Heuser, 1978). Zimmermann and Denston (1977b) strengthened this finding by observing that empty vesicles take up dextran into the lumen during recycling.

The number of vesicles close to the presynaptic membrane at rat diaphragm-phrenic nerve junction is increased after prolonged low frequency stimulation presumably due to the rapid mobilization of vesicles to the release sites. By contrast, vesicles fall in number after prolonged high frequency stimulation because the supply of vesicle appeared not to keep pace with the depletion of vesicles (Jones and Kwanbunbumpen, 1968; Hubbard and Kwanbunbumpen, 1968).

Such evidence collectively is consistent with the vesicular hypothesis of transmitter release which is widely accepted by most research workers.

(b) The cytoplasmic hypothesis. Whittaker (1968), and Israel et al., (1979) demonstrated that in electric organ of Torpedo, ACh is found not only in the synaptic vesicles but also in a cytoplasmic pool.

A continuing controversy was established when these and other authors took the view that quanta originated not from exocytosis of vesicles, but from release of ACh directly from the cytosolic store via a gated channel (vesigate, gated release of cytoplasmic ACh through a channel) or release operator (mediatophore, neuromediator transport system) in the presynaptic membrane (Israel and Dunant, 1979; Tauc, 1982). This somewhat radical proposal stemmed from the

conclusion that some data seemed inconsistent with the vesicular hypothesis. For example, although electron micrographs of stimulated nerve terminals showed vesicles fused with the axolemma, this was a rare event. Expansion of the terminal area in the superior cervical ganglia due to vesicular fusion with the axolemma was also rarely observed (Birks, 1974). Furthermore, CAT was found in the cytoplasm but not in the vesicle (Fonnum, 1966) and the isolated vesicles do not synthesize ACh (Suszkiw, 1976). Thus, as ACh was synthesized in cytoplasm, cytoplasmic ACh, but not vesicular ACh, was supposed to be preferentially released and synthesized when the nerve was stimulated (Dunant et al., 1972). It was postulated that depolarization of the nerve terminal led to the influx of calcium that, in turn, bound to and acted on the ACh channels or protein molecule associated with ACh channels (vesigates) in the membrane. When the vesigates were saturated with cytosolic ACh, the cytoplasmic ACh was liberated in a quantal manner. If nerve stimulation was prolonged, the vesicular ACh began to supply the cytoplasmic store and the empty vesicles sequestered the accumulating calcium. Thereafter the nerve terminal returned to its resting state when the calcium was buffered via different mechanisms (Dunant et al., 1972; Dunant and Israel, 1985). In addition, Tauc et al. (1974) had demonstrated that two hours after the injection of AChE into the cholinergic neuron of the buccal ganglion of Aplysia, the postsynaptic

potential decreased and gradually disappeared. He reasoned that AChE destroyed the cytoplasmic but not the vesicular ACh, the postsynaptic potential itself was due to the cytoplasmic ACh. Therefore, Marchbanks (1978) suggested the cytoplasmic ACh might play an important role in neurotransmission.

However, this hypothesis was challenged by other electrophysiological evidence. Using the patch clamp technique, Neher and Marty (1982) and Fernandez et al. (1984) demonstrated that when the exocytosis occurred, the fusion of mast cell or adrenal chromaffin cell granules with the plasma membrane causes an increase in surface area resulting in an increase in membrane capacitance (C_m). The stepwise increases in membrane capacitance reflects an electric connection between the cell exterior and the vesicle interior, but not the cytoplasm, when membrane fusion begins with the formation of a fusion pore.

In 1987, Young and Chow were unable to demonstrate neuronal membrane channel currents (>2.5 pA) coincident with transmitter release or the peak of the mepps in a cultured Xenopus nerve-muscle system. If such a transmitter channel did exist in the presynaptic membrane, single channel openings should correlate with the occurrence of mepps.

In the face of this evidence, the cytoplasmic hypothesis

of transmitter release finds little support, and the experiments described in this thesis will be interpreted in accordance with the conventionally accepted vesicular hypothesis.

2.5.3. Abnormal release

(a) Non-quantal release. Katz and Miledi (1977) detected what appeared to be a continuous leakage of molecules of ACh from resting frog nerve terminals through a Ca^{2+}-dependent mechanism. The leakage occurred spontaneously and was not enhanced by the arrival of a nerve impulse (Katz and Miledi, 1981). The leakage was estimated to result in a concentration of 10^{-8} M ACh in the synaptic cleft and, in the presence of anticholinesterase, it caused a minor depolarization of the end plate (average 40 μV in the frog and 0.1-1 mV in the mouse). When the postjunctional membrane was curarized, a small hyperpolarization was seen. The hyperpolarizing response (H effect) was the result of the temporary depression of a steady depolarization (Dunant, 1986). From studies at mouse neuromuscular junctions, Vyskocil and Illes (1977) reported that the hyperpolarization was further increased either in K^+-free solution or in the presence of ouabain which inhibited ATPase. Therefore, Vizi and Vyskocil (1979) suggested that membrane ATPase activity might play a role in regulating the non-quantal release.

The exact role of non-quantal release is still unknown. Quantal release (measured as mepp frequency) was greatly increased while the total ACh output was increased only slightly when 2 Hz stimulation was applied to mouse diaphragm (Vizi and Vyskocil, 1979) or when rat diaphragm was depolarized with high K^+ solution (Liley, 1956a), indicating that at rest, transmitter release consists of two fractions, quantal and non-quantal release, and only 1-3% of total release represents quantal release (Mitchell and Silver, 1963). Vizi and Vyskocil (1979) suggested that the large fraction of non-quantal release might have a trophic role in maintaining the muscle fibers but is not responsible for synaptic transmission, as its postsynaptic effect is small even in the presence of anticholinesterase. Bray et al. (1982) and Drachman et al. (1982) supported the idea that non-quantal ACh might play an important role in any trophic effect on the muscle fibers by maintaining the membrane potential, because surgical denervation, botulinium or chronic postjunctional blockade by alpha-bungarotoxin all led to a fall in membrane potential and an increase in the number of extrajunctional ACh receptors (Pestronk et al., 1976; Mathers and Thesleff, 1978; Pestronk et al., 1980).

(b) subnormal quanta. Because of the improvement in recording techniques, small subminiature endplate potentials (submin. epp), about one tenth the size of a normal mepp, were

observed in frog cutaneous pectoris and mouse diaphragm muscle in addition to the normal mepp (Dennis and Miledi, 1971; Cooke and Quastel, 1973a; Kriebel and Gross, 1974; Bevan, 1976). At rest, the submin. epps represent about 2-5% of the total events but under certain experimental conditions or treatments (e.g. heat challenge, tetanic nerve stimulation, colchicine, botulinum toxin, lanthanum ions, etc.), this proportion is increased (Kriebel et al., 1976). Kriebel et al., (1976) proposed that submin. epp is due to the release of the content of a single vesicle whereas the normal mepps result from the synchronous release at one active zone of several subunits i.e., the submin. epps were the fundamental quanta. However, an alternative view is that submin. epp result from the discharge of incompletely filled vesicles (Cooke and Quastel, 1973a), this view is substantiated by the observation that the submin. mepp frequency is increased in rat diaphragm following a period of K^+-evoked transmitter release and after treatment with vesamicol (Searl et al., 1990).

(c) Supra-normal quanta. Unusual giant mepps, classified as Ca^{2+}-independent intermittent non-quantal ACh release representing 2-3% of the total population of mepps, were observed in normal rat neuromuscular junctions (Liley, 1957). The giant mepps were frequently found during synaptic development and when the synaptic transmission was blocked or poisoned, and it appeared that this type of Ca^{2+}-independent

secretion was not involved in the propagation of impulses (Thesleff and Molgo, 1983).

The large mepps could also be induced after the treatment with 4-aminoquinoline (50-250 μM) whereas normal mepps and epps are not affected by 4-aminoquinoline (Molgo and Thesleff, 1982). The 4-aminoquinoline-induced giant mepps were 2 or more times the size of normal average mepp and comprised 15-40% of the total population of mepps. Because the giant mepps could not be altered by nerve stimulation, nerve terminal depolarization, or change in extracellular calcium or magnesium concentration, it was suspected that the release did not originate from an "active zone" (Jansen and van Essen, 1976; Molgo and Thesleff, 1982; Thesleff and Molgo, 1983). Moreover, the time course and shape of giant mepps did not correspond to that of the summation of single mepps (Jansen and van Essen, 1976). For these reasons, the giant mepp was not believed to represent a quantum or multiples of the quanta that normally comprise an epp (Molgo et al., 1982; Lupa et al., 1986).

3. MECHANISM OF TRANSMITTER RELEASE

In the course of normal neurotransmission, the nerve terminal is, of course, depolarized, either directly by the action potential, or by electrotonic spread from the limit of

travel of the action potential (Brigant and Mallart, 1982; Mallart and Brigant, 1982; Mallart, 1985). Release of transmitter is not dependent on either an inward sodium current, as it occurs in the presence of tetrodotoxin (Katz and Miledi, 1966, 1967a, 1969; Bloedel et al., 1966), or an outward tetraethylammonium (TEA) sensitive potassium current (Katz and Miledi, 1967b). It is, however, dependent on an inward current of calcium ions (Katz and Miledi, 1967b; Cooke et al., 1973), which is mediated by voltage dependent channels and is therefore a function of membrane potential (Zucker and Haydon, 1988).

The involvement of calcium in neuromuscular transmission was first reported by Locke (1894) who noted that neuromuscular transmission requires the presence of small amounts of Ca^{2+} in the bathing solution. Feng (1936) suggested that transmitter released at motor nerve terminal depends on the concentration of calcium ions. Del Castillo and Stark (1952) demonstrated that the evoked transmitter is elicited by the calcium influx through voltage-dependent calcium channels. When the extracellular calcium is lowered, either the amplitude of the epp is reduced to that of an mepp or the epp disappears completely. The mepp frequency is also affected by extracellular calcium. When the calcium concentration is decreased the spontaneous frequency of release is depressed (Boyd and Martin, 1956b; Elmqvist and

Feldman, 1965; Hubbard et al., 1968a). The fact that calcium is a link between depolarization and transmitter release led Katz and Miledi (1967b) to propose the "calcium hypothesis of transmitter release".

3.1. Ca^{2+} ENTRY

The detailed steps in the process of transmitter release remain obscure. Certainly Ca^{2+} is central to transmitter release (Smith and Augustine, 1988; Zucker and Haydon, 1988) whether spontaneous, evoked by an action potential, or by the electrotonic spread from the limit of travel of the action potential (Brigant and Mallart, 1982; Mallart and Brigant, 1982; Mallart, 1985). Ca^{2+} is believed to enter the nerve terminal through voltage-dependent channels, although the precise nature of the channel remains unknown (Augustine et al., 1987; Tsien et al., 1988). It is possible that the terminals of many neurons possess a heterogeneity of Ca^{2+} channels that all contribute to the overall inward current on depolarization (Penner and Dreyer, 1986; Dunn, 1988), but others have presented evidence for only one type of channel (Guan et al., 1988). The density of channels is such that, with the anticipated diffusion of Ca^{2+} in the cytoplasm, there may be little overlap in the intracellular domains influenced by ions permeating individual channels (Simon and Llinas, 1985; Zucker and Fogelson, 1986).

The well established fourth power relationship between $[Ca^{2+}]_o$ and ACh release (Dodge and Rahaminoff, 1967; Andreu and Barrett, 1980) may be indicative of co-operativity between 4 Ca^{2+} ions to release one quantum, but the contribution made by local Ca^{2+} concentration within the terminal, and whether the Ca^{2+}-domain of any channel overlaps with the domains of its neighbours to cause non linear Ca^{2+} concentration profiles adds to the basic uncertainty surrounding the process of neurotransmitter release (Augustine et al., 1987).

3.2. Ca^{2+}-CONCENTRATION DECLINE

The resting concentration of $[Ca^{2+}]_i$ is around 0.1 μM. Deviations from this norm are restored by means of energy-driven Ca^{2+} extrusion and sequestration mechanisms together with passive binding to acceptor sites (Di Polo and Beauge, 1983). These mechanisms are most likely located in the plasma membrane and membranes of the subcellular organelles, principally endoplasmic reticulum and mitochodria. The mechanisms cannot be instantaneous, and so a profile of declining $[Ca^{2+}]_i$ with time is to be expected following a single nerve impulse. Although this profile has never been quantified, its effects have been adduced in observations on transmitter release in several instances.

3.3. TRIGGER Ca^{2+} BINDING SITE

Although many Ca^{2+}-binding proteins exist in the nerve terminal, particularly in association with synaptic vesicles (Augustine et al., 1987), no clear candidate for the trigger that is activated by Ca^{2+} to cause vesicle exocytosis has emerged. On the basis of kinetic studies, the release reaction is believed to be simple and probably single staged. Silinsky (1985) proposed an elaborate scheme for vesicular exocytosis involving putative docking proteins, Ca^{2+}-binding proteins and contractile elements such as fodrin and actin which was consistent with the known facts, yet failed to rise above the level of speculation for lack of specific data on release. The process that is initiated by the Ca^{2+} ion and leads to the release of vesicular contents to the exterior remains, at present, unknown.

4. MODULATION OF NEUROTRANSMITTER RELEASE

4.1. FREQUENCY MODULATION

Despite the depletion of the IAS, neurotransmission rarely fails under conditions of rapid repetitive stimulation. Aside from de novo synthesis of transmitter, several mechanisms have been demonstrated to affect either the proportion of the IAS released per impulse, or to replenish

the IAS from deeper, less labile stores.

4.1.1. Facilitation.

Although a rapidly occurring increase in transmitter release at unsuppressed synapses during the initial pulses of a tetanic burst of stimulation has long been recognized (Liley and North, 1953; Hubbard, 1963; Mallart and Martin, 1967; Katz and Miledi, 1968; Balnave and Gage, 1977; Wilson, 1979; Gage and Murphy, 1981; Glavinovic and Narahashi, 1988), it has been studied under conditions of high $[Mg^{2+}]_o$, where virtually no depression or loss of IAS occurs. Brief bursts of stimuli result in an increase in m to a plateau. From the cessation of tetanic stimulation, the value of m from individual episodes of evoked release at varying time intervals declines. Magleby and Zengel (1982) identified four separate exponential declines in m, and defined the two faster as facilitation (F_1 and F_2) (Zengel and Magleby, 1982) similar to those described by Mallart and Martin (1967). The consensus of opinion is that these processes are attributable to Ca^{2+} ions that enter the nerve terminal during rapid stimulation and are incompletely removed from binding sites before successive episodes of release (Lev-Tov and Rahaminoff, 1980; Charlton et al., 1982; Zucker and Lana-Estrella, 1983; Silinsky, 1985). The exponential decay constants, therefore, are expressive of Ca^{2+} sequestration processes, and the most rapid (T_{f1}=60 msec)

is compatible to the time constant of decline seen in the second of paired stimuli at 37°C (Thies, 1965).

4.1.2. Augmentation.

The process of augmentation was defined by Magleby and Zengel (1976) as a process that elevated transmitter release during tetanic stimulation, and was seen to have a decay time constant of 7 sec at Mg^{2+}-suppressed frog nerve terminals. The process has a divalent cation dependence as it can be increased in amplitude by substituting Ba^{2+} for Ca^{2+} in the bathing solution. However, the ion is probably acting as a catalyst for some complicated biochemical reaction. The molecular basis for augmentation is unknown, although Zengel and Magleby (1982) suggested that $[Ca^{2+}]$ at a strategic point within the terminal was involved in an "expression factor", although membrane phosphorylation has also been suggested as the mechanism (Erulkar, 1983).

4.1.3. Potentiation.

Potentiation was in fact recognized for some time, although known by a variety of names, before Magleby (1973a) systematically differentiated the process from the two components of facilitation (Magleby and Zengel, 1975a, 1975b). Again, the diagnostic criterion is the decay time following

the cessation of tetanic stimulation. The time constant of potentiation is long (2 sec to 3 min) and may be influenced by a variety of conditions. Again, Ca^{2+} appears to be necessary, although the enhancing influence of Li^{+} led Silinsky (1985) to suggest that inositol triphosphate (IP_3) and phosphorylation of vesicular proteins may be involved in the process.

4.1.4. Long term potentiation.

The other example of potentiation is long-term potentiation (LTP). In the hippocampus, a long-lasting potentiation of the response to a single stimulus was found after short periods of repetitive stimulation had been applied to the afferent perforant pathway in the dentate gyrus (Lomo, 1966, 1971). The importance of LTP is that its mechanism appears to be similar to memory, and LTP may serve as a model for learning and memory.

In addition to the central nervous system, LTP also occurs in the peripheral nervous system. LTP can be measured in rat superior cervical ganglion (SCG) (Brown and McAfee, 1982), bull-frog sympathetic ganglion (Koyano et al., 1985) and crayfish neuromuscular junction (Baxter et al., 1985). It has not been demonstrated, however, at the vertebrate neuromuscular junction.

4.2. RECEPTOR MODULATION OF NEUROTRANSMITTER RELEASE.

The realisation that the release of neurotransmitter can be modulated by receptors located on the nerve terminal itself was slow to develop, but is now well established for peripheral noradrenergic neurons, and for neurons within the central nervous system (Starke, 1977, 1981, 1987; Langer, 1981; Chesselet, 1984; Starke et al., 1989). Although the existence of cholinoceptors on the motor nerve terminal was first suggested in 1957 (Riker et al., 1957), and repeatedly adduced by a number of authors to explain the phenomena of repetitive antidromic firing (Standaert, 1964, Standaert and Adams, 1965; Riker, 1966) and posttetanic potentiation (Lilleheil and Naess, 1961; Glavinovic, 1979a), the precise complement of presynaptic receptors and their role in regulating transmitter release is still open to controversy.

4.2.1. Autoreceptors.

(a) Nicotinic. Both nicotinic and muscarinic cholinoceptors have been reported to exist on the motor neuron terminal. The nicotinic receptors have two identifiable effects which led to the postulate that there are, in fact, two distinct classes of nicotinic receptor present, subserving different functions. Bowman has recently reviewed the evidence (Bowman, 1990) and classed one population as providing a positive feedback to the

process of transmitter release. Their effects are most obvious when repetitive stimulation is carried out in the presence of curariform drugs or hexamethonium (Bowman et al., 1984, 1988; Ferry and Kelly, 1988; Fletcher and Forrest, 1975; Gibb and Marshall, 1984; Harbourne et al., 1988; Hubbard and Wilson, 1973; Magleby et al., 1981). In such cases the amplitude of the end plate response is less well sustained than in the absence of the antagonist drug, producing a pronounced rundown that results in a fade of the contractile response of the muscle. The snake toxins, alpha-bungarotoxin and erabutoxin b, did not share the property of inducing rundown with the curariform drugs. The conclusion of these observations was that there were nicotinic receptors on the nerve terminal that were different from both the postsynaptic receptors at that synapse, and from the receptors found on the neurons in autonomic ganglia. These prejunctional receptors responded to the retrograde action of ACh released from the nerve by facilitating the further release of the transmitter, thus counteracting the depletion of stores that inevitably accompanies release. The transduction mechanism of these receptors is completely unknown.

The second of Bowman's two classes of presynaptic nicotinic receptors is recognised by the effect already mentioned, in that nicotinic agonists such as decamethonium, or anticholinesterase agents can induce repetitive antidromic

firing in nerves following a single or low frequency stimulus. This response is blocked by alpha-bungarotoxin, and is thought to arise from a prolonged depolarization of generator potential caused by the depolarising effect of agonist on nicotinic receptors that resemble postsynaptic end plate receptors in their drug sensitivity. At higher frequencies of stimulation, release of transmitter is apparently depressed by the actions of anticholinesterases in a manner resembling the phase II block of neuromuscular transmission that is induced by depolarizing blocking drugs (Creese et al., 1982).

There is not widespread acceptance of Bowman's classification, and others contend that the two effects could be obtained from the same type of receptor (Wessler et al., 1987b, 1988a).

(b) Muscarinic. A similar controversy surrounds the existence of muscarinic receptors at the motor nerve terminal. A number of authors have reported a negative effect on transmitter release following exposure to drugs with a predominantly muscarinic preference (Duncan and Publicover, 1979; Michaelson et al., 1979, 1980; Kloog et al., 1980; Strange et al., 1980; Abbs and Joseph, 1981, Dowdall et al., 1981; Dunant and Walker, 1982) although the last group subsequently reported that despite being inhibited by atropine, the effect could only be elicited by oxotremorine,

and not by bethanecol, methacholine or muscarine (Dunant et al., 1982). Others have claimed an enhancing effect of muscarinic receptor stimulation (Das et al., 1978, Ganguly and Das, 1979) while yet more have failed to demonstrate any effect at all (Gunderson and Jenden, 1980; Haggblad and Heilbronn, 1983). Wessler et al. (1987a) concluded that both stimulant and inhibitory effects coexisted at the motor neuron terminal, and that the observed response was dependent on either the appropriate concentration of drug or the appropriate drug with affinity for the receptor in question. They subsequently reported that facilitatory effects could be blocked by pirenzepine, and were thus presumably mediated by M_1 receptors. As a postscript, they proposed that the inhibitory effects might be mediated by M_2 receptors (Wessler et al., 1988b; Wessler, 1989).

The physiological significance of these observations has not been clearly demonstrated. Vizi and Somogyi (1989) suggested that the nicotinic facilitatory effect predominated over the muscarinic inhibitory one, and Arenson (1989) concluded that muscarinic receptors, though present, played no role in the normal regulation of transmitter release at the neuromuscular junction.

Little is known of the transduction mechanisms involved although Pinchasi et al. (1984) reported that activation of

the presynaptic muscarinic receptor in the Torpedo electric organ was associated with increased production of prostaglandin E_2. Such a response might be expected to increase transmitter output, as prostaglandin E_2 is known to have this effect at cholinergic nerve terminals (Madden and Van Der Kloot, 1982; Das and Ganguly, 1984). If such is the case, then the transduction sequence of the putative M_1 receptor is in part revealed.

4.2.2. Heteroreceptors.

Like most other neurons, motor nerve terminals have a population of heteroreceptors present that assist in the modulation of neurotransmitter release (Laduron, 1985). The list must be regarded as incomplete, and still open to a certain amount of controversy, especially as few peptides have been systematically examined in this system.

(a) Adrenoceptors. Evidence for the existence of presynaptic adrenoceptors at the motor nerve terminal has come from both the ability of norepinephrine (NE) and other alpha-adrenoceptor agonists to reverse the partial block imposed by tubocurarine on neuromuscular transmission (Bowman et al., 1962; Bowman and Nott, 1969, Malta et al., 1979) and electrophysiological studies showing that neurotransmitter release, as measured by miniature end-plate potential

frequency (meppf) or quantal content of the epp, was increased in the presence of either NE or epinephrine (Epi) (Krnjevic and Miledi, 1958; Jenkinson et al., 1968; Kuba, 1970; Kuba and Tomita, 1971). Although the effect of NE or Epi was antagonised by phentolamine, dibenamine and phenoxybenzamine, but not by dichloroisoprenaline or pronethalol, the concept that a conventional alpha-adrenoceptor was involved was challenged by Miyamoto and Breckenridge (1974) who demonstrated that meppf was elevated by theophylline as well as by NE, and concluded that the action of NE was to elevate cyclic AMP levels in the motor nerve terminal. As this is normally mediated by a beta-adrenoceptor, the question of receptor type and signal transduction was laid open. Apparent confirmation of the presence of presynaptic beta-adrenoceptors with a modulatory function was provided by Wessler and Anschutz (1988) who noted that propranolol and atenolol blocked the ability of NE and Epi to enhance the release of ^{3}H-acetylcholine (^{3}H-ACh) from stimulated phrenic nerve-diaphragm end-plate strips. A further element of confusion was added by the observation that high concentrations of the alpha2-adrenoceptor agonists, clonidine and xylazine, could enhance meppf. Yohimbine blocked not only this effect, but also the response to phenylephrine (PE) (Lim and Muir, 1983). However, since high concentrations were required for these results, the specificity of the drugs for the alpha2-adrenoceptor was acknowledged to be in doubt. More

recently, a group using a radiolabelled choline washout technique failed to observe any effect of xylazine on ACh release (Somogyi et al., 1987).

(b) Adenosine receptors. Adenosine was first shown to reduce the release of transmitter at motor nerve terminals in 1972 (Ginsborg and Hirst, 1972), an observation that was later extended to many other neuron types (Fredholm, 1988). Subsequent work confirmed this effect to be attributable, at least in the frog motor neuron, to an R-type of purine receptor, a receptor type that is associated with the adenylate cyclase enzyme system (Silinsky, 1980, 1984; Buckle and Spence, 1982; Ribeiro and Sebastiao, 1985). Two subtypes of adenosine receptors have been identified as having opposite effects on the generation of cyclic AMP in a variety of cultured cells (Van Calker et al., 1979; Schwabe, 1983). The subtypes of adenosine receptor are normally identified on the basis of their affinity for each of a pair of adenosine analogues, with the A_1 receptor having a preferential affinity for L-N6-phenylisopropyladenosine (L-PIA) and inhibiting cyclic AMP production, while the A_2 receptor shows greater affinity for 5'-N-ethylcarboxyamido-adenosine (NECA) and stimulates the production of cyclic AMP (Stone, 1984). In an attempt to characterise the adenosine receptor present at the neuromuscular junction, Ribeiro and Sebastiao (1985) found that these two analogues did not inhibit the responses of frog

preparations with sufficiently separated concentration-effect curves for certainty. It should be noted however that they placed greater quantitative reliance on inhibition of twitches than on direct measurements of transmitter release.

The subject was further clouded by the repeated claim that the depression of transmitter release caused by adenosine analogues was accompanied by an increase in the formation of cyclic AMP within the motor nerve terminal (Silinsky, 1984; Silinsky et al., 1987a & b). This contrasted with data already established in this laboratory and by others (Dryden et al., 1988). Further publications have appeared subsequent to the performance of the work described in this thesis, and they will be considered along with the present data in the Discussion.

(c) Adrenocorticotrophin (ACTH) receptors. The use of the immunosuppressant properties of corticosteroid drugs in the treatment of myasthenia gravis led to the successful application of massive doses of the pituitary hormone, ACTH, in the therapy of this disease (Millikan, 1951; Torda and Wolff, 1951; Grob and Namba, 1966; Namba et al., 1971). A possible direct effect of the peptide hormone on neurotransmission was demonstrated by Birnberger et al. (1977) who noted that although mepp frequency was increased in the presence of ACTH, the quantal content of epp was reduced in

the Mg^{2+} depressed rat phrenic nerve. This latter observation was contradicted by Johnston et al. (1983) who found not only an increase in the quantal content of the epp in both Mg^{2+} and tubocurarine treated preparations, but also noted the persistence of the effect. The transduction of the effect of ACTH has been well studied in the adrenal cortex, where it has been shown to increase cyclic AMP and stimulate steroidogenesis (Haynes, 1958; Haynes, et al., 1959). No effect on phosphodiesterase was found (Grahame-Smith et al., 1967) but the presence of cyclic AMP dependent protein kinase in ACTH sensitive cells was confirmed by Murray et al. (1987). At very low concentrations (0.1 nM-1 pM), however, ACTH enhanced the incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol and the production of inositol phosphate in whole adrenal cortex (Farese, 1983; Farese et al., 1986; Whitley and Tait, 1987).

(d) Miscellaneous receptors. In addition to the above receptor types which were further studied in the work described in this thesis, opioids are believed to be present in motor neuron terminals. Both morphine and met-enkephalin have been shown to reduce ACh output both at the frog neuromuscular junction (Pinsky and Frederickson, 1971; Bixby and Spitzer, 1983) and the Torpedo electric organ (Michaelson et al., 1984). Luteinizing hormone (LHRH) on the other hand, caused a prolonged increase in transmitter release at the frog

neuromuscular junction (Akasu, 1986).

5. TRANSDUCTION MECHANISMS

The presence of presynaptic receptors (other than nicotinic receptors) implies the co-existence of transduction mechanisms involving the mediation of a G-protein (Berridge, 1985; Cockcroft and Stutchfield, 1988; Graziano and Gilman, 1987; Milligan, 1988; Stryer and Bourne, 1986; Ui, 1986). While G-proteins may activate enzymes resulting in the production of second messengers, they have also been demonstrated to act directly on membrane structures such as ion channels. In neurons, both a sustained activation of K^+ channels by G_k and a reduction in Ca^{2+} channel conductance by G_o have been described (Dunlap et al., 1987). These effects were observed in the somata of neurons, and the question of similar direct effects of G-proteins in terminals modulating transmitter release is at present unresolved.

Strong evidence for the modulation of transmitter release by second messenger components of transduction systems within the motor nerve terminal has been documented, e.g. cyclic AMP (cAMP) (Dryden et al., 1988) protein kinase C (PKC) (Eusebi et al., 1986; Light et al., 1988; Murphy and Smith, 1987; Publicover, 1985b), and calmodulin, known also to be present, is thought to affect transmitter release when the Ca^{2+}

concentration within the terminal is raised (Publicover, 1983, 1985a; Roufogalis, 1980).

5.1. CYCLIC AMP

While cyclic AMP is generally agreed to enhance the release of transmitter at the motor nerve terminal in vertebrates (Dryden et al., 1988), its role has been studied in Aplysia neurons. Direct injection of cyclic AMP into a sensory neuron, as well as extracellular application of a phosphodiesterase inhibitor resulted in an enhanced action potential and transmitter release from its terminal (Kandel and Schwartz, 1982). More convincingly, the intracellular injection of the activated catalytic subunit of the cyclic AMP dependent protein kinase (ATP:protein phosphotransferase, E.C. 2.7.1.37) (PKA) also enhanced the gill withdrawal reflex in the animals (Castellucci et al., 1980). Such a response was normally associated with the action of serotonin, a transmitter associated with increases in cyclic AMP levels in these neurons (Brunelli et al., 1976). The substrate for the protein kinase was not established unequivocally in these experiments, although the ultimate effect was the closure of a calcium dependent potassium channel, the prolongation of the action potential and an increase in the amount of transmitter released. These effect were recorded in the cell soma, and it is by no means certain that the same mechanism would operate

at the nerve terminal even in Aplysia, let alone vertebrates.

5.2. PHOSPHATIDYLINOSITOL DERIVATIVES

Understanding of the nature of the transduction process at receptors was expanded significantly by the realization that a G-protein mediated link existed between many receptors and phospholipase C (PLC). Two isozymal forms of PLC have been isolated from neuronal systems in the brain (Gerfen et al., 1988; Homma et al., 1988). Most PLC enzymes derived from mammalian tissues are specific for phosphatidylinositol or its phosphorylated derivatives, and in the course of hydrolysis of phosphatidylinositol diphosphate (PIP_2), two active derivatives are produced, inositol triphosphate (IP_3) and diacylglycerol (DAG) (Waite, 1987). These have been the subject of many reviews (Hirasawa and Nishizuka, 1985; Abdel-Latif, 1986; Nahorski, 1988) and have been associated with several responses in neurons.

5.2.1. Inositol triphosphate.

IP_3 is believed to diffuse in the cytosol and to act at a specific receptor on the endoplasmic reticulum to release calcium from the stores there (Ehrlich and Watras, 1988; Fink et al., 1988; Stauderman et al., 1988; Suarez-Isla et al., 1988; Furuichi et al., 1989). Elevated cytosolic Ca^{2+} levels

may affect several systems including Ca^{2+} dependent ion channels and enzymes, and by combining with calmodulin, affect calmodulin dependent systems such as phosphodiesterases and protein kinases (Publicover, 1985a; Veigl et al., 1989).

IP_3 is degraded to inositol and inorganic phosphate by a series of phosphatase enzymes which can be inhibited by the lithium ion (Abdel-Latif, 1986; Nahorski, 1988). The net effect in the tissue is to cause the accumulation of inositol-1 phosphate that would normally be degraded to inositol prior to reincorporation into phosphatidylinositol and reinsertion into membrane phospholipids (Chiu et al., 1987). A corollary is of course that PIP_2 reserves in the membrane might reasonably be expected to decline with repeated activation of the receptor in the presence of Li^+ with an accompanying diminution in the response. Such Li^+ associated reductions in the magnitude of IP_3 mediated responses have been documented (Hokin-Neaverson and Sadeghian, 1984; Kendall and Nahorski, 1987; Worley et al., 1988). However, immediately following the application of Li^+, a period of time should exist when the levels of IP_3 are raised as progress of the molecules through the cascade, $IP_3 > IP_2 > IP_1$, is impeded by product inhibition of enzymes involved. This may explain the observation that within minutes of the application of Li^+ to a tissue, IP_3 mediated responses to agonist substances are amplified (Berridge et al., 1982).

Although IP_3 production is conventionally associated with receptor activation, this may not always be so. Habermann and Laux (1986) reported that depolarization of a rat brain particulate preparation increased its production of IP_3. Thus in addition to receptor activation, repetitive stimulation of nerves with the attendant depolarization might serve to activate PLC.

5.2.2. Diacylglycerol.

DAG is preferentially lipid soluble and remains associated with the cell membrane. Along with phosphatidylserine and Ca^{2+} it serves to activate PKC which, when activated, is membrane associated rather than freely soluble in the cytosol (Niedel and Blackshear, 1986). Exogenous DAG is capable of mimicking the effect of the endogenously produced molecule, and responses to such application are often used in support of arguments for the existence of a particular transduction pathway. For example, Murphy and Smith (1987) reported that a DAG increased both the spontaneous and the evoked release of transmitter from the mouse phrenic nerve, with the implicit but unjustified suggestion that PKC mediated transmitter release rather than modulated it.

5.3. PROTEIN KINASES

The net effect of much second messenger action is activation of a protein kinase. There are three classes of protein kinase that are at present known to be of relevance to neuronal function. Collectively these enzymes are ATP:protein phosphotransferases, and as such, have some features in common. They all bind ATP as the phosphate donor, and therefore are susceptible to inhibition by agents that also bind at that site. This has led to a continuing degree of ambiguity in the literature, as a drug that inhibits one class of kinase may not act exclusively on that class (Garland et al., 1987).

5.3.1. Cyclic nucleotide dependent protein kinases.

Both cyclic AMP and cyclic GMP dependent kinases exist but while the latter is of importance in smooth muscle function its role in neuronal activity has not been defined. On the other hand, PKA is widespread in tissues, including nervous tissue, and its importance is better appreciated (Corbin et al., 1988). Each holoenzyme molecule is comprised of two catalytic and two regulatory subunits. Each regulatory subunit has two cyclic AMP binding sites that show co-operativity, and binding of the nucleotide removes the inhibitory effect of the regulatory subunit on the catalytic

subunit. The catalytic subunit has of necessity an ATP binding site, and may be activated in the absence of cyclic AMP by the drug, forskolin. When cyclic AMP binds to the regulatory sites of PKA in the cytosol, the catalytic subunits dissociate into active states that catalyze the phosphorylation process (Nestler and Greengard, 1984). The gamma phosphate group of ATP is transfered to the hydroxyl group of serine, threonine or tyrosine residues in the target protein. Both cyclic nucleotide dependent kinases are susceptible to inhibition by naphthalenesulfonamides and isoquinolinesulfonamides. The respective drugs, A-3 (N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide) and H-7 (1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine) show particularly high affinity in this regard (Hidaka et al., 1984; Inagaki et al., 1986). High concentration of both PKA and substrates such as synapsin I are present in grey matter. From such observations, Greengard has proposed a central role for protein phosphorylation in neuronal regulation (Nestler and Greengard, 1984).

5.3.2. Protein kinase C.

PKC is a single polypeptide of molecular weight about 82 kD (Kikkawa et al., 1987). It is abundant in brain where it is concentrated in presynaptic terminals (Niedel and Blackshear, 1986). It is an apoenzyme that requires the

presence of phospholipid, preferably phosphatidylserine, Ca^{2+}, and a DAG to be fully active (Kuo et al., 1980; Kaczmarek, 1987). The ternary complex of enzyme, Ca^{2+} and phospholipid is also active but requires 100 fold higher Ca^{2+} concentrations. In its inactive form, the enzyme is cytosolic in location, but in the process of becoming activated, it is translocated to the cell membrane where it exerts many of its effects (Kikkawa et al., 1987; DeReimer et al., 1985a). Many of the substrates of PKC that have been identified are membrane receptors such as the adrenoceptor, as well as structural molecules such as actin-binding protein and myosin light chain and basic protein. Most phosphorylation occurs on the serine and threonine residues of substrates, although some phosphorylation of tyrosine has been reported (Niedel and Blackshear, 1986).

A unique feature of PKC is its response to esters of phorbol, in particular, phorbol 12-myristate 13-acetate. These esters have been recognised as tumor promoters and substitute for DAG in the activation sequence for the enzyme. As a result, effects that can be induced by phorbol esters in tissues are invariably ascribed to PKC. In Aplysia bag cell neurons an increase in voltage dependent Ca^{2+} current has been reported (DeReimer et al., 1985b) while in chick dorsal root ganglion neurons a suppression of the Ca^{2+} current was caused by esters of phorbol (Rane and Dunlap, 1986). In rat

hippocampal cells a broadening of the Ca^{2+} action potential and an attenuation of the Ca^{2+} activated K^{+} current was observed (Baraban et al., 1985; Malenka et al., 1986a; Storm, 1987). In addition the phenomenon of long term potentiation can be mimicked by phorbol esters (Malenka et al., 1986b; Akers et al., 1986). However there are sufficient differences in the duration of the effect and its susceptibility to PKC inhibition to suggest that the relationship is not a simple one (Muller et al., 1988). The inhibition of PKC (or CaMKII, q.v.) blocks the induction of LTP but not its expression once it is established (Malinow et al., 1989). There are thus other steps in the induction of LTP that require prior phosphorylation of protein(s).

At the motor neuron terminal phorbol esters increased both the spontaneous and the evoked release of transmitter at both frog and mouse neuromuscular junctions (Publicover, 1985a; Eusebi et al., 1986; Murphy and Smith, 1987).

A wide variety of compounds have been reported to inhibit PKC on the basis of an inhibition of phorbol ester induced responses e.g. rhodamine 6G (O'Brian and Weinstein, 1986), auranofin (Parente et al., 1986) and triphenylethylenes such as clomiphene and tamoxifen (O'Brian et al., 1986, 1988a, 1988b). This has led to an implicit assumption in the literature that the compounds are specific for PKC, an

assumption that has been decried to little avail (Garland et al., 1987).

The isoquinolinesulfonamide, H-7, is equiactive against PKC and the cyclic nucleotide dependent protein kinases (Hidaka et al., 1984) but the naphthalenesulfonamide, A-3, has about one tenth the affinity for PKC that it has for PKA or PKG (Inagaki et al., 1986). A natural inhibitor system in the form of sphingoid bases such as sphingosine, derived from the action of sphingomyelinase has also been reported (Kolesnick and Clegg, 1988). Paradoxically, DAG appeared to activate the sphingomyelinase, thus providing a means of terminating the activity that it initiated in the first place.

5.3.3. Ca^{2+}/calmodulin dependent protein kinases.

The Ca^{2+}-binding protein, calmodulin, is apparently present in all eukaryotic cells, and on binding Ca^{2+} ions, the molecule is activated into an effector or agonist molecule with affinity for other proteins within the cell. Calmodulin is present in the nerve terminal, apparently in association with synaptic vesicles and there has been considerable speculation that it played a role in transmitter release (De Lozenzo et al., 1979; De Lozenzo, 1980, 1982). Asynchronous or spontaneous release is more susceptible to inhibition of calmodulin by drugs than evoked release giving rise to the

speculation that cytosolic calmodulin differed in its properties from vesicle associated calmodulin (Publicover, 1985b).

Of more particular relevance is the observation that [illegible] at least fifteen Ca^{2+}-calmodulin dependent kinases ([illegible]ulman, 1988; Veigl et al., 1989), each one of which [illegible]vated by an increase in the concentration of Ca^{2+} in the cytosol. While some kinases in this group are dedicated in that they are restricted in substrate specificity, e.g. myosin light chain kinase, phosphorylase kinase and probabl, CaM kinase III, two have apparently less rigid substrate requirements and are of interest in the area of transmitter release.

CaM kinase I was identified in synaptosomes and phosphorylated synapsin I at site 1, located in the globular domain of the molecule, and the same site phosphorylated by PKA. It is known to be present in the cytosol of all cell types. In addition to synapsin I, it also accepts another vesicle associated protein, protein III, as a substrate. CaM kinase II, the multifunctional CaM kinase or simply CaM kinase, is also widespread in distribution, but has a wider spectrum of substrates. Among these is also synapsin I, although the site of phosphorylation is in the vesicle associated domain of the molecule.

No specific inhibitors of the CaM kinases have been developed, although inhibitors of calmodulin will inevitably inhibit the enzyme system. Many calmodulin inhibitors exist (Veigl et al., 1989) including sphingosine (Jefferson and Schulman, 1988), phenothiazines (Weiss, 1983) and the naphthalenesulfonamides and their analogues (Tanaka et al., 1982; Hidaka and Tanaka, 1983; MacNeil et al., 1988). The naphthalene sulfonamides such as W-7 are about 10 times more potent inhibitors of calmodulin dependent reactions than of PKC mediated ones (Hidaka and Hagiwara, 1987; O'Brian et al., 1987), an observation that can be used to advantage in identifying the enzyme system responsible for a reaction.

5.3.4. Substrates.

Although synapsin has figured prominently in the work on kinase substrates in nerve terminals, this is merely a reflection of the predilections of one research group (see below), and by no means rules out the involvement of other phosphorylated protein in transmitter release. The identity of these target proteins is unknown, but constitutes a fundamental question in the area of transmitter mobilization and release. While many proteins can be phosphorylated by the non-dedicated protein kinases thus far identified, PKA PKC and CaM-kinases I and II, synaptic vesicle-associated proteins represent one of the most intriguing possibilities. In light

of Greengard's hypothesis (Greengard and Browning, 1988, Introduction 2.3), it would seem that there are duplicate if not multiple systems for making reserves of neurotransmitter available at times of maximum release.

6. AIMS OF THIS STUDY

The foregoing evidence indicated that both the ACh autoreceptors on the motor nerve terminal and presynaptic heteroreceptors play an important role in modulating the transmitter output from motor nerve terminals. However, the identity of the presynaptic receptor subtypes, and the nature of transduction mechanisms underlying adenosine receptor, ACTH receptor, and adrenoceptor mediated responses at the motor terminal are still controversial.

The aims of this study are therefore focussed on the identification of the specific receptor subtype on motor nerve terminals by using more specific agonists and antagonists than had been used hitherto, and the investigation of the transduction mechanisms which underlie the modulation of transmitter release at this site.

CHAPTER II

MATERIALS AND METHODS

1. TISSUE PREPARATION

1.1. EXPERIMENTAL ANIMAL

Experiments were performed on isolated phrenic nerve-hemidiaphragm preparations from 25-30 gm mice of ICR strain (University of Alberta breeding colony). The rationale for and advantages of using the mouse phrenic nerve-diaphragm preparation is that it is a mammalian preparation and thin enough for adequate oxygenation and visualization of the nerve branches. The phrenic nerve-diaphragm preparation has a simple, accessible synapse. Although the nerve terminal activity cannot directly be measured by microelectrodes, the electrophysiological data can be deduced by inference from the electric activity recorded from postsynaptic membrane. The amplitude of a potential recorded postsynaptically is inversely proportional to the diameter by a power of 1.5, and is directly proportional to input resistance (Katz and Thesleff, 1957). The muscle fiber in mouse diaphragm is about 10 to 30 um in diameter (Salpeter et al. 1978). Therefore, the diameter of a fiber is small enough to ensure a relatively high resistivity and the mepp amplitude monitored at the muscle is about 1 mV or more, which is large enough for convenient measurement and unambiguously distinct from background noise (Fig. 1).

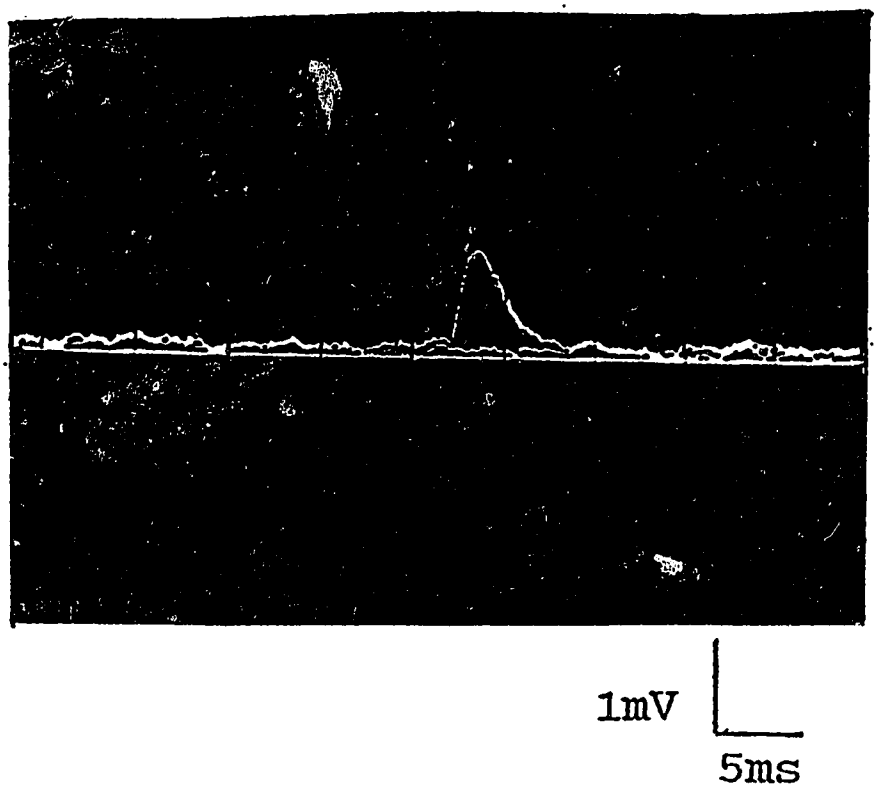


Fig. 1. Signal to noise ratio.

The ratio of the amplitude of an incoming mepp to the peak to peak amplitude of background noise was large enough to distinguish signal from noise. The amplitude of the signal ranged from 0.8 to 2 mV depending on the particular fiber, while that of noise was less than 0.1 mV.

1.2. DISSECTION

Each mouse was sacrified by cervical dislocation and then transferred to a small dissection board where the four limbs were fixed. The ventral rib cage was opened and removed, the complete diaphragm with fragments of costal arch and stumps of phrenic nerves attached was then dissected free. The crura were discarded and the two hemidiaphragms were transferred to a Petri dish filled with physiological solution and separated by cutting through the pars sternalis. The preparations were then pinned to the Sylgard covered base (Sylgard 184 elastomer, Dow Corning Corporation, Midland, Michigan, U. S. A.) of a small plastic petri dish, 35 mm in diameter and with a capacity of 3 ml. Tissues were bathed with oxygenated physiological solution at room temperature (20-21° C). Where necessary one hemidiaphragm was used as control while the other one was used as the test tissue and exposed to drugs of interest.

2. SOLUTIONS FOR ELECTROPHYSIOLOGICAL STUDIES

2.1. KREBS-HENSELEIT SOLUTION

Krebs-Henseleit solution (Krebs and Henseleit, 1932) of the following composition (mM) was used for adenosine analogue studies: sodium chloride, 118; potassium chloride, 4.7;

magnesium sulfate, 1.2; calcium chloride, 2.5; potassium phosphate (monobasic), 1.2; sodium bicarbonate 25; and dextrose, 11.1. The solutuion was buffered at pH 7.2 with 5% CO_2 in the bubbling gas.

2.2. BRETAG'S SOLUTION

Bretag's solution (Bretag, 1969) was used for catecholamine and ACTH experiments. The solution had a pH of 7.2 and was of the following composition (mM): sodium chloride, 107.7; potassium chloride, 3.48; magnesium sulfate, 0.69; calcium chloride, 1.53; sodium dihydrogen phosphate (monobasic), 1.67; sodium hydroxide, 9; sodium gluconate, 9.64; dextrose, 5.55; sucrose, 7.6; HEPES, 18.

2.3. BRETAG'S SOLUTION CONTAINING 15 mM K^+

The high potassium concentration was obtained by substitution of sodium chloride of normal Bretag's solution with an equal molar concentration of potassium chloride to maintain the osmotic strength. That is the molar concentration for sodium chloride is reduced from 107.7 to 96.18 mM, while potassium chloride is increased from 3.48 to 15 mM.

3. ELECTROPHYSIOLOGICAL STUDIES

The Petri dish with the isolated diaphragm in place in physiological solution was then mounted on the stage of a compound microscope and the preparation was superfused with physiological solution by gravity feed from a reservoir at a rate of approximately 3 ml/min. Removal of solution was achieved by suction. A complete change-over of physiological solution or drug solution in the bath was accomplished in 3-4 min. Experiments were performed at room temperature (20-21° C).

Conventional electrophysiological techniques using glass microelectrodes (Borosilicate glass, CD 1.5 mm, ID 0.8 mm, Hilgenberg Glass, Mulsfeld, West Germany) filled with 3 M KCl and having a resistance of 5-20 megohms were used to penetrate the muscle fibers in the region of the end plate (Graham and Gerard, 1946; Ling and Gerard, 1949).

Signals were amplified by a DC preamplifier with an input impedance in excess of 10^{11} ohm, and displayed on a Textronix R5031 dual-beam storage oscilloscope for mepp frequency monitoring and a Textronix 502A dual beam oscilloscope for membrane potential recording. When the preparation was stable with a resting membrane potential of -65 mV (-40 mV for tissue in 15 mM K^+) or more, mepp(f) was monitored by a gated

frequency analyzer (Ortec model 406A) and dual counter/timer (Ortec model 715). Readouts at minute intervals were automatically transferred via an interface to a recording printer (Roland DG PR-1010A). Fig. 2 illustrates a schematic arrangement of equipment for monitoring and recording. Recordings where the mepp rise time was greater than 1 ms (Fig. 3) or the frequency of quantal release over the range of 40 per minute (3000 for accelerated tissue) were discarded. Signal to noise ratio was greater than 10 (Fig. 1). Once a stable control reading of mepp frequency was obtained for at least 5 minutes the perfusate was changed to a physiological solution containing the drug under investigation. Observation at a single end plate was maintained for periods in excess of 30 minutes. Experiments performed without drug confirmed that transmitter release was unaltered over this time period. Tissue was exposed to drug in one experiment only and then discarded. In this way each fiber served as its own control, and complications from interfiber variance in mepp frequency could be reduced. Mean mepp(f) measured over the initial five minutes prior to addition of drug was taken as 100% and the mepp count of each minute thereafter expressed as a percentage of this control value. Each experiment was repeated in at least six different tissues.

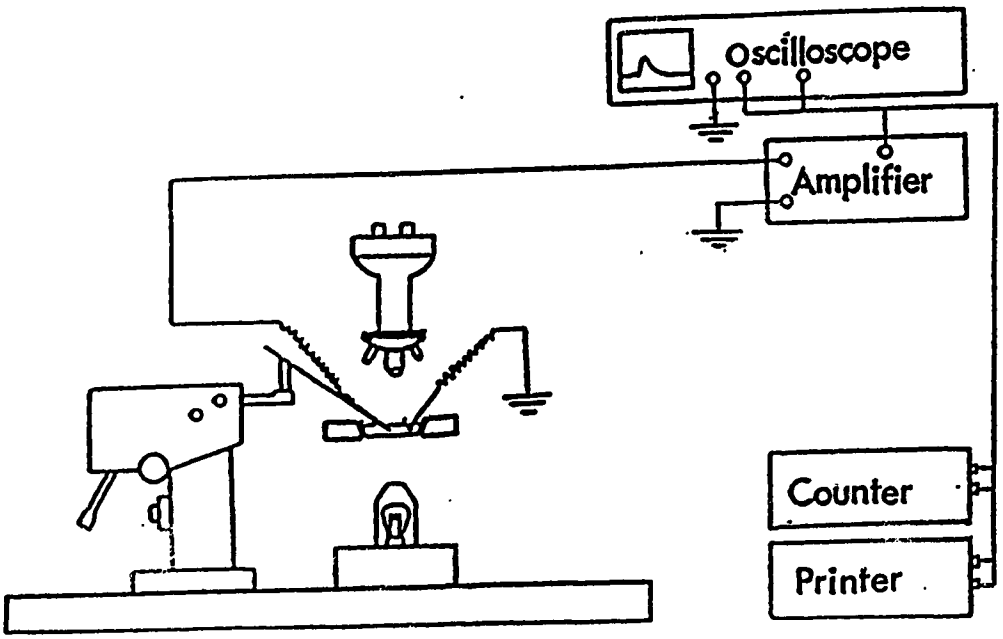


Fig. 2. Schematic representation of the arrangement of the monitoring and recording apparatus.

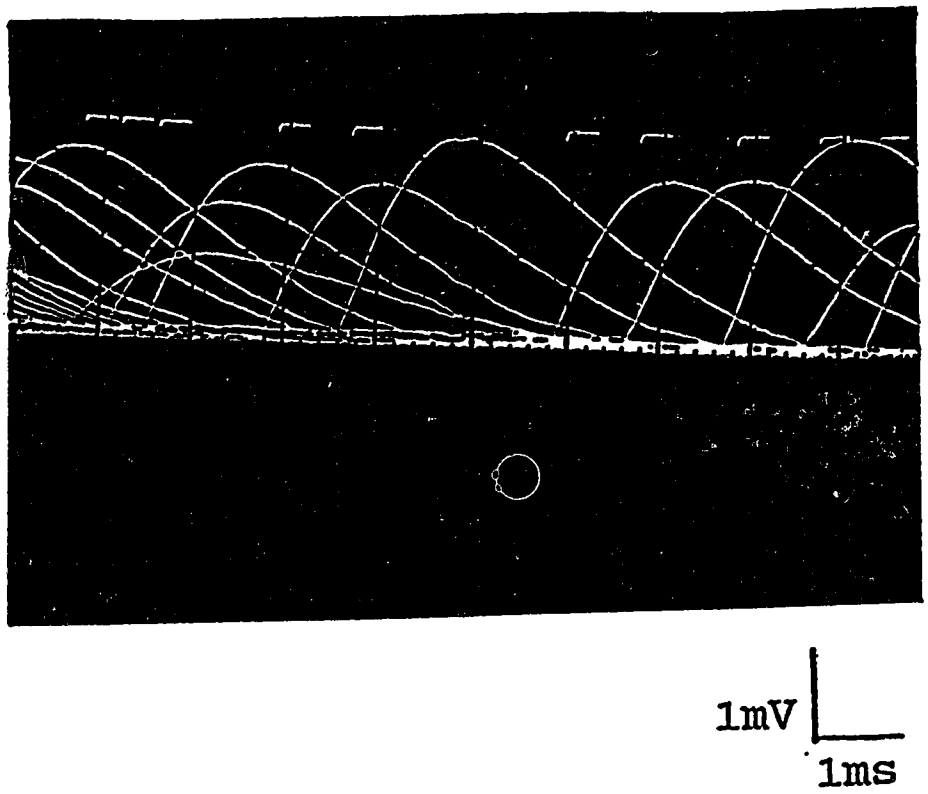


Fig. 3. The rise time of a single mepp.

The spontaneous resting and accelerated transmitter release were determined by monitoring the frequency of mepp at a single end plate where the rise time was less than 1 ms. This picture represented about 33 sweeps and mepps were recorded under conditions of accelerated release (15 mM $[K^+]_o$). The discontinuous line (upper trace) is the top of 500 μS pulses initiated as the rising membrane potential (lower trace) crossed the preset threshold, and is therefore indicative of a single mepp. These pulse were fed to the counter for recording.

4. EXPERIMENTAL PROTOCOLS

4.1. ADENOSINE RECEPTOR EXPERIMENTS

In experiments designed to determine the effect of adenosine analogues, agonist drugs were added to the preparations as described in the preceding section. Where the antagonism by theophylline was under investigation, agonist drug was added and the mepp frequency observed until new stable values for mepp frequency were attained. Theophylline was then added, still in the presence of agonist, and the mepp frequency monitored for a further period of time.

In experiments designed to elucidate the transduction mechanism of the adenosine receptor, a period of preincubation was added to the protocol. Tissues were incubated in oxygenated Krebs-Henseleit solution containing 50 μM of either H-7 or piperazine at room temperature for 60 min prior to replacement with normal Krebs-Henseleit solution and perfusion with 1 μM 2-chloroadenosine as before. In control experiments, the tissue were treated similarly, except that preincubation was in Krebs-Henseleit solution alone. Where preincubation with Pertussis toxin (PTX) was involved, a different modification to the protocol was required. As uptake of PTX into the cell is an active process requiring an appreciable time to accomplish, tissues were pre-incubated at

37° C for 3-4 hours in oxygenated Kreb-Henseleit solution containing 2.5 μg/ml PTX (Katada and Ui, 1982). Control tissues were similarly pre-incubated using Krebs-Henseleit solution alone. After the preincubation period, tissues were pinned on a Sylgard base and exposed to 1 μM 2-chloroadenosine as before.

4.2. ADRENOCEPTOR AND ACTH EXPERIMENTS

In these experiments tissues were exposed to agonist drugs in HEPES buffered Bretag's solution. As the frequency of spontaneous release in Bretag's solution is very low, and no response to these drugs was detected at normal concentration of salts, it was necessary to carry out the experiments under the conditions of accelerated transmitter release caused by elevating the potassium concentration of the bathing solution. The following protocol was therefore adopted.

Dissected diaphragms were treated for 20 minutes with 1.5 M formamide in Bretag's solution at room temperature (del Castillo and Escalona de Motta, 1978), before being washed in normal Bretag's solution for 10 minutes and immersed in Bretag's 15 mM K^+ solution. After 30 minutes, depolarization of the terminals and the rate of transmitter reached a steady state (Gage and Quastel, 1965; Cooke and Quastel, 1973b) and

exposure to agonist or antagonist drugs could proceed as before. Treatment with formamide was necessitated by the tendency of the muscle to fibrillate when depolarized by the high K^+ concentration. Formamide is believed to uncouple the excitation-contraction process by interference with either the t-tubular system, or the sarcoplasmic reticulum without affecting the resting membrane potential or the action potential (Escalona de Motta, 1982; Califano et al., 1982).

Where adrenoceptor antagonists were under investigation, tissues in 15 mM K^+ were preincubated at room temperature with the particular antagonist for 30 minutes before 10 μM Epi was introduced to the bathing fluid.

For the investigation of transduction mechanism, a 60 minute preincubation period at room temperature with an enzyme inhibitors in 15 mM K^+ Bretag's solution, followed by washout and perfusion of 10 μM NE or 10 μg/ml ACTH as before was incorporated in the protocol. In the experiments involving the use of lithium, the equivalent molar concentration of NaCl was replaced by 10 mM LiCl in 15 mM K^+ Bretag's solution. After a 5 min period of stable transmitter release without Li^+, the Li^+ containing solution was perfused through the Petri dish and the mepp frequency observed. When the stimulatory effect of Li^+ reached a plateau, 0.1 μM NE or 10 μg/ml ACTH was added to the perfusion solution, and the

effect of these substances on mepp frequency in the presence of Li^+ observed. Experiments involving PTX were prefaced by a period of incubation with 2.5 μg/ml PTX in oxygenated Krebs-Henseleit solution at 37° C for 3-4 hours as before. Thereafter, experiments were performed in 15 mM K^+ Bretag's solution. Control tissues were similarly treated with omission of the PTX from the preincubation solution.

5. DRUGS USED

5.1. AGONISTS*

Adrenocorticotrophin (adrenocorticotropic hormone, ACTH), porcine (Sigma)

L-Arterenol bitarrrate (noradrenaline, norepinephrine bitartrate) (Sigma) (adrenoceptor)

2-Chloroadenosine (2-ClA) (Sigma) (adenosine receptor)

(-)-Epinephrine (+)-bitartrate (adrenaline bitartrate) (Sigma) (adrenoceptor)

5'-N-Ethylcarboxamidoadenosine (NECA)# (Boehringer Mannheim) (adenosine receptor)

(±) Isoproterenol HCl (isoprenaline HCl) (Sigma) (beta-adrenoceptor)

2-(p-Methoxyphenyl)-adenosine (CV-1674)# (Takeda Chemical Industries, Osaka) (adenosine receptor)

Phenylephrine HCl (Sigma) (alpha 1-adrenoceptor)

L-N6-Phenylisopropyladenosine (PIA)# (Boehringer Mannheim) (adenosine receptor)

5.2. RECEPTOR ANTAGONISTS

Nadolol (Squibb Canada) (beta-adrenoceptor)

Prazosin HCl (Pfizer Canada Inc.) (alpha 1-adrenoceptor)

Theophylline (Sigma) (adenosine receptor)

Yohimbine HCl (British Drug Houses Ltd) (alpha 2-adrenoecptor)

5.3. ENZYME INHIBITORS

(N-(6-Aminoethyl)-5-chloro-1-naphthalene sulfonamide) (A-3) (Gift from Dr. Hiroyoshi Hidaka, Dept. of Pharmacology, Nagoya University School of Medicine) (calmodulin, cyclic nucleotide dependent kinases)

N-(6-Aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) (Sigma) (calmodulin)

Auranofin^ (Smith, Kline and French) (PKC, calmodulin)

Clomiphene citrate (Sigma) (PKC, calmodulin)

Lithium chloride, hydrated (British Drug Houses Ltd) (phosphatase)

1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) (Sigma) (PKC, cyclic nucleotide dependent kinase)

Pertussis toxin (PTX) (Connaught Laboratories) (some

G-proteins)

Piperazine citrate (Mann Research Laboratories, Inc.)

Polymyxin B sulphate (Sigma) (PKC)

* Unless specified otherwise, all the drugs were directly dissolved in either Krebs-Henseleit or Bretag's 15 mM K^+ solutions according to the experimental requirements. Where necessary, solution was accelerated by sonication.

NECA, PIA, CV-1674 were first dissolved in dimethyl-sulfoxide (DMSO) and then diluted in Krebs-Henseleit solution before use, the final concentration of DMSO was less than 1%. To compensate for small changes in mepp(f) caused by DMSO, all control tissues were bathed in the physiological solution containing the same concentration of DMSO as the drug solution.

^ Auranofin was first dissolved in polyethylene glycol 400 (PEG 400) (Sigma), to a concentration of 20 mM, and dilution of this stock solution was made with Bretag's solution. The final concentration of PEG 400 was 0.1%, which had no discernable effect on mepp frequency. Nonetheless, all control experiments for the auranofin group were performed in the presence of 0.1% PEG 400.

6. DATA ANALYSIS AND STATISTICS

Data appearing in this thesis were "normalized" for all experiments. All values at minute intervals were expressed as percentage of initial control value, which itself was the mean of at least five successive readings at minute intervals before agonist drug was admitted to the dish. For comparison of drug effects, the peak response, measured over several minutes and averaged for each experiment was combined with similar results from other tissues to provide a mean response. Where appropriate, values presented are the mean ± standard error of the mean for the number (n) of tissues used. Unless otherwise specified, n indicates the number of fibers used and equals the number of hemidiaphragms used in a particular experimental series (5-6). Unpaired or paired Student's t values as appropriate were determined to estimate the level of significance level of the difference between two means. Multiple comparisons were made using Duncan's multiple Range Test. A probability < 0.05 was taken to indicate a significant difference.

CHAPTER III

RESULTS

1. ADENOSINE RECEPTORS

1.1. THE INHIBITORY EFFECT OF ADENOSINE ANALOGUES ON TRANSMITTER RELEASE

All four adenosine analogues tested (2-ClA, L-PIA, CV-1674, and NECA) reduced the frequency of spontaneous transmitter release from the motor nerve terminal of mouse phrenic nerve. The maximum inhibitory effect was attained 10 minutes after application of the drugs. Fig. 4 shows the typical time course of CV-1674 at three different concentrations. Results presented in Fig. 7 indicate that 2-ClA shares the same time course as CV-1674. Mepp frequency is depressed to a steady state in a concentration-dependent manner 10 minutes after introduction of agonist to the perfusing solution.

1.2. COMPARISON OF POTENCY OF ADENOSINE ANALOGUES ON TRANSMITTER RELEASE.

The concentration-effect relationships for the four drugs are shown in Fig. 5. L-PIA produces maximal inhibition in the concentration range of 0.1-0.3 μM, with a reduction in its effect at higher concentration (10 μM). However, the other three drugs reach their maximal inhibitory effect at 10 μM. As seen in Fig. 5, the curve for PIA parallels with that of NECA,

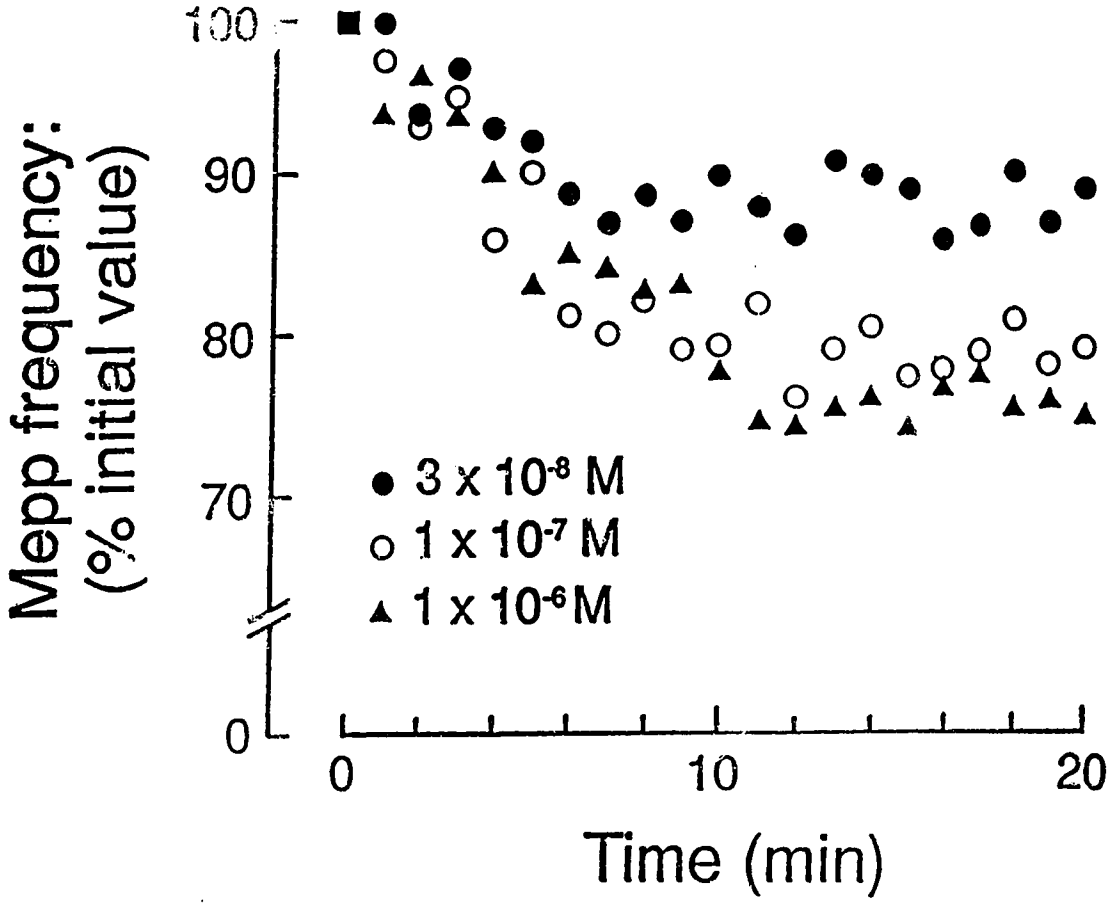


Fig. 4. Time course of the effect of three different concentrations of the CV-1674 on mepp frequency in the mouse hemidiaphragm preparation.

The control value was obtained for at least 5 minutes preceding application of CV-1674. Mean control mepp frequency was 56.86 per minute (■). Each point represents the mean value from five to six preparations. Standard errors of the mean were ±5-11% of initial value and have been omitted for clarity.

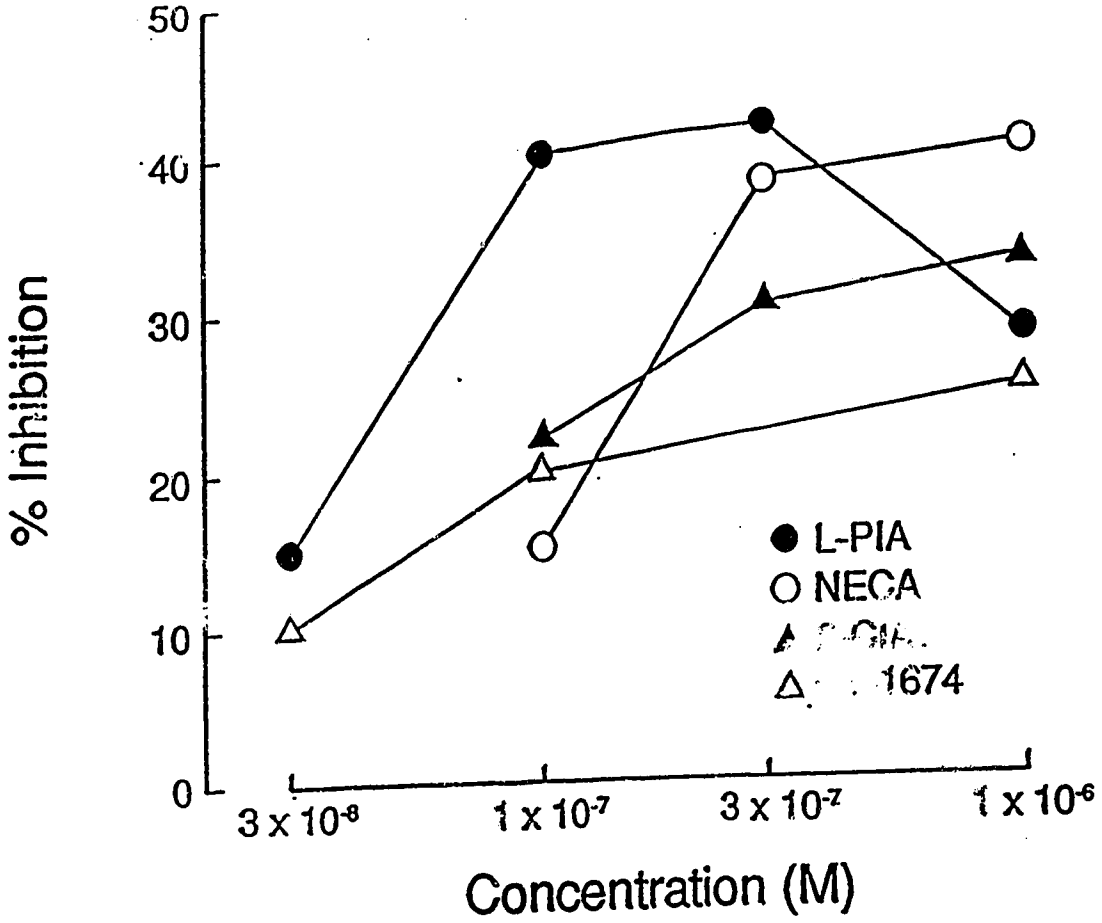


Fig. 5. Effect of L-PIA (●), NECA (○), 2-ClA (▲), and CV-1674 (△) on mepp frequency in the mouse hemidiaphragm preparation, shown as concentration-inhibition lines. Each point represents the mean response from five or six preparations. Standard errors of the mean were ± 6-11% of initial value and have been omitted for clarity.

while the curve for 2-ClA is parallel to that for CV-1674, with an apparently lower asymptote than that observed with PIA and NECA. Furthermore the slopes for PIA and NECA are steeper than those of 2-ClA and CV-1674.

From Fig. 5 it is possible to derive an approximate rank order of potency for the pairs of adenosine analogues, based on ED_{50} values. In this case the order of potency is L-PIA > NECA and 2-ClA $\geq$ CV-1674, though the potency ratio between these drugs is narrow.

1.3. INHIBITORY EFFECT REVERSED BY ANTAGONIST

An adenosine receptor antagonist was used to confirm the presence of a presynaptic adenosine receptor. At the peak of the response to CV-1674, theophylline was introduced to the dish at a concentration that does not affect mepp frequency in the mouse diaphragm (Dryden et al., 1988). Fig. 6 illustrates the effect of theophylline (0.1 mM) on the inhibitory action of a submaximal dose of CV-1674 (0.1 μM). Theophylline was applied 11-12 minutes after the addition of CV-1674, and restored the mepp frequency to a value close to that observed before addition of CV-1674.

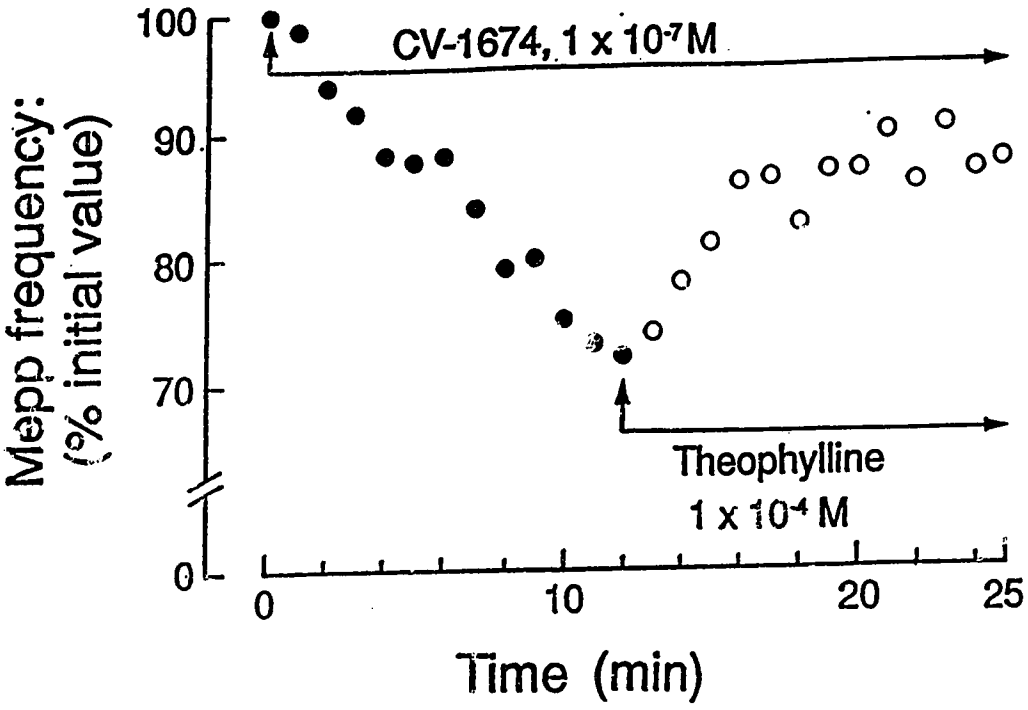


Fig. 6. Effect of theophylline (10^{-4} M) on the depression of mepp frequency induced by CV-1674 (0.1 μM). The control value was obtained for at least 5 minutes preceding application of CV-1674. Theophylline was introduced at 11-12 minutes in the continued presence of CV-1674. Each point represents the mean response from five to six preparations. Standard errors of the mean were ± 5-11% and have been omitted for clarity.

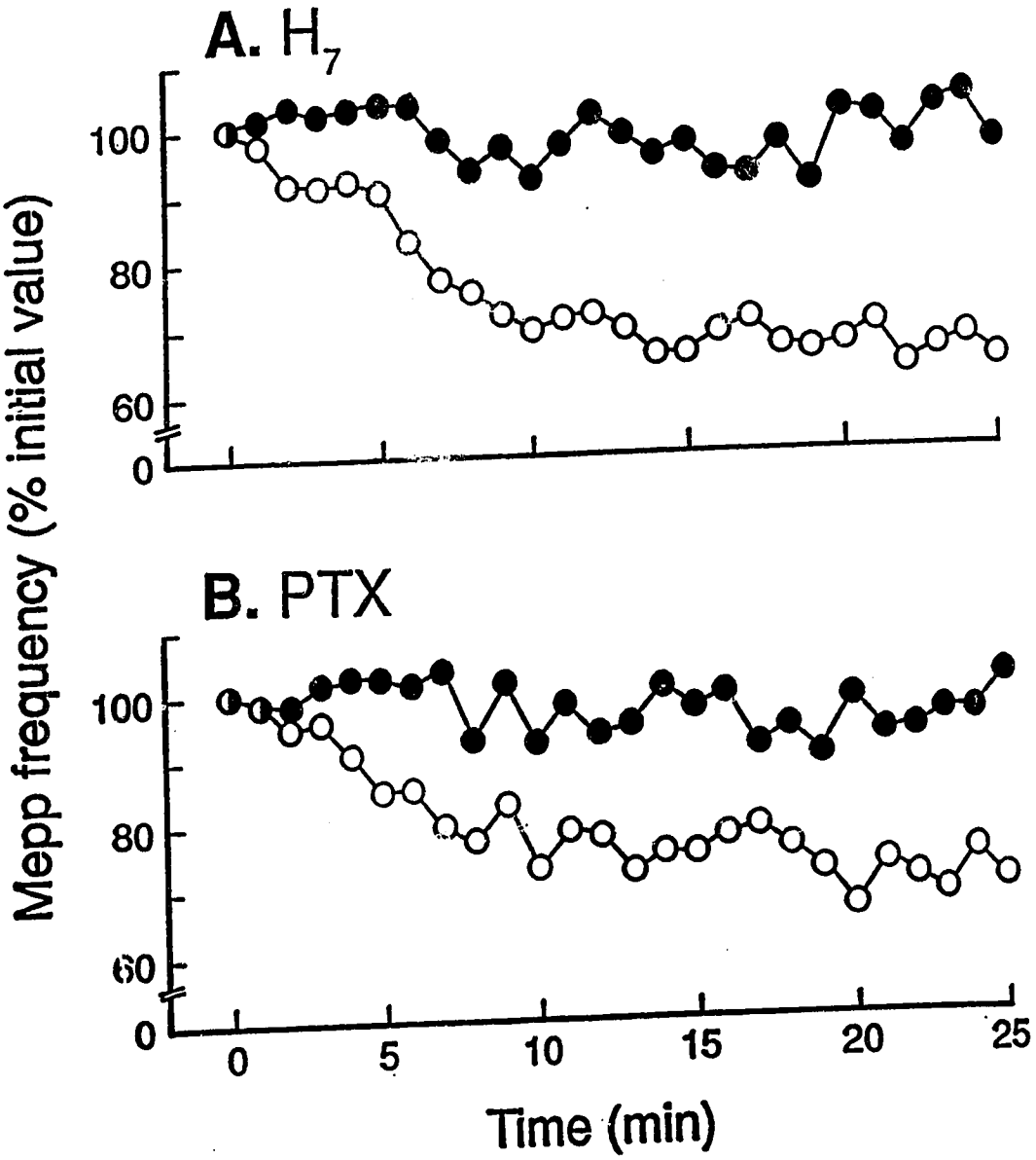
1.4. SIGNAL TRANSDUCTION

As controversy existed concerning the mechanism of transduction of the presynaptic adenosine receptor, two experiments were performed using either the protein kinases inhibitor, H-7, or PTX, active against certain G-proteins. The average resting mepp frequency obtained from 30 muscle fibers in each of six different preparations by random penetration before and after incubation with H-7 revealed that at 50 μM, H-7 alone caused a significant reduction in mean mepp frequency to 65.9 $\pm$ 2% ($\pm$ S.E.M.) of initial control level ($p < 0.05$, n=6, Student's t-test). Untreated control tissues after the same period had a mean mepp frequency of 98.9 $\pm$ 4.4% of initial value.

After the preincubation period with H-7, 1 μM 2-ClA was perfused through the tissue bath. As seen in Fig 7A, about 10 minutes after exposure to 1 μM 2-ClA, mean mepp frequency in control tissues was depressed to 67.4 $\pm$1.0% of preperfusion levels, indicating a 32.6% decrease in resting mepp frequency. In H-7 pretreated tissue, however, 2-ClA evoked no change in the rate of transmitter release as mepp frequency was 97.8 $\pm$ 3.2% of initial control value, significantly different from the value for control tissues ($p < 0.05$, n=6). Pretreatment of tissues with 50 μM piperazine instead of H-7 did not affect the response to the adenosine analogue, indicating that the

bicyclic sulfonamide residue of H-7, recognized as essential for protein kinase inhibition (Garland et al., 1987), was necessary for the molecule's activity. Prior incubation of tissues with PTX was also effective in abolishing the response to 2-ClA. Fig. 7B shows that between 10 and 25 minutes after perfusion of 2-ClA had begun, mean mepp in control tissues was 74.5 $\pm$ 1.5% of preperfusion levels. However, prior incubation with 2.5 μg/ml PTX abolished the inhibitory effect of 2-ClA. Mean mepp frequency was 95.8 $\pm$ 6.6% of preperfusion levels, significantly above values from control tissues ($p < 0.05$, $n=6$).

Fig. 7. Effect of H-7 or PTX on response to 2-ClA. Test tissues (●) were preincubated either with 50 μM H-7 (panel A), or with 2.5 μg/ml PTX (panel B). Control tissues (o) were preincubated in Krebs-Henseleit solution alone. Frequencies are expressed as percentage of the average mepp frequency for each tissue measured for at least 5 consecutive minutes prior to perfusion of the agonist. Each point is the mean of 6 experiments. Standard error bars have been omitted for clarity, but fell within the range of 4-7% of initial average.


A. H7
100
80
60
0
B. PTX
100
80
60
0
0
5
10
15
20
25
Time (min)
Mepp frequency (% initial value)

2. ADRENOCORTICOTROPHIN RECEPTORS

2.1. EFFECT OF ACTH ON SPONTANEOUS AND ACCELERATED TRANSMITTER RELEASE.

The resting mepp frequency was not affected in the presence of 10 μg/ml ACTH (n=6) in normal Bretag's solution. When the potassium concentration of Bretag's solution was increased to 15 mM, the mepp frequency was accelerated (Fig. 8B). The accelerated mepp frequency was significantly increased by 16% in the presence of 10 μg/ml ACTH (116.84 ± 2.67% of the initial control value, $p < 0.01$) in tissues from 12 out of 20 mice (Figs. 9,12), while 8 of them showed no response. Fig. 9 shows the accelerated mepp frequency reached a plateau value 5-7 minutes after application of 10 μg/ml ACTH. Fig. 10 shows both hemidiaphragms of a responding mouse manifested a similar response to ACTH. When one (right) hemidiaphragm exhibited a positive response to ACTH (119.16 ± 5.70% of the control value, n=5), the other one (left) was equally sensitive (121.13 ± 9.77%, n=5). However, when one hemidiaphragm of a particular mouse failed to respond to ACTH, the contralateral was also insensitive (n=4). Therefore, one hemidiaphragm could be used first to determine if both tissues would respond and also as a control tissue, while the other one was used as test tissue.

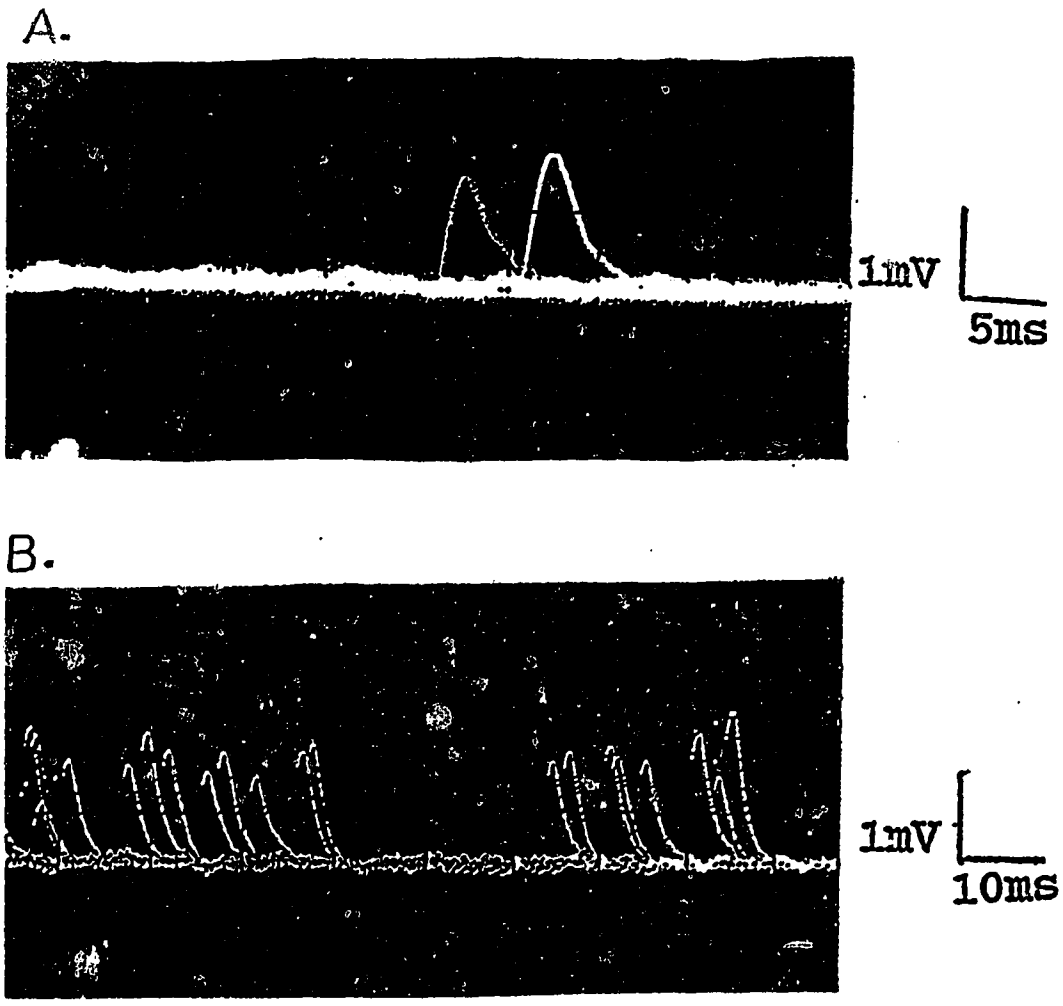


Fig. 8. The effect of 15 mM $[K^+]_o$ on transmitter release.

(A) Resting spontaneous transmitter release in 3.48 mM K^+. (B) Accelerated transmitter release. The nerve terminal was partially depolarized with 15 mM K^+ for 30 minutes, mepp frequency was increased. The raised concentration of potassium is believed to exert its action by opening voltage-sensitive calcium channels and raising cytosolic free calcium. There are an estimated 42 sweeps on panel A and 6-7 sweeps on panel B.

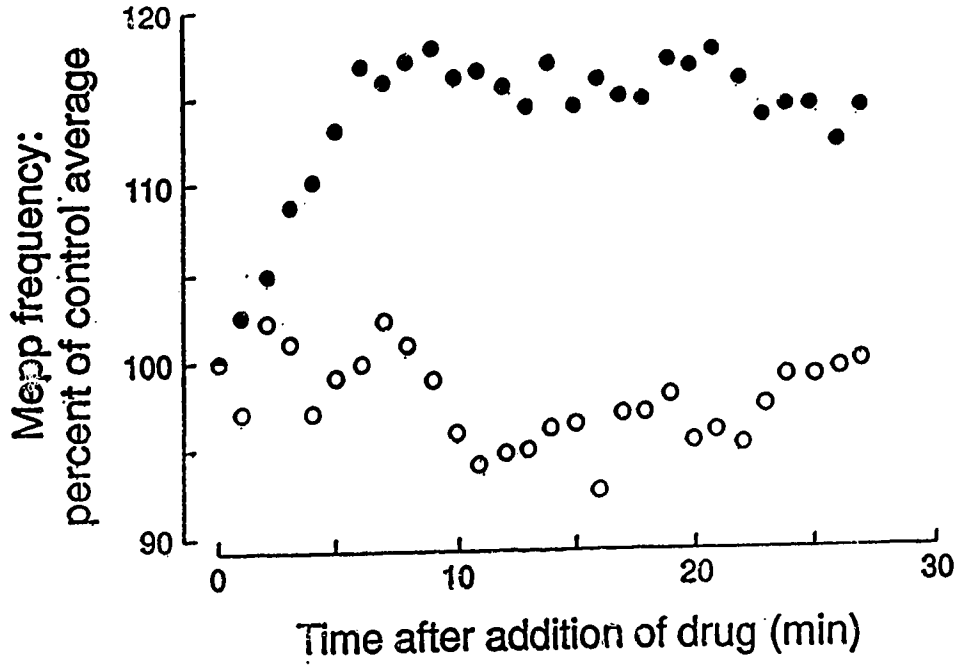


Fig. 9. Time course of the effect of ACTH on accelerated transmitter release.

ACTH (10 μg/ml) was perfused after a control value of at least 5 minutes was obtained. Accelerated mepp was sustained at a plateau 5-7 minutes after addition of ACTH (●) in 12 out of 20 mice tested (see Fig. 12), while no response to the same concentration of ACTH in 8 of 20 mice was seen (o). For clarity, each point is the mean of only 6 determinations, standard errors fell between 1 and 6% of control value and have been omitted. The mean control frequency measured over 5-6 min. before addition of drug was 1911 per minute (●).

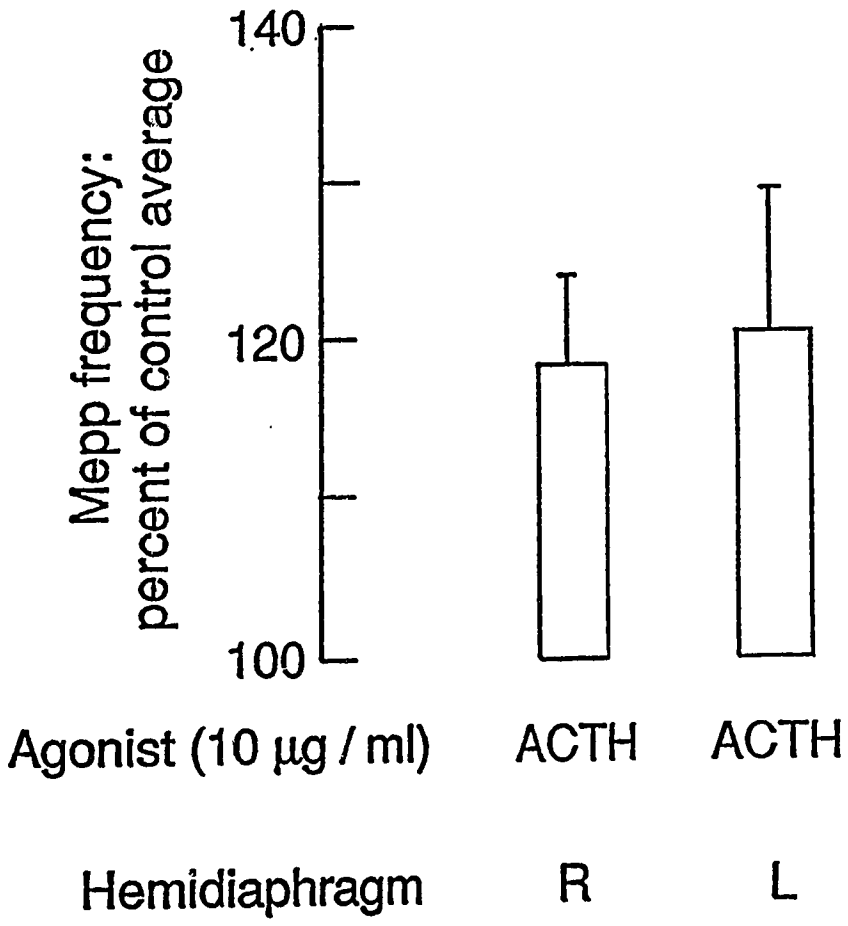


Fig. 10. Comparison of response of contralateral hemidiaphragms to ACTH.

Only 60% of diaphragms tested responded to ACTH, but where a response was observed, it was bilateral and comparable. Mean right hemidiaphragm response to ACTH (R) was 119.16 ± 5.70%, n=5, and mean left hemidiaphragm response (L) was not significantly different (121.13 ± 9.77%, n=5).

2.2. TRANSDUCTION MECHANISM OF THE PRESYNAPTIC ACTH RECEPTOR.

As demonstrated above, ACTH caused an increase in accelerated mepp frequency. As ACTH is believed to act on adenylate cyclase in the adrenal cortex, the mechanism by which ACTH modulated transmitter release from motor nerve terminals was postulated also to involve adenylate cyclase and PKA. As a test of this postulate the following experiments were performed. The ACTH sensitivity of one hemidiaphragm was tested while the other was incubated in 2.5 μg/ml pertussis toxin at 37° C for at least three hours before treatment with 10 μg/ml ACTH. Sensitive untreated controls responded with a 13.91 $\pm$ 2.7% increase in mepp frequency but in the PTX incubated contralateral tissues, the response to ACTH was larger (122.36 $\pm$ 1.36% of initial average, n=6) (Fig. 11). As PTX is known to affect sensitive systems under these conditions (Fig. 7B) it appeared that a PTX-sensitive system was indirectly but not directly involved in the transduction of the presynaptic ACTH receptor. Further studies showed that in H-7 (50 μM) treated tissues, the ACTH-induced increase in transmitter release was significantly attenuated to 101.58 $\pm$1.33% of initial value in 5 out of 7 tissues compared with 115.24 $\pm$ 4.35% caused by 10 μg/ml ACTH alone ($p < 0.05$, n=5) (Fig. 12). The involvement of cyclic AMP- or cyclic GMP-dependent protein kinases or PKC in this transduction mechanism thus seemed a possibility. A-3 which at a

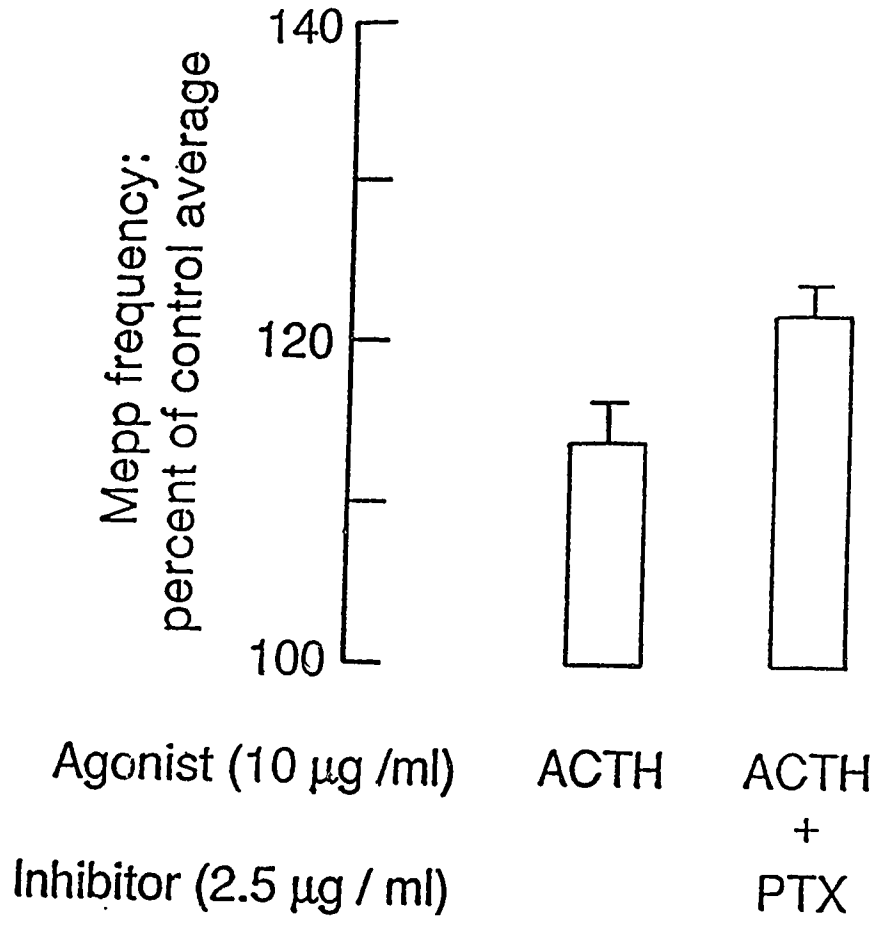
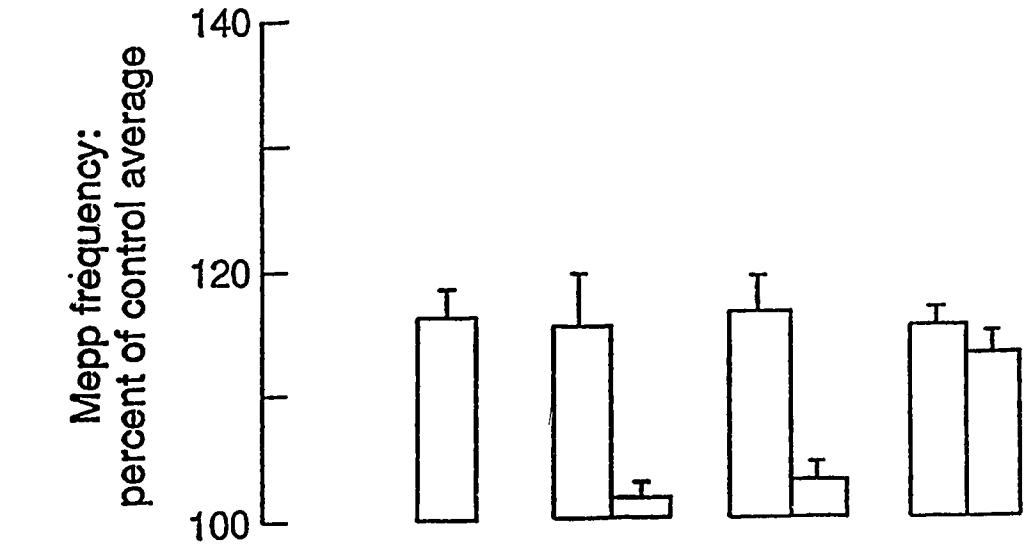


Fig. 11. Effect of PTX on accelerated transmitter release enhanced by ACTH.

Pretreatment of tissues with 2.5 µg/ml PTX for 3-4 hours at 37° C enhanced the response to ACTH (10 µg/ml), this intensification was significantly different from the control response to ACTH ($p < 0.05$, Student's t-test).

Fig. 12. Effect of H-7, A-3, W-7 on the response of accelerated transmitter release to ACTH in paired hemidiaphragms.

The increase in transmitter release induced by 10 μg/ml ACTH was significantly attenuated by pretreatment with 50 μM H7 for 60 minutes ($p < 0.01$, Student's paired t test). The response was also significantly depressed by the prior treatment of 10 μM A-3 for 60 minutes ($p < 0.01$, Student's paired-t test). The effect of 10 μg/ml ACTH was not affected by pretreatment with 10 μM W-7.



Agonist (10 µg / ml)	ACTH	ACTH	ACTH	ACTH
	+	+	+	+
Inhibitors (µM)				
H_7	—	50	—	—
A_3	—	—	10	—
W_7	—	—	—	10
Fractional Response	$^{12}/_{20}$	$^{5}/_{7}$	$^{5}/_{6}$	$^{7}/_{10}$

concentration of 10 μM inhibits cyclic nucleotide dependent protein kinases and CaM-dependent kinase but not PKC was thereafter used to determine whether PKC or one of the cyclic nucleotide dependent kinases was likely involved. The stimulatory effect induced by ACTH in this experiment (116.33 $\pm$ 3.04% of initial value) was significantly reduced to 103.18 $\pm$ 1.48% of initial value in 5 out of 6 tissues which were pretreated with 10 μM A-3 ($p < 0.05$, n=5) (Fig. 12).

As this evidence was far from confirmatory, an attempt was made to demonstrate an intensification of the response in the presence of lithium (see Introduction, 5.2.1.) as described in Methods. Fig. 13 shows that mepp frequency was significantly increased to a value of 119.96 $\pm$ 1.6% of initial value (n=6, $p<0.01$), 5 to 10 minutes after perfusing with 10 mM lithium. The effect of lithium on accelerated mepp frequency was sustained to form a plateau up to the 14th minute of exposure. The effect of ACTH added during the plateau of the lithium effect is shown in Fig. 14. Mepp frequency was increased to 112.20 $\pm$ 4.09% after addition of 10 μg/ml ACTH but without Li^+. However, the effect of ACTH was not significantly different in the presence of Li^+ (109.46 $\pm$ 2.33% of initial value, n=6). From this the tentative conclusion was made that IP_3 did not play a role in the modulation of transmitter release caused by ACTH. ACTH appeared probably, therefore, to enhance the accumulation of

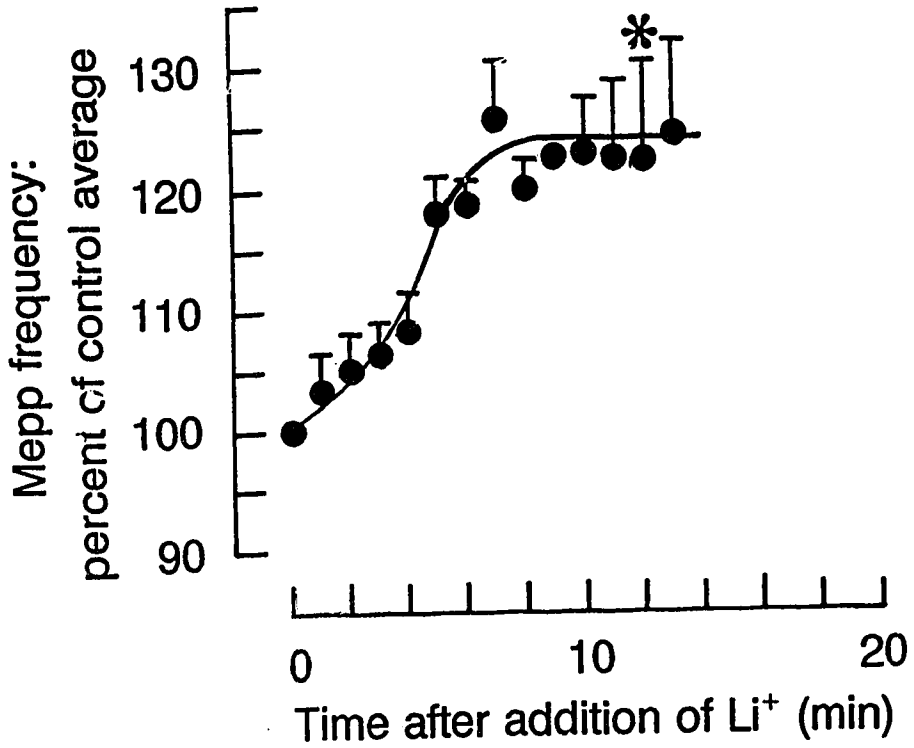


Fig. 13. Effect of 10 mM Li^+ on accelerated mepp frequency.

The 15 mM K^+-evoked mepp frequency rose to a reach a plateau over 5 to 10 minutes when exposed to 10 mM Li^+ (n=5). The potentiating effect of Li^+ on mepp frequency is sustained up to the 14th minute of exposure to Li^+.

* n=3

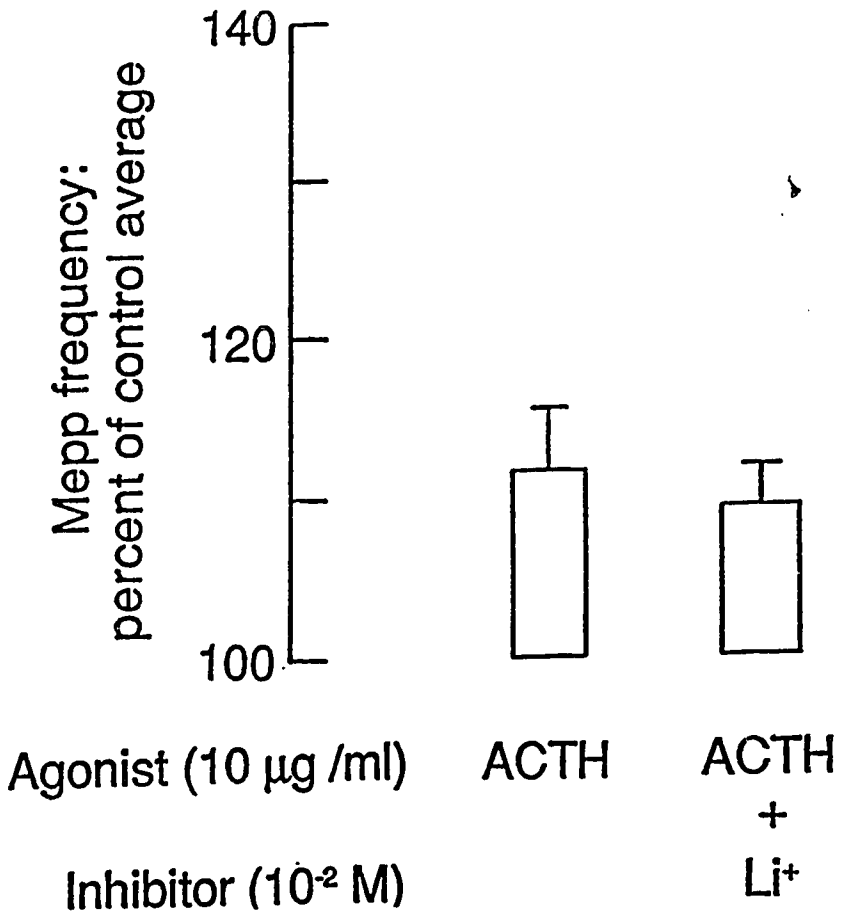


Fig. 14. Effect of 10 mM Li^+ on the increase in accelerated mepp frequency caused by ACTH.
Tissues were pretreated with 10 mM lithium and at the plateau of the response, 10 mg/ml ACTH was added. The additional increase in mepp frequency caused by ACTH in the presence of Li^+ was not significantly different from that produced in the absence of Li^+ (see text for detail).

cyclic AMP within the nerve terminal which in turn activated cyclic AMP-dependent protein kinase. However this transduction sequence is not calmodulin-dependent. In an experiment involving 7 responding tissues out of 10 tested, 10 μM W7 did not affect the response to ACTH (115.49 $\pm$ 1.49% of initial value in after treatment with W7 compared with 113.08 $\pm$ 1.82% of initial value caused by ACTH alone) (Fig. 12).

3. ADRENOCEPTORS

3.1. EFFECT OF ADRENOCEPTOR AGONISTS ON SPONTANEOUS AND ACCELERATED TRANSMITTER RELEASE FROM MOTOR NERVE TERMINALS

The resting mepp frequency was not changed in the presence of up to 10 μM Epi, or 1 μM NE in Bretag's solution containing 3.48 mM K^+ (Fig. 15). When the concentration of K^+ was increased to 5.9 mM which is the same as that in Krebs-Henseleit solution, the agonists again caused no alteration in mepp frequency. As seen in Fig. 15, mepp frequency was recorded continuously before and during application of drugs and the variance after exposure to agonists is similar to that seen in the preperfusion period (see blank). However, when the K^+ concentration was further raised to 15 mM, the accelerated mepp frequency was

significantly increased by both NE and Epi, and by the selective alpha-adrenoceptor agonist, phenylephrine (PE) (Fig. 16). As shown in Fig. 16, both Epi and NE causes a rapid increase in mepp frequency to reach a peak within 5 minutes, and thereafter a slow but definite decline in mepp frequency was observed. However, the effect of PE was slower in onset but better sustained over the period of time.

3.2. POTENCY OF ADRENOCEPTOR AGONISTS

The concentration-effect relationships of adrenoceptor agonists are presented in Fig. 17. Epi and NE were similar in potency while PE manifested a lesser potency. Isoproterenol evoked no response even at high concentration, indicating an absence of beta-adrenoceptors in modulating spontaneous quantal release from the motor neuron terminal.

3.3. ADRENOCEPTOR SUBTYPE

The effectiveness of PE, and lack of response to isoproterenol suggested that an alpha-1 adrenoceptor might be involved. This was confirmed using the selective antagonist, prazosin, effective at alpha-1 adrenoceptor, yohimbine, effective at alpha-2 adrenoceptor, and nadolol, active at both

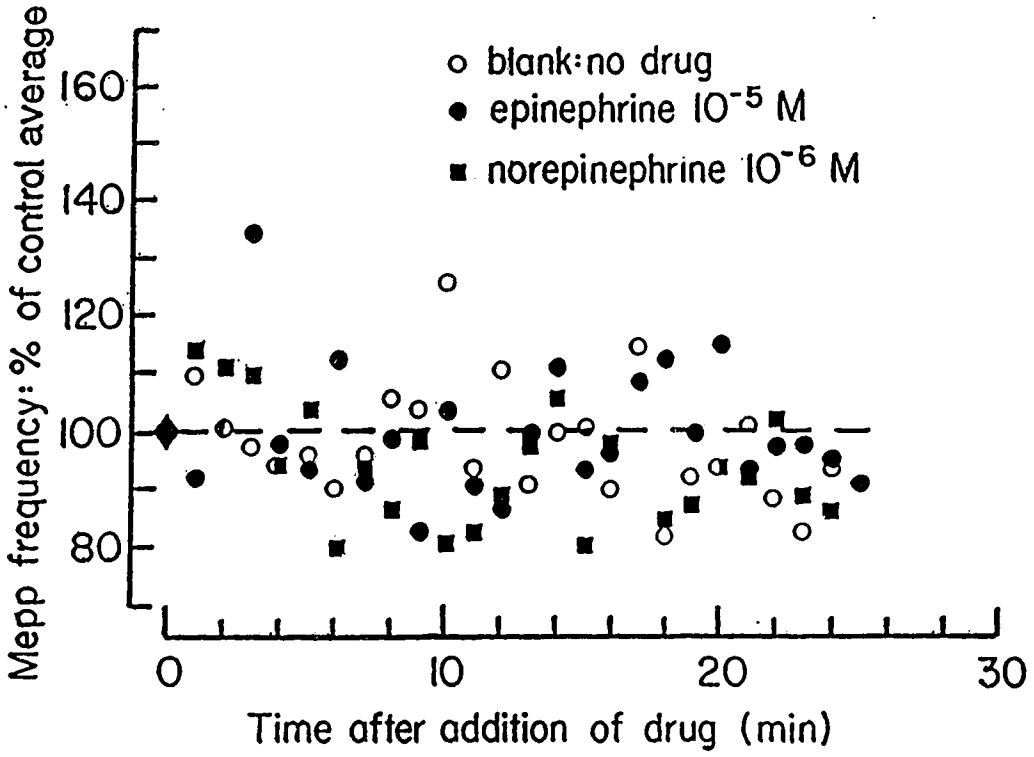


Fig. 15. Resting mepp frequency in 3.48 mM $[K^+]_o$ following exposure to adrenoceptor agonists.

Spontaneous quantal release is a random process with considerable variation about the mean. The presence of either Epi (10 μM) or NE (1 μM) caused no significant change in the mean release or the randomness of this pattern. Points are the mean of observations from 6 different tissues. Mean control mepp frequency measured for 5 minutes before addition of drugs was 27.14 per minute (◆).

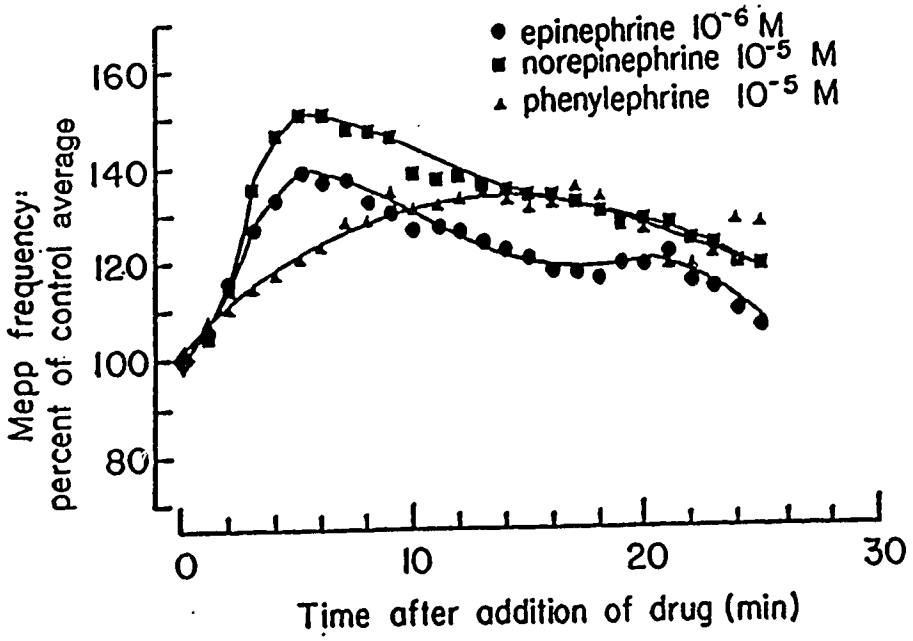


Fig. 16. Mepp frequency in 15 mM $[K^+]_o$ following exposure to alpha-adrenoceptor agonists.

Various alpha-adrenoceptors induced an increase in mepp frequency under conditions of accelerated transmitter release. The response was rapid with Epi or NE, but declined rapidly over a 30-minute period. Onset is slow with phenylephrine but is better sustained. Points are the means of observations from 6 different tissues. The mean control frequency measured over 5 min before addition of drug was 27.91 per second (◆).

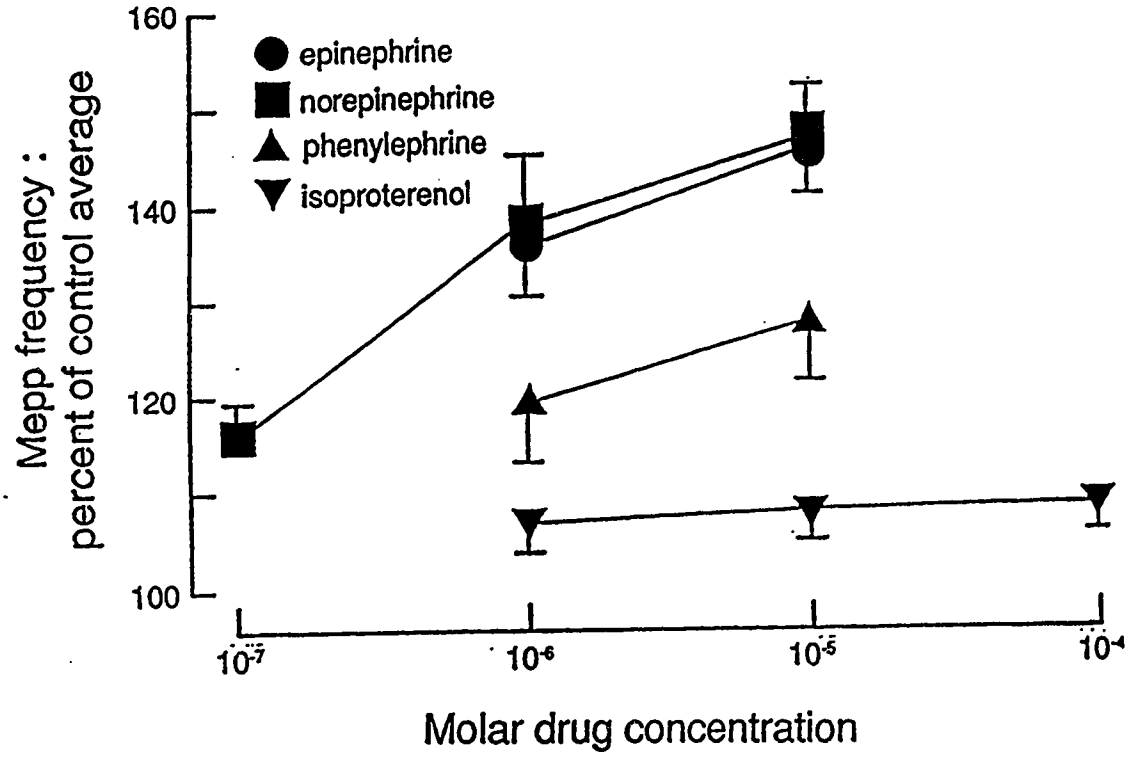


Fig. 17. Concentration-effect curves for adrenoceptor agonists.

Mean mepp frequency was measured between 5 and 10 minutes after addition of agonists. Epi and NE were equipotent, but the phenylephrine was of lesser potency. Isoproterenol had no effect.

beta adrenoceptor subtypes. As shown in Fig. 18, the effect induced by 10 μM Epi which exerts both alpha- and beta-adrenoceptor agonist effects was blocked by 1 μM prazosin but not by 1 μM yohimbine and unaffected by 1 μM nadolol. From this evidence it would appear that the catecholamine-induced increase in accelerated transmitter release at the motor nerve terminal is mediated by an alpha-1 adrenoceptor.

3.4. TRANSDUCTION MECHANISM OF PRESYNAPTIC ALPHA-1 ADRENOCEPTOR

Activation of alpha-1 adrenoceptor in many tissue is believed to initiate the breakdown of PIP_2 into DG and IP_3 (Hirasawa and Nishizuka, 1985). It was of interest, therefore to attempt to determine if this transduction pathway was used by the alpha-1 adrenoceptors on motor nerve terminals also. The response to 1 μM NE was tested after pretreatment with a G-protein inhibitor and enzyme inhibitors which reputedly inactivate protein kinases. Following preincubation with 2.5 μg/ml PTX the response to NE was 145.70 $\pm$ 12.30% of initial value (n=5) (Fig. 19). This was not significantly different from the control response (141.11 $\pm$ 13.22% of initial value) and suggests that a pertussis toxin sensitive G protein is not part of the transduction sequence.

The response to NE was also seen in 50 μM H-7 pretreated

tissue (143.68 ± 10.07% of initial value, n=5) but was attenuated in a concentration dependent fashion by polymyxin B and clomiphene, and by auranofin (Fig. 20). As the response to NE was not affected despite the presence of H-7 the involvement of protein kinase C or a cyclic nucleotide-dependent protein kinase is unlikely in the signal transduction mechanism. The response to NE was, however, reduced when tissues were pretreated with 10 μM W7 (110.11 ± 4.30% of initial value, n=5, $p < 0.01$, Fig. 20). As W-7 is exclusively a calmodulin inhibitor at this concentration, it would appear that the effect of NE on transmitter release is calmodulin dependent, and that calcium might play a role in the increase in transmitter release. Accordingly, the possibility that IP_3 which mobilizes calcium from intracellular pool might be involved in this response was tested. Lithium was employed to raise the cytosolic level of IP_3 by delaying its catabolism as before (Result 2.2.). NE (0.1 μM) applied at the plateau of the Li^+-induced response, caused a further increase in mepp frequency, significantly larger than that caused by 0.1 μM NE alone (129.1 ± 3.11% of initial value i.e. the plateau of Li^+ response vs 115.58 ± 3.56% of initial value, n=6, $p < 0.01$) (Fig. 21). Thus, lithium potentiated the effect of NE on mepp frequency.

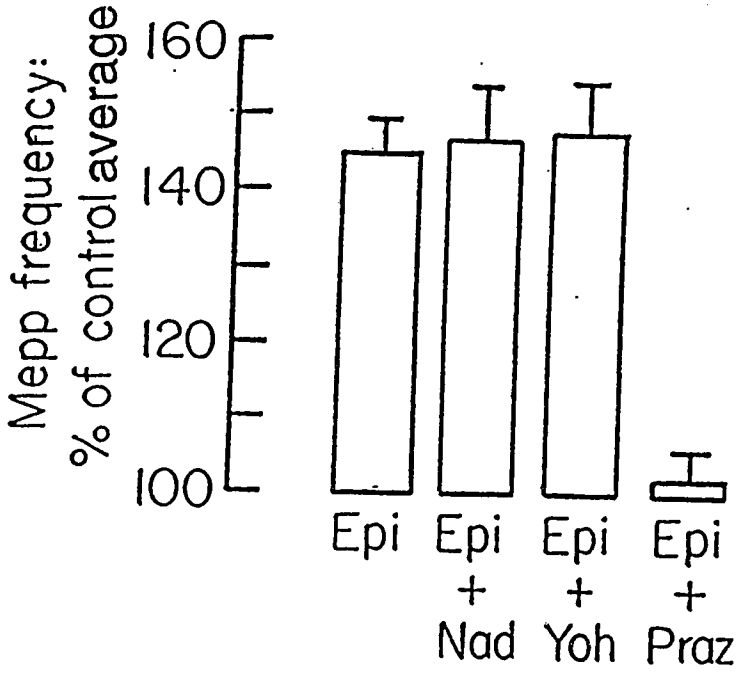


Fig. 18. Effect of specific adrenoceptor antagonists on increase in mepp frequency induced by 10 μM epinephrine. Nadolol, a beta-adrenoceptor antagonist without intrinsic activity or local anesthetic effect, and yohimbine, an alpha-2 adrenoceptor antagonist had no effect at concentration of 1 μM. Prazosin (1 μM), an alpha-1 adrenoceptor antagonist virtually abolished the response to epinephrine.

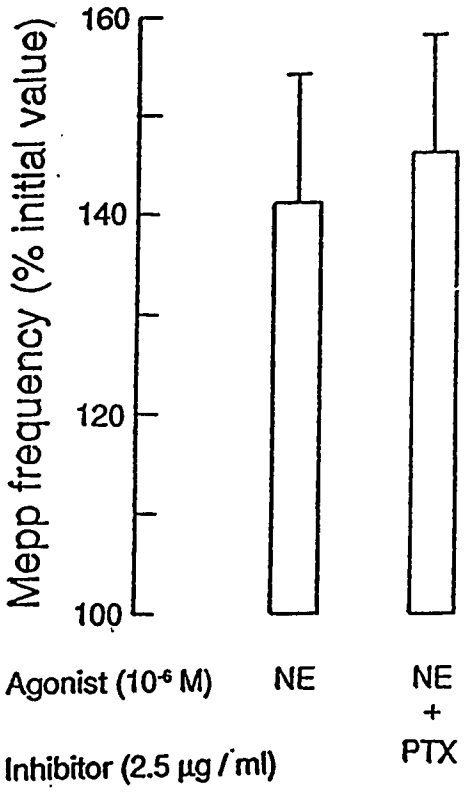


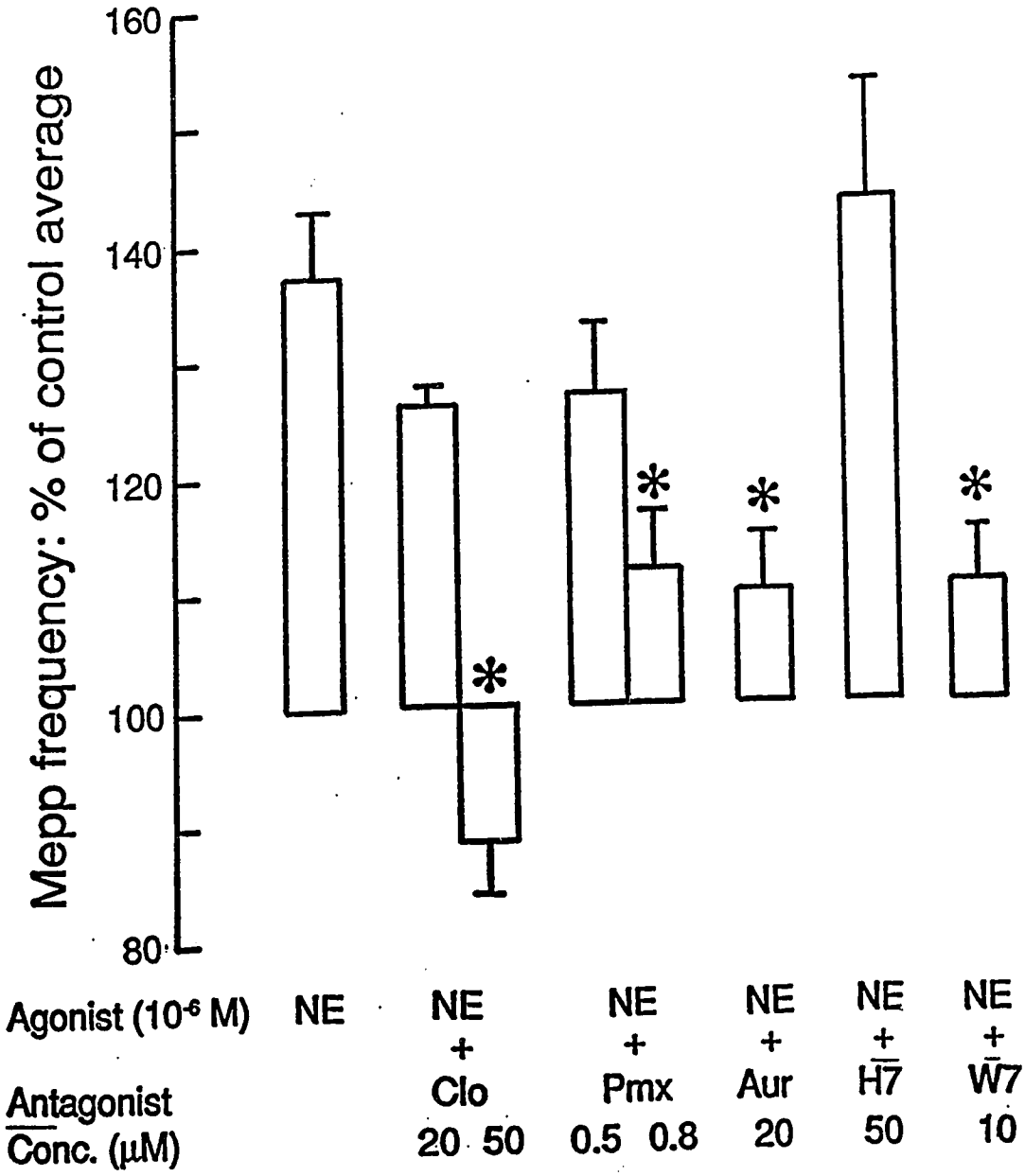
Fig. 19. Effect of PTX on the increase in transmitter release caused by NE.

Tissues were preincubated with 2.5 µg/ml PTX in oxygenated Krebs-Henseleit solution at 37° C for 3-4 hours prior to perfusion of NE as described in Methods. NE alone (1 µM) caused an increase in transmitter release of 41.11 ± 13.22%. The stimulant response to NE is not significantly changed in tissue pretreated with PTX (145.70 ± 12.3% of initial value).

Fig. 20. Effect of protein kinase inhibitors on NE-mediated transmitter release.

The response to 1 μM NE was reduced in a concentration-dependent manner by clomiphene and polymyxin B (PMX) and by auranofin. However, the response is not affected by pretreatment with 50 μM H-7. The response to NE is reduced following preincubatiuon with W-7.

* indicates values significantly different from that produced by NE alone ($p < 0.05$, Duncan's multiple range test).


Mepp frequency: % of control average
160
140
120
100
80
*
*
*
*
Agonist (10^{-6} M)
NE
NE + Clo
NE + Pmx
NE + Aur
NE + H7
NE + W7
Antagonist Conc. (μM)
20 50
0.5 0.8
20
50
10

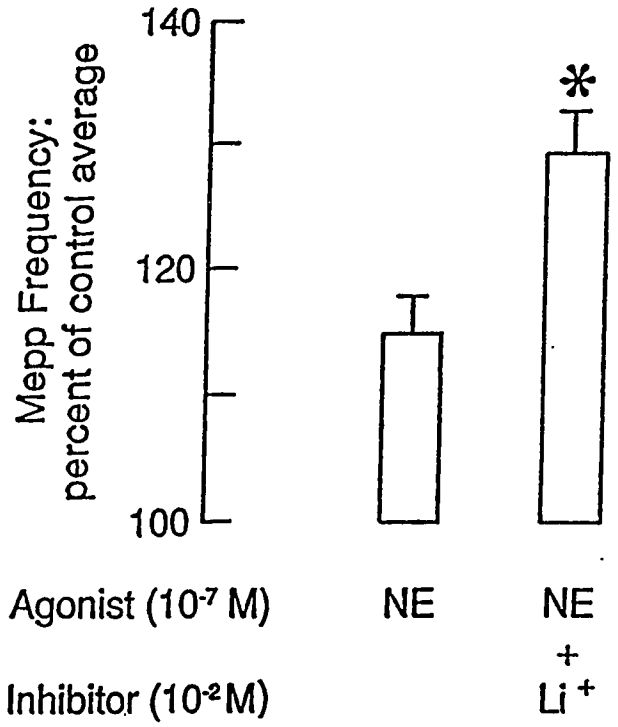


Fig. 21. Effect of 10 mM Li^+ on the increase in mepp frequency induced by NE.

When 0.1 μM NE is perfused at the time of peak Li^+ effect (see Fig. 13), the further increase in mepp frequency is significantly greater than in the absence of Li^+ ($p < 0.05$, Student' t-test).

CHAPTER IV

DISCUSSION

1. METHODOLOGY

In the work described in this thesis, the objective was to study the nature and function of some of the heteroreceptors present on the motor nerve terminal and that modulate quantal transmitter release. To this end, a parameter for study was sought that had the virtue of simplicity of measurement, minimal ambiguity in interpretation and minimal experimental intervention and manipulation. Spontaneous release in the form of mepp frequency was chosen, and has been the consistent parameter in use throughout this study.

The criteria outlined above were largely met in using this parameter, as counting numbers of events takes no account of the magnitude of the event itself, thus obviating any postsynaptic effect of the drug under study. As long as the window settings of the equipment are appropriate, counting is simple and automatic. When the membrane voltage rises above the set threshold, an event is recorded, but a second event cannot be recorded until the membrane voltage falls below the threshold once more. As long as the signal to noise ratio is sufficiently large, and the resting membrane potential itself is stable, there is no ambiguity about the nature of the signal recorded, as is clear from Fig. 3. At low frequencies of discharge, the coincidental release of two quanta is rare

indeed, and contributes virtually no error to the observations. At the accelerated rates of release encountered in the presence of raised $[K^+]_o$ levels, however, a second mepp occurring in the time occupied in measuring the first was not uncommon, and the resulting measurements of numbers of mepp within a given time period were erroneously depressed. The equipment available did not have a second activating threshold to detect the additional amplitude that additive mepp's display and it was necessary to assess the extent of error introduced during experiments in 15 mM K^+. Careful examination of oscilloscope records at a fast sweep speed indicated that at the frequencies encountered in the experiments described in this thesis, the coincidence error was consistently less than 8%. As the results were normalized, with each fibre acting as its own control for the initial mepp frequency, it was decided not to make further correction to the values obtained to allow for putative coincidence errors.

Mepp frequency certainly involves little experimental intervention other than manipulation of extracellular potassium levels (see below). However, the question arises as to whether mepp frequency is in any way representative of the synchronous release of quanta evoked by a nerve impulse, the physiological parameter that largely determines the efficiency of synaptic transmission. While the two processes may

superficially appear to differ considerably, it may be that the fundamental difference lies only in time course. Both are quantal processes, with a dependence on Ca^{2+}. The dependence of synchronous release on an inward Ca^{2+} current has been well documented (see Introduction, Sec. 3.), and this current serves to change the local $[Ca^{2+}]$ in the vicinity of release sites as a necessary prelude to transmitter release. Spontaneous release too, has a dependence on $[Ca^{2+}]_i$ (Baker, 1972; Barton et al., 1983), and despite the apparent difference in the statistics of transmitter release manifested by the two processes, it has been argued that they in fact represent two points on a spectrum of release probabilities, determined by Ca^{2+} occupation of essential sites rather than inward flow of the free ion itself (Silinsky, 1985). Thus spontaneous release can be considered as analogous to evoked release, differing only in that the binding of Ca^{2+} to the essential sites proceeds at a slow pace due to the low $[Ca^{2+}]$ found in the cytosol of the resting cell.

In the course of the work described in this thesis, the arguments set out by Bretag (1969) were assessed and accepted. The gist of his thesis was that the composition of conventional physiological salt solutions was based on the ionic composition of serum. Such a calculation took no cognisance of the osmotic contribution of plasma proteins which must be in osmotic equilibrium with interstitial fluid.

Accordingly, Bretag devised a formulation that reportedly preserved the resting membrane potential of rat diaphragm fibers better than Krebs-Henseleit solution. When the same solution was used in the protocols followed for ACTH and adrenoceptor studies in the present work, the mean resting mepp frequency was noted to be significantly lower (see Results). As the osmolarity of Bretag's solution is about 7 mOsm less than that of Krebs-Henseleit solution, this fall in mepp frequency may be due to the known sensitivity of transmitter release to the osmotic pressure of the bathing solution. Both amphibian and mammalian motor nerves increase transmitter release as the tonicity of the surrounding fluid is increased (Hubbard et al., 1968b; Kita and Van der Kloot, 1977; Narita et al., 1983). The mechanism of this response is not known, but may be related to either changes in terminal volume, or changes in the $[Ca^{2+}]$ in the cytosol in response to changing osmotic gradient.

The $[K^{+}]$ of Bretag's solution is lower than that of Krebs-Henseleit solution (3.48 mM vs 5.9 mM). It is not possible to measure the membrane potential of the nerve terminal directly, and so a number of assumptions must be made. The potassium equilibrium potential, calculated from the Nernst equation, would therefore be higher (-93 mV vs -80 mV). Undoubtedly, the resting membrane potential of the nerve terminal is lower than these figures, but it is doubtful if it

is lower than -60 mV in Krebs-Henseleit solution. The threshold of activation of the T channel, the Ca^{2+}-channel with the lowest threshold found in neurons is -60 mV (Bean, 1989). Therefore, it is unlikely that there is significantly more inward Ca^{2+} current in nerve terminals bathed in Krebs-Henseleit solution than in Bretag's solution, and the difference in $[K^+]$ is not the cause of the difference in resting mepp frequencies.

An additional consideration is that the $[Ca^{2+}]$ of Bretag's solution is 59% of that in Krebs-Henseleit solution. Up to 4 mM $[Ca^{2+}]_o$ there is little change in mepp frequency at frog neuromuscular junctions (Shaver Madden and Van der Kloot, 1978). It is unlikely, therefore, that difference in $[Ca^{2+}]_o$ is the sole cause of the change in mepp frequency. However, it is of significance that no response to catecholamines was obtained in normal Bretag's solution. This conflicts with the observations of others who were able to detect a response at normal $[K^+]_o$ (Jenkinson et al., 1968; Krnjevic and Miledi, 1958; Kuba, 1970; Kuba and Tomita, 1971; Lim and Muir, 1983; Miyamoto and Breckenridge, 1974). Kuba and Tomita (1971) noted that the response to NE was markedly dependent on $[Ca^{2+}]_o$. Authors using relatively high $[Ca^{2+}]_o$ (2.5 - 2.7 mM) were able to obtain a statistically significant increase in mepp frequency (Kuba, 1970; Lim and Muir, 1983). Others, using up to 2 mM $[Ca^{2+}]_o$ either did not apply statistics or,

in the case of Miyamoto and Breckenridge (1974), found that the increase in mepp frequency was not statistically significant. As spontaneous release of quanta (mepp's) for the purposes of this thesis is regarded as equivalent to evoked release on an extended time scale, a means of improving the cytosolic contact with external [Ca^{2+}] was sought.

Mepp frequency can be increased by passing depolarizing current through the tissue and this effect can also be mimicked by raising the extracellular [K^+] (Del Castillo and Katz, 1954a; Liley, 1956a). Blaustein (1975) suggested that depolarized synaptosomes from rat whole brain have an increased permeability to calcium, thus causing intracellular calcium accumulation and enhanced transmitter release. Other work suggested that this interpretation was true at relatively modest levels of potassium (< 25 mM) (Shaver Madden and Van der Kloot, 1978).

In the rat phrenic nerve-diaphragm preparation, raised concentrations of K^+ evoked two distinct phases of accelerated transmitter release (Gage and Quastel, 1965). One component takes a few minutes to complete while the other component is slow in effect, and requires up to half an hour to reach an equilibrium. For this reason, tissues in the present work were bathed in 15 mM [K^+] for at least 30 minutes to equilibrate before experiments were commenced. In order to

determine which concentration of K^+ is suitable for the study of effect of agonists on transmitter release, various concentrations of K^+ (3.48, 5.9, 15 and 25 mM) were examined (data not shown). 15 mM K^+ caused a well sustained increase in mepp frequency and was chosen as the medium for experimentation. Miyamoto and Breckenridge (1974) successfully resorted to the same stratagem when dibutyryl cyclic AMP (4 mM) in normal [K^+] Kreb's Henseleit solution failed to affect transmitter release. Transmitter release in the presence of 3.48 mM $[K^+]_o$ and 15 mM $[K^+]_o$ are depicted in Fig. 8.

2. ADENOSINE RECEPTORS

The results indicate that adenosine receptor agonists exert an inhibitory effect on transmitter release that is inhibited by theophylline. From this it is reasonable to infer that an adenosine receptor exists on the motor neuron terminal. The precise identification of this receptor is not as simple as it would superficially appear, largely on account of the disparity of opinion concerning the classification of the adenosine receptors as a whole. This controversy has received extensive airing recently (Daly, 1985; Dunwiddie, 1985; Dunwiddie and Fredholm, 1985; Phillis and Barraco, 1985; Stiles, 1986; Ribeiro and Sebastiao, 1986, Fredholm and Dunwiddie, 1988a). Briefly, the effect on adenylate cyclase

was the original criterion for classification, with the A_1 receptor having an inhibitory effect on the enzyme, and the A_2 receptor exerting a stimulant action (Van Calker et al., 1978,1979; Schwabe, 1983). A pragmatic distinction between the receptor types could be made on the basis of the potencies of adenosine analogues, with a rank order at the A_1 receptor of L-PIA > NECA (See Introduction, page 36), and at the A_2 receptor NECA > L-PIA (Londos et al. 1980; Stone, 1984). An attempt by Londos et al. (ibid.) to substitute the terms R_i and R_a for A_1 and A_2 did not gain wide acceptance. Dissatisfaction with this scheme in its application to neuronal tissue, led to speculation that and alternative transduction mechanism to cyclic AMP ought to be considered, although the division of receptors into A_1 and A_2 was retained, at least until more definitive proof was available. Direct coupling of a G-protein to an effector such as an ion channel was considered a possibility, with three tentative models proposed. These models postulated either multiple subtypes of A_1 receptor, each with its unique G-protein intermediate, or one type of A_1 receptor with multiple types of G-protein intermediates, or thirdly, only one A_1 receptor type with one G-protein intermediate interacting with multiple effectors such as ion channels (Fredholm and Dunwiddie, 1988a). A proposal for the recognition of a third type of receptor, termed A_3, based on data from neuromuscular studies was made by other workers (Ribeiro and Sebastiao, 1985,1986,

1988; Sebastiao and Ribeiro, 1988a,1988b). NECA and L-PIA were supposedly equipotent at this site, and both more potent than 2-ClA. This proposal is highly speculative, however, relying as it does on results obtained from muscle contraction, and interpreting them in terms of transmitter release. As a consequence, the proposal is not widely accepted as yet (Receptor Nomenclature Supplement, TIPS, January, 1990).

Against this background the results presented in this thesis must be considered. The depressant action of adenosine analogues on spontaneous transmitter release at mammalian motor nerve terminals and the blocking action of theophylline agree well with previously published work carried out on evoked release at the frog neuromuscular junction (Ginsborg and Hirst, 1972; Silinsky, 1980,1984) and subsequently published work on the rat phrenic nerve-diaphragm preparation (Sebastiao and Ribeiro, 1988b), and K^+-accelerated release (Buckle and Spence 1982; Silinsky, 1988). All four adenosine analogues examined had a similar effect but the concentration effect relationships formed two parallel pairs (Fig. 6). Those for L-PIA and NECA were parallel and steeper than the parallel pair for 2-ClA and the lesser known analogue, CV-1674. For this reason 2-ClA ought not to be included with L-PIA and NECA when preparing a potency ratio. L-PIA was, however, only three times more potent than NECA, whereas in

the classical description of the two receptor types, L-PIA was two orders of magnitude more potent than NECA at the A_1 or inhibitory receptor (Londos, 1980). This presents a problem in identifying the receptor type involved as A_1, and some authors decline to accept such a categorization (Ribeiro and Sebastiao, 1985, 1986). Others have accepted this narrow a ratio of potencies with the proviso that some evidence exists that adenylate cyclase is indeed inhibited by the activated receptor (Baer and Vriend, 1985). This may not be easy to establish as more than one receptor type has been postulated to coexist on the same cell, with a range of conflicting transduction mechanisms (van Calker et al. 1979; Wojcik and Neff, 1983). Indeed, Silinsky et al., (1989) reported that after PTX mediated abolition of the inhibitory effect of adenosine on transmitter release, there was unmasked a late potentiating effect which was apparently mediated by a separate transduction mechanism. In addition, the requirement to demonstrate an effect on adenylate cyclase has been superceded by the suggestion that multiple transduction mechanisms may be associated with the presynaptic adenosine receptor (Fredholm and Dunwiddie, 1988a). In the last analysis, potency ratios remain the criterion for the identification of receptor types. The claim that there is an A_3 receptor based on potency ratios derived from muscle twitches (Ribeiro and Sebastiao, 1986; 1988) has been rejected by others on the basis of incomplete equilibration with the

biophase (Fredholm and Dunwiddie, 1988b), and seems difficult to defend, as the parameter of choice is so many steps removed from the primary site of the actions of the drugs used. Muscle twitch is an all or none phenomenon for each muscle fibre, and can only indicate whether a threshold epp was reached rather than provide a parameter for a graded response like transmitter release. For these reasons, the receptor present on the motor terminal can be confidently classed as an A_1, a conclusion that was recently supported by other work (Barry, 1990).

The lack of parallelism between the concentration-effect relationship for 2-ClA and those of L-PIA and NECA unfortunately precludes the use of 2-ClA in establishing a potency ratio comparable with those published for indirectly evoked skeletal muscle twitches (Ribeiro and Sebastiao, 1985; Sebastiao and Ribeiro, 1988b) and for a variety of smooth muscle preparations (Stone, 1983; Baer and Vriend, 1985; Christofi and Cook, 1985). The decreased slope of the lines for 2-ClA and CV-1674 may be indicative of a distinct receptor/transduction mechanism in operation with these two agonists. This seems intuitively unlikely, and the pharmacological precedent at cholinergic and noradrenergic synapses, where synthetic analogues resistant to inactivation have steeper concentration-effect curves than the natural transmitter, may be invoked. Jarvis et al. (1985)

demonstrated that 2-ClA was transported by the adenosine transporter molecule into human erythrocytes, and if such a phenomenon were occurring at the motor nerve terminal, it would explain the lack of parallelism between the concentration-effect relationships for 2-ClA and CV-1674 on the one hand, and L-PIA and NECA on the other.

The effectiveness of PTX in preventing a 2-ClA mediated response corresponds well to similar results obtained from isolated neurons, hippocampal slices and in situ brain (Dolphin and Prestwich, 1985; Fredholm and Lindgren, 1986; O'Regan and Phillis, 1987) and has been recently confirmed at the rat phrenic nerve synapse (Silinsky et al., 1989). From this it can be deduced that a PTX-sensitive G-protein is involved in the transduction sequence at this site. This does narrow the field somewhat, although knowledge of the range of G-proteins and their susceptibilities to PTX is presently incomplete. It is certain, however, that the adenylate cyclase stimulant G-protein, G_s, and one of the phospholipase C activating G-proteins, G_p (also called G_c or G_x), are unaffected by PTX (Ui,1986). Of the remaining known G-proteins, G_i - the adenylate cyclase inhibitory G-protein, and G_o seem the most likely candidates. G_i has also been implicated in the activation of phospholipase C at some locations, while G_o has been identified as acting directly on ion channels rather than activating an enzyme (Hescheler et

al., 1987).

The possible action of adenosine agonists on calcium movements in the nerve terminal has been the source of much speculation. The uptake of $^{45}Ca^{2+}$ into K^+-depolarized synaptosomes from rat cerebral cortex or Torpedo electroplax was reported to be reduced by adenosine and its derivatives (Ribeiro et al., 1979; Wu et al., 1982) but the first of these reports has been criticized on the grounds of lack of statistical significance (Fredholm and Hedqvist, 1980). Voltage dependent inward calcium currents in sympathetic ganglion neurons and dorsal root ganglion neurons were inhibited by adenosine analogues (Henon and McAfee, 1983; Dolphin et al., 1986; MacDonald et al., 1986), a response that was PTX sensitive and therefore required the mediation of a G-protein (Gross et al., 1989). While the suggestion that the action of adenosine at the motor neuron terminal may be due to a direct attenuation of an inward calcium current has some support (Ribeiro and Sebastiao, 1986), this cannot be so in the present work. In the resting nerve terminal membrane, there is no identified calcium conductance. The dependence of mepp frequency on $[Ca^{2+}]_o$ is probably mediated through changes in the rate of Ca^{2+} extrusion from the cytosol, or even changes in the rate of non-specific diffusion into the cytosol. Lack of certainty as to how this latter is effected makes it impossible to comment further on potential drug

effects. Silinsky (1986) examined the case for a direct adenosine mediated effect on inward Ca^{2+} current at the nerve terminal and found it wanting. As the action of adenosine was unaffected by the presence of TMB-8, a drug that prevented Ca^{2+} efflux or uptake at intracellular storage sites (Hunt et al., 1990), his preferred alternative mechanism of action was a change in the Ca^{2+} affinity of a cytosolic binding site that is involved it the quantal release process (*ibid.*; Silinsky et al., 1987b). Because of the sensitivity of the action of adenosine analogues to temperature he concluded that the process required the participation of a second messenger system (Silinsky and Hirsch, 1988). For several years Silinsky had proposed that raised cytosolic cyclic AMP was the cause of depressed transmitter release. This contention arose from the observation that a drug, MDL 12,330A (initially RMI 12,330A), reputedly an adenylate cyclase inhibitor, increased the output of transmitter from stimulated motor nerves (Silinsky, 1984; Silinsky and Vogel, 1986; Silinsky et al., 1987a & b) and was supported by reports of experiments using cyclic AMP loaded liposomes, where a similar response was noted. Such an opinion ran counter to the conclusions of others concerning the relationship between cyclic AMP and transmitter release (Hattori and Maehashi, 1987; Dryden et al., 1988), and seemed untenable when it was considered that MDL 12,330A was equipotent as a cyclic nucleotide phosphodiesterase inhibitor and an inhibitor of adenylate

cyclase (Hunt and Evans, 1980), and in any case Silinsky had used subthreshold concentrations of the drug (Silinsky and Vogel, 1986). In his report on the PTX sensitivity of the adenosine receptor system at motor nerve terminals, Silinsky conceded that depression rather than elevation of cytosolic cyclic AMP levels was the most likely consequence of adenosine analogue action, at least in the rat. He suggested, however, that the reduced Ca^{2+} affinity theory (*vide supra*) could be explained by combination of Ca^{2+}-binding proteins such as calmodulin with the beta-gamma units of the G-proteins liberated following receptor activation.

The loss of response to adenosine receptor activation following incubation with H-7 argues in favour of the involvement of a protein kinase in maintaining normal levels of transmitter release. It is true that at the concentration used, H-7 is equiactive against PKC and the cyclic nucleotide dependent kinases (Garland et al., 1987; Hidaka and Hagiwara, 1987) and adenosine has been shown to inhibit phosphatidylinositol turnover in rat striatal slices (Petcoff and Cooper, 1987), this latter effect seemed to be mediated by an A_2 receptor. Instead the results presented in this thesis are compatible with the conventional view of A_1 receptor activation coupled with the suggestions of Greengard (Greengard and Browning, 1988) that synaptic vesicles can be liberated from entrapment in the cytoskeleton by the action of

PKA. This is not to suggest, however, that synapsin is the only substrate of PKA. On the contrary there are likely to be many more, including the possible sustrates favoured by Silinsky (*vide supra*). More evidence on this point is required before definitive conclusions can be drawn.

A corollary stemming from the observation that the resting mepp frequency declined after H-7 treatment is that there is a basal level of cyclic AMP production and PKA activation in the absence of extraneous stimulation. This conclusion is supported by the fact that phosphodiesterase inhibitors can affect the resting mepp frequency at the neuromuscular junction (Dryden et al., 1988).

3. ACTH RECEPTORS

The stimulant effect of ACTH on transmitter release at the motor nerve terminal agrees well with earlier reports that investigated the response in frog tissues (Birnberger et al., 1977, Johnston et al., 1983). It appears to be a specific effect although it cannot be rigorously tested pharmacologically in the absence of proven specific antagonists of ACTH. The thrust of the investigation was directed towards finding a stimulatory complement to the inhibitory A_1 adenosine receptor investigated in the previous section and so no concentration-effect relationship was

investigated owing to the expense of the agonist material. Instead, the investigation concentrated on the transduction mechanism of the response.

No response was observed in normal Bretag's solution but when transmitter release was accelerated by the use of solution containing 15 mM K^+, a significant response was observed. The implications of this observation have already been considered (Discussion, Section 1). The time course of the onset of the response (Fig. 9) resembles that seen for adenosine analogues (Fig. 4) and for catecholamines (Fig. 16) and would be consistent with a second messenger mediated event. The established positive relationship between ACTH and the cyclic AMP content of adrenal cortical cells (Haynes, 1958, Haynes et al., 1959) was the stimulus for the search for evidence of a similar relationship in the motor nerve terminal, although the possibility of a dual effect involving phosphatidylinositol turnover could not be ignored (Farese et al., 1986). The strategy was to employ inhibitors of the proteins believed to be involved, and to compensate for lack of specificity by judicious choice of concentrations and agents with differing spectra of effect.

The failure of PTX to inhibit the response to ACTH merely serves to eliminate a group of G-proteins from further consideration, but does not confirm the involvement of any of

the PTX resistant G-proteins such as G_s or G_p. On the other hand, the increase in the response seen after PTX incubation is itself of interest. Such a phenomenon has been previously documented in both cell free systems and in intact cells (Jakobs et al., 1985). It was suggested that the activated G_s, resulting from the activation of the stimulant receptor, not only in turn activates the catalytic subunit of adenylate cyclase, but also causes simultaneous activation of G_i and the resultant inhibition of the enzyme. In a system where the G_i is inactivated as by PTX, this concomitant inhibition is removed, with an apparent increase in the stimulant response. The precise molecular mechanism of this mutual antagonism of the G-proteins is not understood, but may involve the beta-gamma subunits which are known to have an inhibitory effect on cyclic AMP production (Birnbaumer, 1990). However, this response is apparently unique to the adenylate cyclase regulatory system, and its appearance in the course of ACTH receptor activation at the motor nerve terminal is a small contribution to the circumstantial evidence that transduction of the signal from this receptor involves cyclic AMP.

The effects of the naphthalenesulfonamides (See Introduction 5.3) provide a more positive indication of the likely transduction steps. Of the three that were investigated, H-7 and A-3 were effective in significantly reducing the effect of ACTH to the point of abolition. H-7

has a K_i of 6 μM against PKC and the cyclic nucleotide dependent kinases, and 97 μM against a calmodulin dependent kinase. A-3 has a K_i of about 4 μM against the cyclic nucleotide dependent kinases, 7.4 μM against a calmodulin dependent kinase, but 47 μM against PKC (Garland et al., 1987; Hidaka and Hagiwara, 1987). Thus at a concentration of 50 μM H-7 or 10 μM A-3, only the cyclic nucleotide dependent kinases will be significantly susceptible to each compound. Tissues exposed to W-7 responded normally to ACTH but, as W-7 has a K_i of between 130 and 170 μM against the cyclic nucleotide dependent kinases and PKC, at a concentration of 10 μM no effect would be expected on a transduction system that relied on any of these enzymes. By the same argument, as W-7 at this concentration might be expected to affect calmodulin dependent kinases to a noticeable extent (K_i = 18 μM) the participation of a calmodulin dependent kinase in the transduction sequence can be discounted.

The transitory stimulant effect of Li^+ on transmitter release has been reported before (Crawford, 1975), but no satisfactory explanation of this phenomenon has been forthcoming. As noted before (Introduction 5.2.1.), in the presence of Li^+, IP_3 accumulates temporarily in the cytosol. Responses that are dependent on IP_3 are expected to be augmented. This seems to be the case for adrenoceptor mediated responses at the motor nerve terminal (Results 3.4).

However, as the response to ACTH at the peak of the Li^+ effect was no different from normal it would appear that IP_3 does not play a role in its transduction.

The evidence reviewed above implicates a system regulated by G_s and G_i proteins and involving a cyclic nucleotide dependent kinase. While cyclic GMP might be involved, the steps in the regulation of its synthesis in neurons are virtually unknown. On the other hand, a cyclic AMP dependent system would be consistent with the results as analysed above, and has already been shown to be associated with the ACTH receptor in adrenal cortical cells (Haynes, 1958; Haynes et al., 1959), and in noradrenergic nerve terminals (Gothert and Hentrich, 1984).

Two aspects of the response remain to be considered. Firstly, there is the failure of tissues from approximately 40% of animals to respond. A similar level of failures was noted in frog sartorius fibers (Johnston et al., 1983) and is more likely to be a true physiological variation rather than a problem arising from limitations to the diffusion of the drug into the synaptic region. Other agonists tested in the same system (adenosine analogues and adrenoceptor agonists) did not reveal a comparable dichotomy in their responses. Also, the failure to respond was bilateral. If the failure were attributable to a mechanical inadequacy, then it is

unlikely that it would only affect pairs of hemidiaphragms, and not be randomly distributed among the tissues examined. The phenomenon seems to be a true state of affairs. Some animals do not possess the ACTH receptor on their motor neurons. The situation was not related to season, sex or strain of animal and so its nature remains unexplained.

The final question to raise is whether this receptor and its response is of physiological significance. Certainly, ACTH is released in times of stress, and at such times, means of increasing transmitter release are advantageous to an organism. The concentration of ACTH used was over 3 orders of magnitude greater than its concentration in plasma, and it is uncertain how much of a 39 amino acid polypeptide can cross the capillary membrane to come into contact with the motor nerve terminal. Certainly it can cross the glomerular membrane and appear in the urine, but the concentration in the vicinity of the neuron terminal cannot be as great as in the plasma. A number of possibilities have been suggested (Johnston et al., 1983). Either ACTH is not the natural ligand, but rather a similar but unidentified peptide with much higher affinity for the receptor may be responsible for a physiological effect similar to that under present study. Alternatively, ACTH or a related compound may be released from adjacent sympathetic nerve endings, and be present at the motor neuron terminal at much higher concentration than in the

plasma. Finally, the stress of handling the animal prior to sacrifice might have caused a release of ACTH which left the receptors desensitized. Johnston et al. (1983) noted that frog motor neuron terminals neither recover from the effect of ACTH nor responded to a second exposure to ACTH for as long as 4 hours after initial exposure. Clearly, much remains to be investigated in the relationship between ACTH and the motor neuron.

4. ADRENOCEPTORS

The data presented in this thesis support the hypothesis that the adrenoceptor involved in facilitating quantal release of neurotransmitter from the motor nerve terminal belongs to the α_1 category. While this is in agreement with previously adduced conclusions (see Introduction 4.2.2), it clearly is in contradiction to the suggestions made by Miyamoto and Breckenridge (1974) and by Wessler and Anschutz (1988), that a link exists between the NE-mediated and cyclic AMP-mediated responses. While there can now be little doubt that a system involving cyclic AMP and protein kinase A does exist in the motor nerve terminal and can influence quantal release of Ach (Dryden et al., 1988), the present results indicate that the NE effect on quantal release is mediated not by protein kinase A but by a CaM dependent process. The failure of isoproterenol to evoke a response (Fig. 17), and of nadolol to

antagonise the responses to Epi (Fig. 18) strengthen the opinion that no ß-adrenoceptor modulation of the quantal release mechanism exists at this site. Nadolol has the virtue of being a pure, if non-selective, β-adrenoceptor antagonist, devoid of intrinsic activity and local anaesthetic properties.

Techniques such as radiolabelled choline washout as used by Wessler and Anschutz (1988) do not distinguish between quantal and non-quantal release, and it is possible that the popranolol sensitive facilitatory responses to NE and isoproterenol noted by these authors were evoked by a non-quantal mechanism. If such is the case, then the ß-adrenoceptor involved must have a compartmentalized locus of action and not contribute to a general increase in axoplasmic cAMP levels as proposed by Bowman (1990). General increases in cAMP levels in motor neuron terminals are known to increase quantal release (Dryden, et al., 1988).

The failure of yohimbine to modify the response to Epi suggests that α_2 adrenoceptors also are not involved in the presynaptic response to catecholamines at the neuromuscular synapse, as also concluded by others (Malta et al., 1979; Somogyi et al., 1987).

In the motor nerve terminal, the α_1-adrenoceptor does not appear to require a PTX sensitive link for the transduction of

its signal (Fig. 19). Of the known PTX insensitive G-proteins, G_s acts as a stimulant to adenylate cyclase, and is normally activated by ß-adrenoceptors among others, while G_p is one of the two proteins known to activate phospholipase C (the other, G_i, is PTX sensitive). As the α_1-adrenoceptor is widely recognised to be linked to phosphatidylinositol turnover (Hirasawa and Nishizuka, 1985) and as in the present experiments the exaggerated response to NE in the presence of Li^+ suggests that IP_3 was produced (Fig. 21), G_p would seem to be the intermediate involved in the transduction sequence.

Identification of the next stage in the transduction sequence is more tentative, depending as it does on the imperfect specificities of protein kinase antagonists and the necessity to rely on the different effective concentrations against the various protein kinases (Tanaka et al., 1982; Garland et al., 1987; O'Brian and Ward, 1989). Several authors have established the existence of protein kinase C, and its association with increased neurotransmitter release, at the myoneural synapse by applying either phorbol esters or diacylglycerol (DAG) (Eusebi et al., 1986; Murphy and Smith, 1987; Publicover, 1985a). In addition, CaM is also believed to affect neurotransmitter release in conditions where Ca^{2+} concentrations in the cytoplasm of the nerve terminal are raised (Roufogalis, 1980; Publicover, 1983, 1985b). CaM could thus combine with Ca^{2+} released from the endoplasmic reticulum

by IP_3 (Majerus et al., 1986). In this way, both DAG and IP_3, compounds released by hydrolysis of PIP_2 following activation of α_1-adrenoceptors (Hirasawa and Nishizuka, 1985), could theoretically induce an ultimate increase in transmitter release. Although polymyxin B, clomiphene and auranofin have all been regarded as inhibitors of protein kinase C on the basis of their ability to inhibit phorbol ester induced responses, such activity is far from specific and may not even be directed at the enzyme itself (Chaffman et al., 1984; Hidaka and Hagiwara, 1987). Polymyxin B and triphenylethylenes such as clomiphene are also CaM antagonists (Mazzei et al., 1982; Lam, 1984) and it is impossible to discount this possible mechanism of action in the present experiments.

Failure of H-7 at a concentration of 50 μM to affect the NE-induced response argues strongly for the absence of PKA and PKC enzymes in the transduction sequence when the K_i is 6 μM (Garland et al., 1987; Hidaka and Hagiwara, 1987). On the other hand, the K_i of W-7 against a CaM activated system (myosin light chain kinase) is 18 μM, and yet at a concentration of 10 μM, the response to NE was reduced by 72%. K_i values for other known actions of W-7 are greater by at least one order of magnitude (Tanaka et al., 1982; Hidaka and Tanaka, 1983; O'Brian and Ward, 1989). This provides support for the concept that a CaM dependent process is involved.

α_1-Adrenoceptors have been reported to elevate cytosolic Ca^{2+} from either internal or external sources (Han et al., 1987), but the potentiation of the response in the presence of 10 mM Li^+ (Fig. 21) suggests that intracellular release in response to (1,4,5)IP_3 metabolism may be the link in the present case. The enhanced response to NE in the presence of Li^+ is therefore consistent with the supposition that IP_3 is the active intermediate in the transduction sequence.

The target substrate of the Ca^{2+}/CaM complex formed as a result of presynaptic α_1-adrenoceptor stimulation is uncertain. Activated CaM is important for regulating a wide range of known cellular reactions with the possibility that others remain to be discovered (Cheung, 1980; Veigl et al., 1989). A number of potential substrates such as phosphodiesterase, phosphatase and adenylate cyclase can be eliminated as their activation is known either not to increase transmitter release (Dryden et al., 1988; Nahorski, 1988) or to be susceptible to H-7 (Chen et al., 1989). However many protein kinases are CaM dependent, and they are plentiful in nerve terminals (Nairn et al., 1985; Schulman, 1988).

5. CONCLUSION

The work described in this thesis provides evidence for the existence of three classes of heteroreceptor at the

mammalian motor neuron terminal, together with incomplete evidence for the transduction mechanisms involved. This is not to suggest that these are the only receptors present, or that their existence is of importance in the overall description of synaptic transmission at this site. If the teleological argument is accepted that the receptors are there for a purpose, it becomes difficult with present knowledge to identify the purpose of the ACTH receptor as indicated above. It is possible to envisage the rise of adrenaline levels in the interstitial fluid around the motor nerve terminal in times of stress, with a corresponding increase in the efficiency of synaptic transmission. Such a circumstance would confer Darwinian advantages to organisms in the course of evolution. On the other hand, exercising muscle generates large amounts of adenosine from ATP metabolism, and the adenosine is readily transported out of the fibers into the interstitial space. Thus during extremes of muscle activity, a self limiting system is in place to reduce the overall excitatory stimulus to the muscle, and so confer a degree of protection. Other receptors for transmitters associated with neurons with no recognised terminals in the vicinity of the motor neuron terminal presents more serious problems for the teleologist. Of course, functional receptors may exist with no known physiological role. An example is the muscarinic receptor on endothelial cells. Alternatively, the presence of the receptors on the nerve terminal may be a reflection of the

complement of receptors present on the soma and dendrites of the motor neuron. While directed insertion of protein structures into the membrane likely does exist, at least for potassium ion channels on myelinated axons (Chiu and Ritchie, 1980, 1981), the possibility of multiple systems designed to localize insertion of each receptor type demands a level of intracellular organization that, though possible, has hitherto been unrecognised. More information on the receptor complement of the motor neuron soma is required before a definitive opinion can be formed.

A feature of the receptor systems examined here is their demonstrated or postulated dependence on kinase systems. While direct G-protein activation of ion channels undoubtedly occurs in some cell types, phosphorylation of one or more target substrate seems to be a more universal phenomenon. The targets in the motor nerve terminal remain the object of much speculation and outright controversy. Ca^{2+} channels are a favourite, but as indicated, cannot be the only target if at all. The intracellular Ca^{2+} binding site is highly speculative, but feasible. Lastly there is the possibility that the size of the pool of available quanta may be varied by phosphorylation of synapsin (Greengard and Browning, 1988), some evidence for which has already been presented by this laboratory (Dryden and Marshall, 1989). Our knowledge of modulation of transmitter release remains limited, and is

likely to remain so until the real sequence of events that underlie the release of quanta is defined.

CHAPTER IV

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