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CALCITONIN GENE-RELATED PEPTIDE AND THE GUINEA PIG LONGITUDINAL MUSCLE-MYENTERIC PLEXUS: STUDY OF THE STIES AND MECHANISMS OF ACTION

BY

YA-DING SUN 

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

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DATED 3 December 1992

DEDICATION

To my wife, Xu Zhang

To my son, Bo-Yang (Sun)

To my mother and father

for their love, encouragement and understanding.

ABSTRACT

Actions and sites of actions of rat calcitonin gene-related peptide (rCGRP) on longitudinal muscle-myenteric plexus (lm-mp) of the guinea pig ileum (GPI) were investigated. rCGRP has biphasic effects on the tension of non-stimulated strips, i.e., an initial contraction followed by relaxation. rCGRP produces similar effects on the contractions induced by electrical field stimulation (EFS). rCGRP acts on both the myenteric neurons and the longitudinal muscle. It excites the enteric neurons to release both acetylcholine and another, as yet undefined, stimulatory substance, resulting in contraction of the smooth muscle. This peptide may also release an inhibitory neurotransmitter(s) which is responsible for part of its inhibitory/relaxation effects on smooth muscle.

Direct inhibitory effects on the smooth muscle constitute part of the inhibitory actions of the peptide on the lm-mp preparation. This is the first investigation of the direct actions of rCGRP in guinea pig plexus-free longitudinal muscle (lm). This peptide shows only inhibitory/relaxant effects on the muscle. It inhibits the contraction induced by depolarization, activation of histaminergic receptors or G-proteins. The contraction induced by activation of muscarinic receptors is unaffected. The rCGRP-induced muscle relaxation effect is competitively antagonized by $hCGRP_{8-37}$, a CGRP receptor blocker.

The mechanisms of the actions of rCGRP on smooth muscle have been studied. Effects of rCGRP on the intracellular free calcium concentration ($[Ca^{2+}]_i$,

as well as the tension have been examined in muscle strips by using techniques which record $[Ca^{2+}]_i$ and tension simultaneously. Histamine increases both tension and $[Ca^{2+}]_i$. Nifedipine, a calcium channel blocker, inhibits the histamine-induced tension and $[Ca^{2+}]_i$ in a nearly parallel manner, while forskolin, a direct activator of adenylate cyclase, shows a stronger inhibition of $[Ca^{2+}]_i$ than of tension. rCGRP, like forskolin, greatly inhibits the histamine-induced contraction but decreases $[Ca^{2+}]_i$ to a much lesser extent, showing a dissociation between tension and $[Ca^{2+}]_i$. rCGRP increases intracellular cAMP levels ($[cAMP]_i$) of the lm. The intracellular cGMP level of the muscle is not affected by the peptide. These results indicate that the direct inhibitory effects of this peptide on smooth muscle may be attributed to its ability to stimulate production of cAMP. Desensitization of the contractile elements to calcium and the $[Ca^{2+}]_i$-lowering effect may be involved in the mechanisms of the actions of CGRP on the smooth muscle.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary dioxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CGRP-IR	CGRP-immunoreactive/CGRP-immunoreactivity
C-terminus	Carboxyl terminus
CNS	Central nervous system
db cAMP	Dibutyryl cyclic adenosine monophosphate
DMPP	1,1-Dimethyl-4-phenyl-piperazinium
DNA	Deoxyribonucleic acid
ED_{50}/EC_{50}	Median effective dose/median effective concentration
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EFS	Electrical field stimulation
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N,N-tetraacetic acid
ENS	Enteric nervous system

Epi	Epinephrine
F^-	Fluoride ion
F_{340}	Fluorescence excited at wavelength of 340 nm
F_{380}	Fluorescence excited at wavelength of 380 nm
Fura-2/AM	Fura-2/acetoxymethyl ester
G-protein	Guanosine triphosphate binding protein
GDP	Guanosine diphosphate
G_i	Inhibitory guanosine triphosphate binding protein
GI tract	Gastrointestinal tract
GPI	Guinea pig ileum
G_s	Stimulatory guanosine triphosphate binding protein
GTP	Guanosine triphosphate
hCGRP	Human calcitonin gene-related peptide
5-HT	Serotonin
i.p.	Intraperitoneally
i.v.	Intravenously
IC_{50}	Median inhibitory dose
IP_3	Inositol triphosphate
K_{ATP}	Adenosine triphosphate-sensitive potassium channel
Kd	Equilibrium dissociation constant
Ki	Inhibitory constant

lm	Longitudinal muscle
lm-mp	Longitudinal muscle-myenteric plexus
MLCK	Myosin light chain kinase
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
N-terminus	Amino terminus
pA_2	A concentration of antagonist that produces a two-fold shift to the right in the dose-response curve of agonist
Q effect	Quiescence effect
R effect	Retraction effect
rCGRP	Rat calcitonin gene-related peptide
R_{max}	Maximal ratio of fluorescence
R_{min}	Mimimal ratio of fluorescence
snRNPs	Small nuclear ribonucleoprotein particles
SR	Sarcoplasmic reticulum
TTX	Tetrodotoxin
$[Ca^{2+}]_i$	Intracellular free calcium concentration
$[cAMP]_i$	Intracellular cyclic adenosine monophosphate level

CHAPTER 1. GENERAL INTRODUCTION

I. CALCITONIN GENE-RELATED PEPTIDE (CGRP)

A. Introduction

Calcitonin gene-related peptide (CGRP) is a peptide with 37 amino acid residues (Rosenfeld *et al.*, 1983). This peptide has two forms in both human and rat, i.e. α- and β-forms (Amara *et al.*, 1985; Steenbergh *et al.*, 1985, 1986; Alevizaki *et al.*, 1986). αCGRP, calcitonin, and katacalcin are all encoded by the calcitonin gene, or the calcitonin/αCGRP gene, in a tissue-dependent manner (Amara *et al.*, 1982; Crenshaw *et al.*, 1987; Leff *et al.*, 1987). CGRP is mainly produced in the nervous system (Rosenfeld *et al.*, 1983, 1984; Kawai *et al.*, 1985) while calcitonin and katacalcin are produced mostly in the thyroid C-cells (Jacobs *et al.*, 1981; Rosenfeld *et al.*, 1983; Sabate *et al.*, 1985). β-CGRP is encoded in the nervous system by a second gene which is sometimes referred to as the calcitonin/βCGRP gene, although it does not actually produce calcitonin (Amara *et al.*, 1985; Steenbergh *et al.*, 1985, 1986; Alevizaki *et al.*, 1986). Another peptide, called islet amyloid polypeptide (IAPP) or amylin, was found to share 46% homology in structure with CGRP and is therefore included in the calcitonin-CGRP gene family (Cooper *et al.*, 1987; Westermark *et al.*, 1987). Amylin mRNA is abundant in the islets of Langerhans but not present in brain, lung, heart, intestine, kidney, liver, adrenal or testes (Leffert *et al.*, 1989).

B. Discovery of CGRP

Calcitonin was discovered in 1962 (Copp and Cheney, 1962; Copp *et al.*, 1962). The sequence of rat CGRP was predicted by the application of recombinant DNA technology in 1983 (Evans *et al.*, 1983; Rosenfeld *et al.*, 1983). Rosenfeld and coworkers (1981), using serially transplanted rat medullary thyroid carcinoma cell culture, observed a spontaneous and permanent switching from high to low calcitonin production. The calcitonin mRNA was found to be replaced by an mRNA with 200 more nucleotides. Analysis of genomic DNA and RNA showed that it has a 128 amino acid precursor polypeptide (Rosenfeld *et al.*, 1983). The latter molecule contained a 37 amino acid cleavage peptide, CGRP.

C. Calcitonin/CGRP gene

1. Calcitonin/α-CGRP gene

a. Splicing

The primary transcription product of a gene is termed a *pre-mRNA* or *primary transcript*. The pre-mRNA undergoes a variety of post-transcriptional modifications to produce the *functional* or *mature messenger RNA*. One type of modification is the precise removal of internal sequences by the cleavage-ligation reaction called *splicing*.

A gene contains nucleotide sequences including those encoding proteins (*exon*) and other sequences important for regulation (e.g. *intron*). Intron refers to the sequences which are located between exons and have some special short sequences.

These special sequences in the introns play important roles in splicing and they are splice sites at both 5'- (*donor*) and 3'-(*acceptor*) ends and *branch points*. The 3'-splice site consensus sequence is an important sequence element for splicing. Deletion or mutation of this sequence can greatly affect splicing. Eukaryotic cells can diversify their gene products by using a process termed *alternative splicing* which involves the joining of different combinations of exons from a primary transcript to give mature mRNAs that encode different proteins. This process is known to be used by more than 50 genes (Breitbart *et al.*, 1987). Certain sequences in pre-mRNA (*cis-acting factors*) and external factors (*trans-acting factors*, e.g. splicing factors) both appear to be involved in the alternative splicing process. A class of particles located in the nucleus has been reported to participate the splicing (Green, 1986). This type of particle, termed *small nuclear ribonucleoprotein particles (snRNPs)*, is one type of trans-acting factor. One of the snRNPs, *U_1snRNP*, has been reported to be a splicing factor involved in the splicing of some pre-mRNAs. This is based on the fact that there is a base-pairing potential between the 5' end of U_1snRNP and the pre-mRNA splice junction. Addition of antibody against the particle inhibits the splicing, and RNAase H-directed cleavage of the 5'-end of U_1snRNA abolishes the splicing *in vitro* (Kramer *et al.*, 1984). Calcitonin and CGRP are the proteins produced from the calcitonin/αCGRP gene which uses the alternative splicing process (Amara *et al.*, 1982; Crenshaw *et al.*, 1987; Leff *et al.*, 1987).

b. Calcitonin/αCGRP gene and splicing model

The present understanding of the organization of the gene is illustrated in Fig. 1-1 from Zaidi *et al.* (1987b). The human calcitonin/αCGRP gene has six exons. Between those six exons are five introns. Among the six exons, the first three exons are common to both calcitonin and CGRP: exon 1 is non-coding and exon 2 encodes the signal peptide, exon 3 encodes the N-terminal flanking peptide. Exon 4 contains sequences for the last seven amino acids of the N-terminal flanking peptide of CGRP, as well as calcitonin and katacalcin, the C-terminal flanking peptide, and ends with an unusual polyadenylation signal, AATAAA. The remaining two exons are a CGRP-coding exon (5), and a non-coding exon (6) which is also for CGRP and also has a polyadenylation signal, ATTAAA, at its end. Splicing of the first three exons to the fourth exon results in the production of mature calcitonin mRNA which encodes the calcitonin precursor. Alternative splicing of the first three exons together with the fifth and sixth exons by excluding the fourth exon creates the mature CGRP mRNA from which the αCGRP precursor is produced. In addition to calcitonin or CGRP, both precursors contain a signal peptide and an amino (N)-terminal flanking peptide. αCGRP is released by cleavage of the paired dibasic amino acids at both the N-terminus and the carboxyl (C)-terminus. Tissue-specific alternative mRNA splicing of the gene results in production of calcitonin mainly by thyroid C-cells (Jacobs *et al.*, 1981; Rosenfeld *et al.*, 1983; Sabate *et al.*, 1985), and also by scattered neuroendocrine type cells (Nylon *et al.*, 1987). αCGRP is primary produced in both

the central and peripheral nervous system (Rosenfeld *et al.*, 1983, 1984; Kawai *et al.*, 1985).

c. Mechanisms for tissue-specific splicing

As mentioned in the preceding section, calcitonin is mainly produced in thyroid C cells while αCGRP is encoded mostly in the nervous system. When the wild type calcitonin/αCGRP gene is introduced into cells of neuronal or non-neuronal origins which normally do not express calcitonin or CGRP, or only produce a low level of the peptide, differential expression is also seen. By using transgenic mice, Crenshaw *et al.* (1987) found that in those tissues which do not express the endogenous gene normally, most of the transgenic mRNA encodes for calcitonin in non-neuronal tissues, while CGRP mRNA is detected in the neurons of brain and heart. More than 98% of calcitonin mRNA is expressed in Hela cells (human epithelial cells) and lymphocytes (Leff *et al.*, 1987; Emeson *et al.*, 1989, 1991) and more than 90% of αCGRP mRNA is detected in adrenal medullary carcinoma cells (PC_{12}), mouse tetracarcinoma cells (F_9) and Ewing 1B cells (Hoppener *et al.*, 1987; Leff *et al.*, 1987; Emeson, 1989, 1991). For convenience, Hela cells and lymphocytes are called calcitonin-producing cells and PC_{12}, F_9 and Ewing 1B cells are called CGRP-producing cells in this thesis. There must be specific mechanisms for regulating the levels of different mRNAs. Based on the reports mentioned above the control could be at the level of transcription.

The exact mechanism of tissue-specific mRNA splicing is still unclear. Both the sequence of pre-mRNA (*cis-acting factors*) and external factors (*trans-acting factors*, e.g. splicing factors) appear to be involved in the alternative splicing process (Green, 1986). Working on the possible *cis*-acting factors, Leff *et al.* (1987) have proposed a model for neuron-specific CGRP production. In their model, both *cis*-acting factors and *trans*-acting factors, referred to as a "splice commitment regulatory factor" or "machinery", were introduced. There has been speculation that a) the *cis*-acting factors are the sequences of exon 4 and neighbouring regions at both ends of this exon; b) the *trans*-acting factor is a factor specific to CGRP-producing neurons; c) the *trans*-acting factor could associate with heterogeneous nuclear RNA; d) the *trans*-acting factor could promote the formation of a pre-mRNA secondary structure that is required for efficient splicing of exon 3 to exon 5, and at the same time, this machinery could inhibit the utilization of the exon 4 (calcitonin) poly (A) site. A gene mutated properly at the exon 4 poly (A) site expressed neither calcitonin nor CGRP in calcitonin-producing cells (Leff *et al.*, 1987). Therefore this failure to express CGRP, according to this model, was explained by the absence of the *trans*-acting factor in the cell line used. This would promote the necessary secondary structure for the alternative mRNA processing.

Considerable progress has been made in identifying the *cis*-acting factor, i.e. key sequences for regulating the alternative processing in the gene. Either deletion or mutation of certain sequences in the gene, or both, are the usual methods used

to identify the key sequences regulating the splicing. These modified genes are then introduced into either calcitonin- or CGRP-producing cells to determine whether there is any change in expression pattern compared to the wild type gene.

In CGRP-producing cells, according to the model by Leff *et al.* (1987) there is a *trans*-acting factor that would promote the necessary secondary structure for CGRP-pattern splicing, and also inhibits the utilization of the poly (A) site at the end of exon 4 (calcitonin exon), resulting in production of CGRP and inhibition of the expression of calcitonin. Deletion of the 3'-splice acceptor of exon 5 stops the production of CGRP mRNA in F9 cells but does not lead to the production of calcitonin in this cell line, while the formation of calcitonin mRNA in Hela cells remains unaffected (Emeson *et al.*, 1989). Emeson *et al.* (1991) found that mutations in nucleotides between -52 and -28 but not between -127 and -52 or between +1 and +13, relative to the calcitonin-specific exon (exon 4) result in an increase in calcitonin mRNA. A combination of deletion and mutation in the sequence consisting of part of the 3' end of intron 3 and part of the 5' end of exon 4 could reverse the ratio of calcitonin mRNA and CGRP mRNA in CGRP-producing cells (in F_9 cells, calcitonin mRNA production was as high as 82% by the modified gene and only 6% by the wild type gene) (Emeson *et al.*, 1989). These results can be explained by the model of Leff. The 3'-splice acceptor of exon 5 is essential for the CGRP-pattern splicing but may not be important for the interaction with the *trans*-acting factor. Deletion of this sequence stops the production of CGRP but does not affect the

inhibitory interaction of *trans*-acting factor with the gene, and, therefore, does not lead to the production of calcitonin. Deletion of nucleotides between -52 to -28, or a combination of deletion and mutation in the sequence including part of intron 3 and part of exon 4 (as in the case reported by Emeson *et al.*, 1989) might change the structure of the cis-acting factor, and could then weaken the interaction between *cis*- and *trans*-acting factors to different extents. This weakening then probably relieves some inhibition of the production of calcitonin, and inhibits the production of CGRP. Several groups have been trying to isolate the possible *trans*-acting factor or splicing factor for calcitonin/αCGRP gene in CGRP-producing cells (Li *et al.*, 1989; Cote *et al.*, 1992).

In calcitonin-producing cells, on the other hand, there is no such *trans*-acting factor to promote a secondary structure of the pre-mRNA in favor of the production of CGRP. Therefore, these cells display a calcitonin-pattern of expression. Large deletions in intron 3 and 4 do not affect the pattern of expression of the calcitonin gene in Hela and F_9 cell lines (Emeson *et al.*, 1989). Mutation of the poly (A) site at exon 4 prevents calcitonin production but does not stimulate CGRP formation (Leff *et al.*, 1987). In another study, Cote *et al.* (1990) observed that a large deletion of the sequence including part of intron 4 and exon 4 at the 3′ end does not have any apparent influence on the splicing of calcitonin-pattern in nuclear extracts of Hela cell (calcitonin-producing cell); however, deletion of four more nucleotides (-5 to -2) in the 3′ -splice acceptor of exon 4 brings about the CGRP-pattern of splicing. The

Leff and his co-workers (1987) reported that deletion of a sequence in intron 3 including part of the 3' -splice acceptor sequence leads to a disappearance of calcitonin mRNA in calcitonin-producing cells and an increase in the CGRP mRNA in the same cell line. Inability to produce calcitonin due to a mutation of the poly (A) site, at the end of exon 4, or deletion of a sequence including this site could be explained as destruction of the essential structure for the calcitonin-pattern splicing. A significant increase in CGRP mRNA in calcitonin-producing cells resulting from the deletion of the 3'-splice acceptor of exon 4 seems impossible according to the model proposed by Leff *et al.* (1987) because there is no *trans*-acting factor to promote a secondary structure of pre-mRNA to favor production of CGRP. At least two possibilities may exist: a) the mutation or deletion leads to a conformational change of the pre-mRNA in favor of a structure suitable for CGRP-pattern splicing even if an external promoting factor is not present, or b) a mechanism other than that described in the model proposed by Leff *et al.* (1987) is involved in the tissue-specific alternative processing of mRNA from calcitonin/αCGRP.

d. Regulation of differential expression

Expression of calcitonin/αCGRP (either the absolute amount or the relative proportions of the two peptides) can be regulated in different cell types *in vitro* and also *in vivo*. Agents known to activate protein kinase C and A are reported to enhance the production of calcitonin and CGRP mRNA, and to increase calcitonin

secretion (deBustros *et al.*, 1985, 1986). 1,25-Dihydroxyvitamin D_3 and, perhaps, increases in serum calcium concentration, inhibit transcription of the calcitonin gene, resulting in decreased production of calcitonin (Cote *et al.*, 1989). These factors also lower calcitonin and CGRP mRNA levels in a time and concentration dependent manner in cultured TT cells, a human cell line derived from a medullary thyroid carcinoma (Cote *et al.*, 1987). Moreover, differentiation of transplanted rat medullary thyroid carcinoma cells is associated with a switch to a low calcitonin/high CGRP-producing state (Rosenfeld *et al.*, 1981). Similarly, Nelkin *et al.* (1989) found that in cultured TT cells, the pattern of alternative mRNA processing changed with the time of culture. The ratio of calcitonin mRNA over CGRP mRNA is 0.2 at the begining of culture and reaches 1.0 at days 6 to 9.

2. *Gene for βCGRP*

A gene for βCGRP was identified in rat and human in the mid 1980's (Amara *et al.*, 1985; Steenbergh *et al.*, 1985, 1986; Alevizaki *et al.*, 1986). Similar to the calcitonin/αCGRP gene, this gene has been localized on the short arm of chromosome 11 (Alevizaki *et al.*, 1986). This peptide is also produced mainly in neuronal tissues and consists of six exons, including a fifth exon for CGRP. High homology has been found in the region for CGRP in both genes and weak homology exists between the exon 4-like regions and CGRP-non-coding regions of both genes. A calcitonin-like (exon 4-like) exon exists, but for some reason does not appear to produce a

biologically active peptide.

Substitution of specific regions from the calcitonin/αCGRP gene into the β-gene can reconstitute some, but not all, aspects of alternative RNA processing (Bennett and Amara, 1991). The divergence at the 3' end of the gene allows the generation of cDNA probes that are either specific for the α- or the β-gene. (Alevizaki *et al.*, 1986; Steenbergh *et al.*, 1986). Peripheral axotomy selectively increases α- but not β-CGRP mRNA in motor neurons of the rat spinal cord, but non-selectively decreases the levels of both forms in the dorsal root ganglia. This indicates that α- and β-CGRP are regulated independently in motor neurons of the spinal cord (Noguchi *et al.*, 1990a).

3. Amylin gene

Amylin (also known as islet amyloid polypeptide, IAPP or diabetes-associated peptide, DAP) is, together with CGRP and calcitonin, said to arise from the same ancestral gene. Its gene is a single-copy gene with an intron-exon pattern similar to the CGRP genes (Nishi *et al.*, 1989). This gene is located on the short arm of chromosome 12 (Mosselman *et al.*, 1988; Nishi *et al.*, 1989), whereas the calcitonin/CGRP genes are located on the homologous chromosome 11 (Alevizaki *et al.*, 1986). Amylin mRNA is abundant in the islets of Langerhans but not present in brain , lung, heart, intestine, kidney, liver, adrenal, or testes (Leffert *et al.*, 1989). The mature peptide is known to be generated by proteolytic processing of an 89-

amino acid precursor. Amylin shares 46% homology in its sequence with the known CGRPs, and also shares some of the effects of CGRP, such as inhibition of glycogen synthesis in muscle (Cooper *et al*, 1988; Leighton and Foot, 1990), inhibition of insulin-stimulated glucose disposal (Molina *et al.*, 1990; Sowa *et al.*, 1990) and vasodilating effects (Brain *et al.*, 1990; Gardiner *et al.*, 1991).

D. General distribution of CGRP

Capsaicin, a pungent ingredient in a variety of red peppers of the genus *Capsicum*, is a tool which is used in studies of the distribution of CGRP and other neuropeptides in certain types of sensory neurons. It is, therefore, necessary to discuss capsaicin before begining a consideration of the distribution of CGRP-immunoreactive (CGRP-IR) neurons and nerve fibers. This compound selectively affects certain types of sensory neurons through both acute excitatory and long term neurotoxic actions. The capsaicin-sensitive neurons are primary afferent sensory neurons (Winter, 1987). These neurons have their cell bodies located in the spinal and cranial sensory ganglia, and give rise to either unmyelinated or thinly myelinated nerve fibers (Hiura and Sakamoto, 1987). These fibers are in the C- or A(δ)-fiber range, respectively (Szolcsányi *et al.*, 1988). The excitation of sensory neurons by capsaicin leads to the release of neuropeptides, including CGRP, from the activated nerve endings, and at the same time the nerve activity is conducted to the central nervous system (Holzer, 1988).

1. Differential distribution of CGRP and calcitonin

It has been established that the regulation of the alternative production of calcitonin or CGRP mRNA is tissue specific (Amara *et al.*, 1982; Leff *et al.*, 1987; Crenshaw *et al.*, 1987). Calcitonin mRNA is found almost exclusively in the thyroid C cells and CGRP mRNA in the nervous system (Rosenfeld *et al.*, 1983; Sabate *et al.*, 1985). In the nervous system, the proportion of calcitonin mRNA has been shown to be less than 0.5% of the level of CGRP mRNA and this proportion has been found to be reversed for the normal thyroid (Lee *et al.*, 1985b; Nitta *et al.*, 1986; Sabate *et al.*, 1985; Sikri *et al.*, 1985). The highest levels of CGRP mRNA in the nervous system have been identified in the trigeminal ganglia, while lower amounts have also been noted in the hypothalamus, lateral medulla, and motor neurons of the spinal cord (Sabate *et al.*, 1985). A certain amount of CGRP coexists with calcitonin in normal thyroid C cells in certain species, e.g. thyroid C cells of dog, cat, cattle, monkey, rat and rabbit. The thyroid C cells in pig, mouse, hamster and guinea pig show weak or no CGRP-IR (Kameda, 1987). In addition, CGRP is also found in mucosal endocrine cells of the human small intestine (Timmermans *et al.*, 1992).

2. Differential distribution of αCGRP and βCGRP

Some reports indicate the differential distribution of CGRP peptides encoded by the α- and β-genes. For example, there is more βCGRP mRNA than αCGRP mRNA in the motor nuclei of the III, IV, and V cranial nerves, while in the

trigeminal ganglia the situation is reversed. αCGRP mRNA and βCGRP mRNA are equivalent in the VII, X, and XII cranial nerves (Amara *et al.*, 1985). In the dorsal root ganglia of the rat, small and medium sized cells express both the αCGRP mRNA and βCGRP mRNA while the α-form is predominant in the large neurons (Noguchi *et al.*, 1990b). In the intestine, βCGRP concentrations are up to seven times greater than αCGRP concentrations. Only βCGRP is detected in the intestines when sensory neurons have been depleted of CGRP by capsaicin. Experiments using Northern blot and *in situ* hybridization to αCGRP- and βCGRP-specific probes have shown that while both αCGRP and βCGRP messenger RNA occur in the dorsal root ganglia, only βCGRP messenger RNA is detected in the intestine. There, it is localized to enteric neurons confined to the myenteric and submucous plexuses of the both small and large intestine. Sensory neurons and enteric neurons have been identified as populations which preferentially express αCGRP and βCGRP, respectively (Mulderry *et al.*, 1988; Sternini and Anderson, 1992).

3. Distribution of CGRP

CGRP is present in sensory, motor and enteric neurons (Amara *et al.*, 1985; Mulderry *et al.*, 1988; Noguchi *et al.*, 1990a,b; Sternin and Anderson, 1992). In rat brain, extensive mapping of CGRP-IR structures has revealed a wide distribution of the peptide (Kawai *et al.*, 1985). Tracts for CGRP-IR fibers have been identified, e.g. tracts between the insular cortex and the ventromedial nucleus of the thalamus

(Shimada *et al.*, 1985), between the hypothalamus and lateral septal area (Sakanaka *et al.*, 1985) and connecting the ventral surface of the hypothalamus and the caudate nucleus (Kawai *et al.*, 1985).

In the spinal cord, CGRP-IR cell bodies are found in sensory neurons (dorsal root ganglia), the fibers of which are found to be localized to laminae I, II and X of the dorsal horn and motor neurons (Gibson *et al.*, 1984; Noguchi *et al.*, 1990a,b). In the sensory (dorsal root, nodose and trigeminal) ganglia, nearly 40% of the cells show CGRP-IR and 50% of those co-store substance P. Together with substance P, CGRP-rich fibers appear to form a part of the primary afferent neuron system comprised of capsaicin-sensitive A(δ)-, C-fiber afferent nerves, and type B (medium-sized) cells (Lee *et al.*, 1985a,b).

CGRP-IR fibers are found in the autonomic sympathetic and parasympathetic ganglia. CGRP-positive cell bodies have been found only in parasympathetic (pterygopalatine and myenteric) ganglia.

In the cardiovascular system, CGRP-rich nerve fibers have been found to be particularly prominent in the papillary muscle, sinoatrial and atrioventricular nodes and to lie in association with coronary arteries (Mulderry *et al.*, 1985). The nodal innervation might be of physiological significance in the regulation of heart rate. CGRP-IR has also been found in intrinsic neurons of the heart (Gerstheimer and Metz, 1986). In blood vessels, there is generally a dense varicose mesh of CGRP-positive nerve fibers in the adventitia with occasional penetrating branches in blood

vessels. Two types of CGRP-IR fibers have been identified in the wall of the cerebral arterial system (McCulloch *et al.*, 1987; Wanaka *et al.* 1987). One type forms dense periadventitial fiber bands and the other forms a meshwork in the adventitia.

In the gastrointestinal (GI) tract, those CGRP-containing fibers innervating the small intestine are of both extrinsic and intrinsic origin, while only extrinsic innervation was seen in other areas of the GI tract (Clague *et al.*, 1985; Gibbins *et al.*, 1985; Mulderry *et al.*, 1988; Sternin and Anderson, 1992). In the mammalian (rat, cat and monkey) oesophagus, both sensory (capsaicin-sensitive) and motor fibers have been described (Rodrigo *et al.*, 1985). In addition, high levels of immunoreactive-CGRP exist in the pylorus, the descending colon and the rectum, with lesser amounts in the pancreas.

CGRP-IR has also been found in intrinsic neurons of the bladder (Moss, 1988).

E. General actions

1. Actions of CGRP

Pharmacological actions of CGRP have been observed in several systems: the cardiovascular, the skeletal muscle and the GI systems. CGRP also exerts some effects on the central nervous system and the immune system. Moreover, CGRP could display some actions through calcitonin receptors with CGRP showing much lower potency than calcitonin.

a. Actions on the cardiovascular system

Intracerebroventricular administration of CGRP selectively stimulates noradrenergic outflow accompanied by intense tachycardia and hypertension (Fisher *et al.,* 1983; Nguyen *et al.,* 1986). Systemic injection or infusion causes hypotension and tachycardia in both experimental animals and man (Fisher *et al.,* 1983; Tippins *et al.,* 1984; Gennari and Fischer, 1985; Lundberg *et al.,* 1985; Struthers *et al.,* 1986). In conscious rats, *i.v.* bolus injection of rCGRP induces decreases in mean arterial pressure and total peripheral resistance and increases in heart rate and cardiac output. The tachycardia response to rCGRP is reduced but not abolished by the β-adrenoceptor antagonist, propranolol, indicating both a reflex mechanism and a direct action are involved in the actions of CGRP on the heart (Lappe *et al.*, 1987). Tachycardia and positive inotropic effects of CGRP observed in rat isolated auricle are partially blocked by a high dose of propranolol (Tippins *et al.,* 1984). A positive inotropic action on porcine ventricular muscle is produced by hCGRP (Miyauchi *et al.,* 1988), but not by capsaicin which has been reported to release CGRP from intracardiac nerves (Miyauchi *et al.*, 1987). All these results indicate a direct action of CGRP on heart muscle.

CGRP receptors are found to be most dense in the atrium, and much less dense in the ventricle (Wimalawanasa and MacIntyre, 1988). Receptors on both ventricular muscle (Miyauchi *et al.,* 1988) and atrial cardiocytes (Dennis *et al.,* 1989) show high affinity to CGRP (with K_ds in the picomolar range). CGRP evokes positive

inotropic and chronotropic effects accompanied by an increase in $[cAMP]_i$ (Fisher *et al.*, 1988; Ishikawa *et al.*, 1988) and calcium currents (Ono *et al.*, 1989) through a pathway other than one involving adrenoceptors.

The hypotensive effect of the peptide is clearly due to its effect on blood vessels. CGRP shows potent vasodilating effects with a long duration of action. The vasodilating effect of this peptide has been confirmed in both *in vivo* and *in vitro* experiments. It dilates arteries including critical coronary and cerebral vessels and systemic vasculature in an endothelium-dependent or -independent manner (see more detail in "Actions of CGRP on smooth muscle"). CGRP itself does not induce edema or cause protein extravasation but does potentiate edema evoked by other agents, such as histamine, bradykinin, etc. (Brain *et al.*, 1986).

b. Actions on gastrointestinal tract

Central administration of CGRP inhibits gastrointestinal motor function, for example, inhibition of gastric emptying, restoration of the fasting pattern of intestinal motility and inhibition of forestomach contractions (Raybould, 1991). A direct inhibitory effect on gastric smooth muscle has been reported (Katsoulis and Conlon, 1989; Maton *et al.*, 1988). In addition, this peptide also affects gastrointestinal secretion. CGRP inhibits secretion of gastric acid (Morley *et al.*, 1981; Hughes *et al.*, 1984, Lenz *et al.*, 1985) and stimulates the secretion of somatostatin in the stomach (Dunning and Taborsky, 1987; Zdon *et al.*, 1988). Specific CGRP binding sites, having a Kd

value of 18 nM, have been found on cells of exocrine pancreas. CGRP also stimulates the production of cAMP in these cells. Interaction of CGRP with its receptors can induce theophylline-dependent amylase secretion from acinar cells of the guinea pig pancreas (Seifert et al., 1985b). CGRP also affects intestinal transport by evoking transient and cyclical increases in chloride secretion in the distal colon of guinea pig (Cook, 1991). The actions of CGRP on the small intestine will be discussed in a later section in this chapter.

c. Other actions of CGRP

CGRP also shows a hypocalcemic effect at 1000 times higher dose than calcitonin in the rat (Tippins *et al.*, 1984; Morita *et al.*, 1989) (see following sections). CGRP has been reported to inhibit spreading, motility and resorption of bone by isolated osteoclasts (Zaidi *et al.*, 1987c,d, 1988). A hypophosphatemic action is seen in rat (Morita *et al.*, 1989). NE-precontracted splenic strips can be relaxed by rCGRP with an EC_{50} value of 50 nM (Sigrist *et al.*, 1986).

2. Actions of calcitonin

The main action of calcitonin is on the bone. The acute inhibitory effect on the osteoclast results in the marked fall of plasma calcium (Robison *et al.*, 1967; Woodhouse *et al.*, 1971). The calcitonin effect on the osteoclast can be resolved into at least two major components: quiescence ("Q " effect) and retraction ("R" effect).

The "Q" effect is characterized by a gradual slowing down of cell motility and finally the cessation of all cellular motile processes. The "R" effect is characterized by gradual pseudopodial retraction leading to an absolute reduction of cell surface area. Other actions of calcitonin include the enhancement of the production of 1,25-dihydroxy-vitamin D_3 (Hass *et al.*, 1971; Rasmussen *et al.*, 1972), and a central analgesic effect (Pecile *et al.*, 1975; Fraioli *et al.*, 1982; Allan, 1983).

F. Structure-activity relationship

1. CGRP from different species

Until now, researchers have sequenced or deduced with molecular biological methods, CGRP from human (Steenbergh *et al.* 1984, 1985), rat (Rosenfeld *et al.*, 1983, 1984), chicken (Minvielle *et al.*, 1986, 1987), pig (Kimura *et al.*, 1987), cow (Collyear *et al.*, 1991) and rabbit (Eysselein *et al.*, 1991) tissues (Fig. 1-2). As mentioned before, in addition to the calcitonin/αCGRP gene, there is another gene encoding another form of CGRP, i.e. βCGRP, in the human and rat (Amara *et al.*, 1985; Steenbergh *et al.*, 1985, 1986; Alevizaki *et al.*, 1986).

A high percentage of homology exists between αCGRP and βCGRP, and also among the peptides from different species. Only one amino acid (3%) difference exists between the rat α- and β-forms, and a difference of three amino acids (8%) between the human α- and β-form. About 84% of the sequence is conserved among the peptides of mammalian origin. There is 46% homology between human amylin

and CGRP (hβ-, rCGRP etc.). However, the homology between calcitonin and CGRP is very low.

Some structural characteristics of these peptides are as follows: a) there is a disulphide bridge between Cys^2 and Cys^7; b) there are several regions of the sequence which are identical in peptides from all mammalian sources, these being positions 4-21, 26-30, 31-34 and 36-37; c) there is a carboxy-terminal phenylalanine amide. Chicken CGRP differs from the peptides of mammalian sources by having the three amino acids, Asp^{14}, Phe^{15} and Gly^{23} instead of Gly^{14}, Leu^{15} and Val^{23}.

The structure-activity relationship of CGRPs from different species has been studied in terms of the affinity to the receptors in binding experiments and the potency of biological actions. Three CGRP sequences, hαCGRP, rαCGRP and hβCGRP, at equimolar concentrations, [illegible] an equivalent elevation of blood flow in the rabbit skin and enhance the contractility of the rat atrium to a similar extent (Brain *et al.*, 1985, 1986; Tippins *et al.*, 1986; Zaidi *et al.*, 1990). In binding experiments on rat liver membranes, the potency of those peptides is in the following sequence: chicken CGRP > rCGRP > hCGRP (Yamaguchi *et al.*, 1988a). Chakder and Rattan (1990) reported that the potency sequence in decreasing the resting tone of the opossum internal anal sphincter is hβCGRP > rCGRP > hαCGRP. Chicken CGRP has a higher potency and longer duration in both hypocalcemic and hypophosphatemic activities than does hCGRP (Morita *et al.*, 1989). The difference in potency of biological actions among the CGRPs reflects the minor changes in amino

acid residues in these peptides.

In certain bioassay systems, αCGRP from the calcitonin gene and its homolog, βCGRP, have similar actions with about equivalent potency. Examples are their actions on intraocular and arterial blood pressure of the rabbit (Krootila *et al.*, 1991), rabbit skin blood flow (Brain *et al.*, 1986), rabbit atrial preparations (Tippins *et al.*, 1986), hypocalcemic and hypophosphatemic actions in the rat (Dennis *et al.*, 1990), osteoclastic bone resorption (Zaidi *et al.*, 1990).

Despite the fact that the α- and β-forms show similar biological potency in most studies, they do have different actions in some assays, for example, in dilating skin blood vessels (Brain *et al.*, 1986). With systemic administration, hαCGRP increases the blood flow in blood vessels in the stomach, duodenum, heart, brain and skeletal muscle, while hβCGRP selectively acts on the vessels in the stomach and pancreas at same dose. In humans, the human β-form inhibits pentagastrin-induced acid secretion while the α-form is ineffective (Beglinger *et al.*, 1988). In the rabbit, the α-form increases the pentagastrin-stimulated acid secretion while β-form decreases the acid secretion (Bauerfeind *et al.*, 1989). It is, therefore, reasonable to speculate that the two peptides from separate genes could play different physiological roles.

2. *Two key structural factors for biological activity: disulphide bridge and full length*

The disulphide bridge between Cys at positions 2 and 7 is critical for

biological activity. Destruction of this bridge greatly reduces or abolishes the biological activity, e.g. actions on the rat atrium (Tippins *et al.,* 1986), guinea pig atrium and rat vas deferens (Morita *et al.,* 1989).

The intact peptide seems essential for full activity. Even the loss of two amino acids at the C-terminus of the molecule results in a marked decrease in activity (Zaidi *et al.,* 1990). Synthetic or enzymatically digested N-terminal and C-terminal fragments of CGRP are weak or inactive relative to the parent peptide, or they become antagonists for CGRP. Although human-α-CGRP fragments, $CGRP_{1-12}$, $CGRP_{1-15}$ and $CGRP_{1-22}$ also produce hypotension and tachycardia in rats, they are much weaker, and the duration of the actions are much shorter than the parent peptide (Maggi *et al.,* 1990b). None of the fragments, $CGRP_{1-11}$, $CGRP_{1-16}$, $CGRP_{1-18}$, $CGRP_{12-18}$, $CGRP_{17-37}$, $CGRP_{19-35}$ and $CGRP_{19-37}$, are effective in the isolated rat atrial preparation (Tippins *et al.,* 1986), the osteoclast bone resorption assay, the rabbit skin blood flow or the edema formation assay (Zaidi *et al.,* 1990). The series of short N-terminal fragments such as $h\alpha CGRP_{1-7}$, $h\alpha CGRP_{1-8}$, $h\alpha CGRP_{2-7}$ and $h\alpha CGRP_{2-8}$ displays no stimulated chronotropic activity in the right atrial preparation (Dennis *et al.,* 1989).

3. Other structures important for biological activity

In the region of the sequence from positions 3 to 21, the peptides from mammalian sources are identical. However, chicken CGRP differs from the

mammalian peptide by two amino acid residues: Asp^{14} and Phe^{15} instead of Gly^{14} and Leu^{15}. Chicken CGRP has been shown to be more potent than rat and human CGRP in several bioassays (Yamaguchi *et al.*, 1988a,b; Dennis *et al.*, 1989). Dennis *et al.* (1989) demonstrated that the amino acid residue at position 14 most influences the potency and duration of the activity. Replacement of Asp^{14} in chicken CGRP by Gly (as in hCGRP) results in a great reduction in both potency and duration of action. In contrast, the replacement of Gly^{14} with Asp in hCGRP enhances the potency and prolongs biological activity.

Some modifications at the N-terminal end, which are presumably made to protect the peptide from inactivation by peptidases, actually increase the strength and duration of action. A pseudo-peptide bond between Ala^1 and Cys^2 in [Ala^1 (CH_2NH)Cys^2] increases the potency of action on the guinea pig atrium and rat vas deferens by 20 to 36% (Morita *et al.*, 1989). Des-1-Ala-des-α-amino-hCGRP (Otaka *et al.*, 1986), des-1-Ala-des-α-amino-chicken CGRP (Dennis *et al.*, 1990) and $Asu^{2,7}$hCGRP (Noda *et al.*, 1987) all of which have no α-amino group in their amino terminal (and therefore are expected to be resistant to amino peptidase) show long-lasting activity. Acetylation of the N-terminal amino group and Lys at positions 24 and 35 diminishes the activity (Tippins *et al.*, 1986).

4. C-terminus: the key structure for receptor affinity

As mentioned above, both N-terminal and C-terminal fragments of CGRP

show very weak or no biological activity. However, some C-terminal fragments exhibit high affinity to the CGRP receptor, and, therefore, become antagonists, while the N-terminal fragments display little or no affinity for receptors.

The disulphide bridge is critical for the biological activity but not critical for binding to receptors in the membrane preparations of central nervous system (CNS), guinea pig atrium and rat vas deferens. Destruction of the bridge as in [Cys(ACM)^{2,7}]-hαCGRP or substitution of the bridge by an amide bond as in the analog cyclo^{2,7}-[Asp^2,Lys^7]hαCGRP only slightly decreases the affinity for the receptors (Morita *et al.*, 1989).

Modification of Asp^3 of hαCGRP with 4-azidoaniline or N-biotinyl-1,8-diamino 3,6-dioxaoctane, and the naturally occurring substitution of Asp^3 by Asn in hβCGRP, are well tolerated with respect to receptor binding (Stangle *et al.*, 1991). However, the N-terminal fragments such as hαCGRP$_{1\text{-}7}$, hαCGRP$_{1\text{-}8}$, hαCGRP$_{2\text{-}7}$ and hαCGRP$_{2\text{-}8}$ retain little or no affinity for [^{125}I]hCGRP binding sites in both brain and spleen membrane preparations of rat (Dennis *et al.*, 1989).

Some C-terminal fragments show relatively high affinity for the receptors and are competitive antagonists . They are hαCGRP$_{8\text{-}37}$, hαCGRP$_{9\text{-}37}$, hαCGRP$_{10\text{-}37}$, hαCGRP$_{11\text{-}37}$, hαCGRP$_{12\text{-}37}$ and a modified fragment, Tyr^{0}rCGRP$_{28\text{-}37}$ (Morita *et al.*, 1989). Among these fragments, hCGRP$_{8\text{-}37}$ and hCCGRP$_{9\text{-}37}$ have the highest affinity. Their affinities are almost identical to the parent peptide for [^{125}I-iodohistidyl^{10}]hCGRP binding sites in both right and left rat atrium, and 3 to 4 times

greater than those of the native peptide in both brain and vas deferens (Mimeault *et al.*, 1991). $hCGRP_{10-37}$ and $hCGRP_{11-37}$ show 4 to 8 times lower affinity in the atrium and 10 to 33 times lower affinity in the brain and vas deferens compared to $hCGRP_{8-37}$ and $hCGRP_{9-37}$. However, $hCGRP_{8-37}$ and $hCGRP_{9-37}$ display antagonistic properties ten times stronger than those of the other two fragments in bioassays of both the right and left atrium. In the vas deferens, while $hCGRP_{8-37}$ and $hCCGRP_{9-37}$ have pA_2 values of 6.24 to 6.33, $hCGRP_{10-37}$ and $hCGRP_{11-37}$ are ineffective. Therefore, the presence of the amino acid residue at position 9 is important for the peptide to retain high affinity for its receptor.

G. Receptors

1. CGRP receptors

a. Distribution

CGRP binding sites or receptors have been demonstrated in many different systems: the central and peripheral nervous system, cardiovascular system, GI tract, blood cells, liver, kidney, etc.

CGRP receptors are distributed widely throughout the nervous system of rat (Goltzman and Mitchell, 1985; Seifert *et al.*, 1985a; Inagaki *et al.*, 1986; Foord and Craig, 1987; Wimalawansa *et al.*, 1987; Yoshizaki *et al.*, 1987; Chatterjee *et al.*, 1991), human (Tschop *et al.*, 1985; Inagaki *et al.*, 1986; Sigrist *et al.*, 1988; Chatterjee and Fischer, 1991; Stangle *et al.*, 1991), dog (Foord and Craig, 1987) and pig (Hiroshima

et al., 1988; Sano *et al.,* 1989).

CGRP and salmon calcitonin binding sites have different distributions and densities in the brain (Goltzmann and Mitchell, 1985). In the rat CNS, the highest density of CGRP binding sites is in spinal cord while that for salmon calcitonin is in the hypothalamus. The highest ratio of CGRP binding sites to calcitonin binding sites is observed in the cortex and the spinal cord, while in the hypothalamus calcitonin binding sites are predominant. The cerebellum usually has a higher density of CGRP binding sites (Inagaki *et al.,* 1986; Foord and Craig, 1987; Wimalawansa *et al.,* 1987).

In the peripheral nervous system, CGRP binding sites have been found on myenteric neurons (α-binding sites; Gates *et al.,* 1989).

CGRP receptors are also present in a variety of peripheral non-neuronal tissues including both atrial and ventricular muscle of the heart (Sigrist *et al.,* 1986; Wimalawansa *et al.,* 1987; Yoshizaki *et al.,* 1987; Miyauchi *et al.,* 1988; Wimalawansa and MacIntyre, 1988; Sano *et al.,* 1989). Wimalawansa and MacIntyre (1988) examined the heart and nine different blood vessels. They found that in the heart, the density of CGRP binding sites is highest in the right atrium, and is also high in the left atrium, while the ventricle has seven- to ten-fold less binding sites than the right atrium.

Specific CGRP binding sites are found in various blood vessels. They are present in aortic arch and abdominal aorta (Wimalawansa and MacIntyre, 1988). The coronary, superior mesenteric, femoral, renal arteries and arteries of the uterus, distal

limbs and the GI tract express CGRP binding sites (Kubota *et al.,* 1985; Sigrist *et al.,* 1986; Power *et al.,* 1988; Wimalawansa and MacIntyre, 1988; Gates *et al.,* 1989; Sano *et al.,* 1989). The inferior vena cava and femoral vein contain CGRP binding sites (Wimalawansa and Macintyre, 1988). CGRP binding sites are also found in gastric smooth muscle (Maton *et al.,* 1988).

CGRP receptors are also found in rat liver (Yamaguchi *et al.,* 1988a,b), spleen, adrenal gland, limb arteries, lung, bladder, pancreas, tongue, intestine (Wimalawansa *et al.,* 1987), kidney (Wohlwend *et al.,* 1985; Seitz *et al.,* 1986; Wimalawansa *et al.,* 1987), pituitary gland, kidney and bone (Goltzmann and Mitchell, 1985).

The pituitary gland and the spleen have a high density of CGRP binding sites. Adrenal glands and pituitary glands mainly express CGRP binding sites while salmon calcitonin binding sites are predominant in both the kidney and bone (Goltzmann and Mitchell, 1985).

CGRP binding sites in most tissues investigated including CNS, liver, skeletal muscle, cardiocytes, lymphocytes and kidney show high affinity for the peptide (with the K_D values from 0.04 to 0.9 nM) (Seifert *et al.,* 1985a; Inagaki *et al.,* 1986; Henke *et al.,* 1987; Yoshizaki *et al.,* 1987; Mak and Barnes, 1988; Yamaguchi *et al.,* 1988a,b; Jennings and Mudge, 1989; Umeda and Arisawa, 1989; Dennis *et al.,* 1990; Chatterjee *et al.,* 1991). CGRP binding sites have moderate to low affinity (1 to 3 nM) in spleen (Dennis *et al.,* 1990) gastric muscle (Maton *et al.,* 1988) and a murine macrophage

cell line (Abello *et al.*, 1991), and low affinity (18 nM) in the guinea pig pancreas (Seifert *et al.*, 1985b). Receptors on cultured rat aortic muscle cells and bovine aortic endothelial cells show very low affinity (120 and 260 nM, Hirata *et al.*, 1988) while those on the coronary artery display the affinity at picomolar levels (6 pM, Sano *et al.*, 1989).

b. Cross-reactivity with the calcitonin receptor

CGRP binding sites and salmon calcitonin binding sites have different distributions in terms of the density of the binding sites in brain (Goltzmann and Mitchell, 1985). Calcitonin has been found not to cross-react with the CGRP receptor in the vascular tree (Kubota *et al.*, 1985; Sigrist *et al.*, 1986) and the atria, ventricles and valves of the heart (Sigrist *et al.*, 1986). These results from both binding and bioassay data demonstrate clearly that CGRP receptors and calcitonin receptors are distinguishable (Goltzman and Mitchell, 1985; Wohlwend *et al.*, 1985; Henke *et al.*, 1987; Yamaguchi *et al.*, 1988a,b; Dennis *et al.*, 1989, 1991; Zaidi *et al.*, 1990; Stangle *et al.*, 1991). However, cross-reactivity has been found between calcitonin and CGRP receptors in several tissues. Several forms of CGRP share some of the effects of calcitonin on the osteoclast and renal calcitonin receptors. However the actions of CGRP are seen at several hundred-fold higher molar concentrations than are those of calcitonin (Zaidi *et al.*, 1987a,b, 1988). In the CNS, salmon calcitonin and human amylin can inhibit the CGRP binding to CGRP receptors at a 100 to 10,000-fold

higher concentration (Goltzman and Michell, 1985; Yoshizaki *et al.*, 1987; Sigrist *et al.*, 1988; Chatterjee *et al.*, 1991; Stangle *et al.*, 1991). Human calcitonin usually shows no affinity for the CGRP receptor. Seitz *et al.* (1986) reported that ^{125}I-salmon calcitonin binding sites on calvarial cells cannot be displaced by rCGRP, and ^{125}I-rCGRP binding sites on these cells are inhibited by salmon calcitonin only at a high dose. In renal cortical membranes, no specific binding sites for ^{125}I-rCGRP can be detected while ^{125}I-salmon calcitonin binding is minimally inhibited by a high dose of rCGRP (Seitz *et al.*, 1986).

c. *Relevance to adenylate cyclase*

CGRP has been reported to stimulate production of cAMP in different tissues where specific CGRP binding sites are present. These tissues include guinea pig pancreatic acinar cells (Seifert *et al.*, 1985b), rat kidney (perhaps through calcitonin receptors, Goltzman and Mitchell, 1985), guinea pig atria (Ishikawa *et al.*, 1988), cultured rat heart cells (Fisher *et al.*, 1988), porcine coronary arteries (Shoji *et al.*, 1987), rat aortic smooth muscle cells (Kubota *et al.*, 1985), T-lymphocytes (Umeda *et al.*, 1988) and liver (Yamaguchi *et al.*, 1988a,b). GTP binding proteins may be involved in this stimulation. GTP can potentiate the effect of CGRP on cAMP levels in the liver (Yamaguchi *et al.*, 1988b), and GTPγS decreases the number and affinity of CGRP specific binding sites in the rat cerebellum (Chatterjee *et al.*, 1991).

d. Subtypes of CGRP receptors

Dennis *et al.* (1990) reported that hαCGRP$_{8-37}$ displays different potencies against the action of hαCGRP in bioassays in various tissues. It potently antagonizes the effect of CGRP on the right atrium, on the CNS-mediated inhibition of food intake, and on the guinea pig ileum. However hαCGRP$_{8-37}$ is less potent in the vas deferens, and ineffective against CGRP-induced hyperthermia. Therefore, these authors proposed the existence of CGRP1 and CGRP2 receptors on the basis of sensitivity to hαCGRP$_{8-37}$. Those receptors more sensitive to hαCGRP$_{8-37}$ (receptors on the right atrium and ileum) are the CGRP1 subtype and the less sensitive are the CGRP2 subtype (those on the vas deferens). We have noted from the data reported by Dennis *et al.* (1990) that hαCGRP$_{8-37}$ actually shows a similar potency in the guinea pig ileum and in the rat vas deferens, although it is much more potent in right atrium. There are some other facts in favor of this classification. [Cys(ACM)^{2,7}]-hCGRP loses most of its cardioexcitatory effects while it retains its agonistic activity in the rat vas deferens (Dennis *et al.*, 1989). It seems that this compound acts as a fairly selective agonist in tissues resistant to the antagonistic properties of hCGRP$_{8-37}$, i.e. those tissues containing CGRP2 receptors.

In spite of the similarities between the α-form and the β-form of CGRP (Tippins *et al.*, 1986; Brain *et al.*, 1986; Zaidi *et al.*, 1987, 1990; Dennis *et al.*, 1989; Krootila *et al.*, 1991), they are as mentioned above encoded by separate genes (Steenbergh *et al.*, 1985; Amara *et al.*, 1985; Alevizaki *et al.*, 1986; Steenbergh *et al.*,

1986). α- and β-CGRP also have different biological actions in certain bioassay systems (Bauerfeind *et al.*, 1989; Beglinger *et al.*, 1988; Brain *et al.*, 1986). Moreover, differences between the α- and β-forms have also been reported with respect to binding characteristics. With the use of both ^{125}I-hα- and ^{125}I-hβCGRP as ligands, hαCGRP exhibits a 3 to 5 times higher affinity for the receptor than does hβCGRP in the human cerebellum and spinal tissues (Dotti-Sigrist *et al.*, 1988; Henke *et al.*, 1987). There is a subtle difference in the regional distribution of the binding sites for the two ligands in the ventromedial hypothalamus and the ^{125}I-hαCGRP binding is more dense than that for ^{125}I-hβCGRP in the inferior colliculus and the cerebellum (Henke *et al.*, 1987). Although the two peptides could have high cross-reactivity with receptors for each other, it is tempting to speculate that there might be different subtypes of CGRP receptors for these two forms of CGRP.

2. Calcitonin receptors

Calcitonin receptors were first found in the physiological target cells of the hormone: osteoclasts. The receptors occur at a high density in these cells (Mark *et al.*, 1972; Luben *et al.*, 1976; Warshawsky *et al.*, 1980; Nicholson *et al.*, 1986). A variety of normal and malignant tissues, most importantly the kidney, have been found to possess receptors for calcitonin (Mark *et al.*, 1972, 1973; Goldring *et al.*, 1978). Other normal tissues possessing receptors are the mammalian brain (Rizzo and Goltzmann, 1981; Tschopp *et al.*, 1985), testes (Chausmer *et al.*, 1982), pig lung

(Fouchereau-Peron *et al.*, 1981a), fish gill (Fouchereau-Peron *et al.*, 1981b), and a variety of lymphoid cell lines (Mark *et al.*, 1974; Moran *et al.*, 1978). Binding sites specific for salmon calcitonin, which show very little cross-reactivity with human calcitonin and CGRP, have been found in the central nervous system (Goltzman and Mitchell, 1985).

3. Amylin receptors

A single specific binding site with high affinity for amylin has been reported by Bhogal *et al.* (1992). In the rat, specific binding sites for ^{125}I-amylin are found to be highest in the lung followed in decreasing order of occurance by the stomach fundus, spleen, brain stem, hypothalamus, and the liver. Specific binding in the lung has a dissociation constant value of 10 nM. The order of potency for displacing the ^{125}I-amylin by different peptides is as follows: r-amylin (5.75 nM) > hαCGRP (38 nM) > h-amylin (55 nM) = rβCGRP > rαCGRP (900 nM).

Amylin cross-reacts with CGRP receptors in both skeletal muscle (Chantry *et al.*, 1991; Zhu *et al.*, 1991) and liver plasma membranes (Morishita *et al.*, 1990; Chantry *et al.*, 1991; Zhu *et al.*, 1991). Amylin shows a 100 to 1000-fold lower affinity than CGRP for CGRP binding sites and is much weaker than CGRP in stimulating the formation of cAMP in both tissues. The ability of amylin to stimulate production of cAMP can be blocked by the CGRP antagonist, hCGRP$_{8-37}$. In addition, amylin can displace ^{125}I-CGRP binding in the rat brain with a K_i value of 37 nM.

II. ACTIONS OF CGRP ON SMOOTH MUSCLE

A. Vascular musculature

Brain *et al.* (1986) first recognized the vasodilator action of CGRP. These investigators found that the peptide induces intense microvascular (mainly arteriolar) dilation with increased blood flow and a persistent flare when injected intradermally in the human. CGRP has a similar potency to prostaglandin E_2, and is several-fold more potent than ACh, ADP, adenosine, 5-HT, Substance P, isoprenaline, or vasoactive intestinal peptide (Williams, 1982; Brain *et al.*, 1986).

The vasodilatory effects of CGRP may be observed *in vivo*. Systemic injection or infusion into healthy human volunteers leads to marked diastolic hypotension accompanied by tachycardia, intense skin flushing, a rise in skin temperature, and a reflexly-mediated release of both NE and Epi. Local infusion of this peptide causes marked dilation of human circumflex, proximal, mid, and distal anterior descending coronary arteries (McEwan *et al.*, 1986). In anesthetized rabbits, intravenous hαCGRP elevates regional blood flow in the stomach, duodenum, heart, brain, and skeletal muscle blood vessels, while hβCGRP only affects the stomach and pancreas (Bauerfeind *et al.*, 1989). In conscious rats, systemic administration of CGRP results in a pronounced increase in blood flow in mesenteric, renal, and hinderquarter skeletal muscle blood vessels. Branchial artery infusion of CGRP increases human forearm blood flow with a longer duration of action than substance P. This is accompanied by dilation of the arteries but not the veins (McEwan *et al.*, 1988).

CGRP has also been reported to relax *in vitro* the vasculature of rat and bovine aorta (Grace *et al.*, 1987; Vallotton *et al.*, 1989; Fiscus *et al.*, 1991; Wang *et al.*, 1991; Gray and Marshall, 1992), pulmonary arteries, and veins of human and guinea pig (McCormack *et al.*, 1989; Maggi *et al.*, 1990a), coronary arteries of the pig, sheep, cow, dog and human (Greenberg *et al.*, 1987; Shoji *et al.*, 1987; Beny *et al.*, 1989; Foulkes *et al.*, 1991; Prieto *et al.*, 1991), cerebral arteries of human, cat and pig (Hanko *et al.*, 1985; Mejia *et al.*, 1988; Saito *et al.*, 1989), mesenteric arteries of guinea pig and human (Marshall *et al.*, 1988; Edvinsson *et al.*, 1989), rat tail artery (Kline and Pang, 1988), hepatic arteries (Brizzolara and Burnstock, 1991), visceral arteries (Bauerfeind *et al.*, 1989; Persson *et al.*, 1991), and pig splenic and skeletal muscle arteries (Pernow, 1989).

B. Visceral musculature

In the isolated vas deferens, CGRP inhibits the electrically-induced twitch response in a dose-dependent manner, and $hCGRP_{8-37}$ antagonizes the action of CGRP (Dennis *et al.*, 1990). CGRP causes relaxation of mouse distal colon which is not blocked by the classical pharmacological antagonists (Cadieux *et al.*, 1990). In rabbit distal colon, CGRP induces a TTX-resistant transient inhibition followed by a stimulation of the longitudinal muscle and a TTX-resistant inhibition of circular muscle (Mayer *et al.*, 1990). CGRP produces sustained relaxation of longitudinal muscle from the rat fundus and guinea pig gastric corpus. The action is unaffected

by TTX, adrenoceptor and purinergic receptor antagonists, somatostatin, apamin and Tyr^0-rat α-$CGRP_{28-37}$ (Katsoulis and Conlon, 1989; Maton *et al.*, 1988). The actions of CGRP on the motility of the intestine will be discussed in a later section.

C. Mechanisms of actions on smooth muscle

1. Involvement of endothelium

Involvement of the endothelium in the vasodilating action of CGRP is a tissue-specific event. CGRP exerts an endothelium-dependent relaxation of the agonist-precontracted vascular muscle of rat and bovine aorta (Grace *et al.*, 1987; Vallotton *et al.*, 1989; Fiscus *et al.*, 1991; Wang *et al.*, 1991; Gray and Marshall, 1992) and some of the coronary arteries (Prieto *et al.*, 1991). The endothelium, however, is not involoved in the actions of CGRP on other coronary arteries (Greenberg *et al.*, 1987; Shoji *et al.*, 1987; Beny *et al.*, 1989; Foulkes *et al.*, 1991; Prieto *et al.*, 1991), cerebral (Hanko *et al.*, 1985; Mejia *et al.*, 1988; Saito *et al.*, 1989), hepatic arteries (Brizzolara *et al.*, 1991), visceral arteries (Persson *et al.*, 1991) and pig splenic and skeletal muscle arteries (Pernow, 1989). Involvement of the endothelium in the CGRP-induced relaxation is observed only in some rat coronary arteries. CGRP induced relaxation of the proximal epicardial artery is dependent on the endothelium, and relaxation of the distal intramyocardial artery is not (Prieto *et al.*, 1991). A systemic study carried by Hughes *et al.* (1988) showed that the relaxing effect of CGRP depends on the site and size of the human vasculature. They found that

CGRP relaxes conduit arteries (internal diameter > 1 mm: splenic, superior thyroid, colic, gastric, brachial, radial, femoral, renal, pulmonary, uterine and coronary arteries) in an endothelium-dependent manner, while in resistance arteries (internal diameter between 100- 400 μm: omental and subcutaneous arteries) the peptide acts in an endothelium-independent fashion. It seems, therefore, that CGRP relaxes the vasculature in a size-dependent manner.

2. Receptor on the smooth muscle

By using autoradiographic techniques, specific CGRP binding sites have been identified in both the intima and media of vessels (see details in "Receptor" section). Receptors on cultured rat aortic smooth muscle cells and bovine aortic endothelial cells show very low affinity (120 and 260 nM; Hirata *et al.*, 1988), while those on the coronary artery display picomolar affinity (6 pM, Sano *et al.*, 1989) Both high and low affinity binding sites are found on the gastric muscle cells (with a Kd of 3 and 3700 nM respectively). The studies of CGRP receptors on smooth muscle are far less extensive than those on other tissues.

3. Proposed mechanisms of actions of CGRP

Cyclic nucleotides in the muscle cells may be involved in the action of the CGRP on the vasculature.

CGRP relaxes aorta in an endothelium dependent manner. In muscle strips

of aorta, CGRP is reported to increases the cAMP of cultured bovine endothelial cells (Crossman *et al.*, 1990) and to stimulate the production of both cAMP and cGMP in aorta strips (Fiscus *et al.*, 1991) in a endothelium- dependent manner. It appears that CGRP acts on the endothelium of the aorta and releases endothelium derived relaxing factor (EDRF) which stimulates the formation of cyclic nucleotides, resulting in relaxation of the muscle. Others report that CGRP can stimulate the formation of cAMP but not cGMP (Grace *et al.*, 1987; Wang *et al.*, 1991). Cyclic AMP, but not cGMP, is increased in co-cultures of bovine endothelium and muscle cells (Crossman *et al.*, 1990). However, CGRP receptors have also been found to be present on smooth muscle cells (Hirata *et al.*, 1988), and this peptide increases cAMP in cultured rat and bovine aortic muscle cells (Kubota *et al.*, 1985, Vallotton *et al.*, 1989; Crossman *et al.*, 1990). Because the CGRP-induced relaxation of both rat and bovine aorta is totally endothelium-dependent (Grace *et al.*, 1987; Vallotton *et al.*, 1989; Fiscus *et al.*, 1991; Wang *et al.*, 1991; Gray and Marshall, 1992), the following questions then arise: a) is the cAMP-elevating effect of CGRP the result of action on smooth muscle cells or endothelial cells or both; b) what is the physiological role of the receptors on the smooth muscle cells; c) is cAMP the mediator of the relaxing effect of CGRP. Further study is needed to elucidate the pathway leading to the increase in cAMP in smooth muscle cells and also to determine the unequivocal mediator of the action of CGRP. CGRP causes a significant increase in cAMP but not in cGMP in porcine coronary arteries and relaxes the muscle in a strip without

endothelium (Shoji *et al.,* 1987). CGRP also increases the cAMP level in the gastric muscle cells with an EC_{50} of 0.4 nM which is close to the EC_{50} of 0.1 nM for inhibition of carbachol-induced contraction by CGRP (Maton *et al.,* 1988).

Nelson *et al.* (1990) reported that CGRP activates the ATP-sensitive potassium channels (K_{ATP}) in muscle cell membranes from the rabbit mesenteric artery, and suggested that activation of K_{ATP} channels underlies a substantial part of the vasodilation produced by this peptide. However, this does not appear to be the case in the rat coronary arteries, where the K_{ATP} channel blocker, glibenclamide shows no effect on the actions of CGRP (Prieto *et al.,* 1991). It has also been reported that CGRP produces hyperpolarization of smooth muscle cells (Saito *et al.,* 1989).

III. CGRP AND THE INTESTINE

A. Structure of the small intestine

1. General structure and enteric nervous system

The wall of the small intestine is composed of muscle layers, the enteric nervous system and the mucosa (Fig. 1-3). Two muscle layers are present in the wall. They are the longitudinal muscle layer located at the outside of the wall and the circular muscle layer located at the inside of the wall. The enteric nervous system (ENS) is a complex neuronal network embedded in the wall of the digestive tube and composed of a collection of intrinsic neurons (enteric neurons) and processes of both

afferent (spinal and cranial sensory neurons) and efferent (sympathetic and parasympathetic) extrinsic neurons. The myenteric (Auerbach's) plexus is located between the longitudinal and circular muscle layers, and the submucous plexus lies beneath the circular muscle layer. These are the two main plexuses of the enteric nervous system. The ganglia are connected by small bundles of nerve fibers (internodal strands) from these two ganglionated plexuses, and smaller bundles of nerve fibers emerge to form non-ganglionated plexuses in the longitudinal muscle, in the circular muscle, around blood vessels, in the muscularis mucosae, around the bases of the mucosal glands, and within the cores of the villi (Gabella, 1981). The functions of the GI tract, such as transport, secretion, motility and blood flow are under the control of the enteric nervous system. The number of enteric neurons present along the whole digestive tract is of the same magnitude as the number of neurons present in the spinal cord of any given mammalian species (Furness and Costa, 1980).

2. *Types of preparations used in studying the motor activity*

There are three types of preparations of the intestine which are usually used in tension studies. They are the segment of the intestine, the innervated strips (Kilbinger, 1982) and the plexus-free strips (Paton and Aboo Zar, 1968). The segment is an intact piece of the intestine of full thickness, and can be used to measure the tension changes in both longitudinal and circular directions. Innervated

strips are the longitudinal muscle with the attached myenteric plexus. Compared to the segment, the innervated strip is: 1) a relatively simpler model with only one nerve plexus. This preparation is free of nerves extrinsic to the intestine and neurons originating in submucosal plexuses or in other sections of the intestine; 2) very thin and drugs which are added to this preparation do not face large diffusion distances as in the segment. Although these two preparations are useful in studying mechanisms of peripheral cholinergic transmission and its modification by drugs, a preparation ideal for the study of direct effects of drugs is needed. The plexus-free lm preparation is a preparation of this type. This preparation is very thin and, to some extent, transparent and has few cell types other than smooth muscle cells.

B. Detailed CGRP-fiber innervation

1. Source and types of CGRP

All CGRP-containing nerve fibers are of extrinsic origin in the gastrointestinal tract except in the intestine (Mulderry *et al.,* 1988). In this region they are afferent sensory neurons and enteric neurons.

The vast majority of CGRP-containing afferent neurons supplying the stomach, proximal duodenum, and pancreas are located in the dorsal root ganglia at the middle and lower thoracic and the upper lumbar levels. Only a small portion are derived from vagal CGRP containing neurons (Sternini and Anderson, 1992). Submaximal relaxation of the histamine-precontracted guinea pig ileum lm evoked

by capsaicin is reversed by hCGRP(8-37) (Barthó *et al.*, 1991). Anti-CGRP serum inhibits the twitch depression induced by capsaicin, and also blocks the effects of exogenous CGRP in guinea pig ileum (Maggi *et al.*, 1988b). Capsaicin produces a concentration-related relaxation of the longitudinal muscle of the rat isolated duodenum, which is reduced markedly by CGRP desensitization (Maggi *et al.*, 1986). The action of capsaicin is completely prevented by removal of the coeliac ganglia plus the superior mesenteric ganglion (Maggi *et al.*, 1986). These reports indicate that capsaicin releases endogenous CGRP from CGRP-containing nerves extrinsic to intestine. In the small intestine of capsaicin-treated rats, there is still a large amount of CGRP remaining and the CGRP is present in the enteric neurons (Mulderry *et al.*, 1988).

The extrinsic sensory neurons and enteric neurons seem to express different forms of CGRP. CGRP-IR cell bodies are found in the plexuses of small intestine in mammalian species, including the rat, guinea pig and mouse (Ohtani *et al.*, 1989), myenteric and submucosal ganglia of the rat jejunum (Ekblad *et al.*, 1987), and duodenum (Lee *et al.*, 1987). βCGRP is produced by enteric neurons, and αCGRP may be produced by neurons extrinsic to the intestine (Mulderry *et al.*, 1988; Sternini and Anderson, 1992). In addition, CGRP is also found in mucosal endocrine cells of the human small intestine (Timmermans *et al.*, 1992).

2. Distribution of nerve fibers

In the rat jejunum, CGRP-containing nerve fibers emanating from the myenteric ganglia terminate on other myenteric ganglia and in the smooth muscle layers, and those emanating from the submucous ganglia terminate mainly on other submucous ganglia and in the mucosa (Ekblad *et al.,* 1987). The long processes of the myenteric cells also run through the circular muscle layer to the submucosa and mucosa of the guinea pig small intestine (Furness *et al.,* 1985). There is a very dense meshwork of CGRP containing fibers in the circular and longitudinal muscle layers and in the myenteric and submucous plexuses of rat duodenum (Lee *et al.,* 1987). There is a small proportion of CGRP containing fibers in the circular and longitudinal outer smooth muscle in the human small intestine. The majority of the CGRP-IR nerve fibers run within the ganglionic plexus (Timmermans *et al.,* 1992). In the guinea pig small intestine, in addition to the fibers in the submucosa and mucosa, many of the labelled nerve fibers are around blood vessels, especially the arterioles. In the inner circular muscle layer, the immunoreactive nerve processes are found in close association (sometimes less than 40 nm) to smooth muscle cells. CGRP-positive terminals contain a predominance of electron-lucent synaptic vesicles (35-40 nm in diameter) together with a few large granular vesicles (80-120 nm in diameter). CGRP-IR is localized in the large granular vesicles, 80-92 nm in diameter. (Feher *et al.,* 1986). Dense and regular networks of CGRP fibers are also found in the smooth muscle layers of the small intestine of mammals other than the guinea

pig, and also that of some non-mammals (Ohtani *et al.,* 1989). CGRP containing fibers are also distributed beneath the epithelium and associated with smooth muscle fibers of the canine ileum (Ichikawa *et al.,* 1991).

C. Actions of CGRP on the intestine

1. CGRP receptors

CGRP receptors or binding sites have been studied mostly in GI tract tissues other than small intestine (Maton *et al.,* 1988). The only investigation of CGRP binding sites in the small intestine to date was conducted with canine small intestine using autoradiography. CGRP binding sites were present on the neurons in both the myenteric and submucous plexus and also in arterioles. No binding sites were found on smooth muscle cells (Gates *et al.,* 1989). There have been no reports on the biological actions of CGRP on canine small intestine.

All these observations confirm that CGRP-containing cell bodies and nerve fibers exist in the small intestine and suggest that they may participate in the regulation of the smooth muscle activity, mucosal cell secretion and blood flow. By analogy with other systems, a sensory role also seems likely.

2. Actions on circular motility

Holzer *et al.* (1989) reported that CGRP induces phasic contractions in the ileum and the jejunum of the guinea pig. These contractions induced by CGRP are

abolished by TTX and atropine and partially inhibited by hexamethonium. CGRP appears to activate neuronal pathways including the cholinergic motor neurons. Cholinergic interneurons also partially participate in the pathway. CGRP also inhibited TTX-insensitive spontaneous circular contractions of the guinea pig ileum (Barthó, 1991).

3. Actions on longitudinal motility

a. Effects on segment preparation

Studies by various laboratories of the longitudinal tension of the guinea pig ileum have produced inconsistent results. Tippins *et al.* (1984) reported that h- and rCGRP causes a dose-dependent contraction of the guinea pig ileum which is antagonized by an antihistamine, mepyramine, and an anticholinergic compound, hyoscine. However, Barthó *et al.* (1987a) observed that CGRP induces a small and transient contraction which disappears in the presence of atropine alone, and a relaxation of non-stimulated guinea pig ileum. CGRP also shows a potentiation followed by depression of electrical field stimulation (EFS) -induced twitches. CGRP also inhibits histamine and bethanechol-induced contraction in the segment (Barthó *et al.*, 1987a). rCGRP induces a transient relaxation of rat duodenum. The segment shows a rapid desensitization to repeated addition of CGRP in terms of both amplitude and duration of the effect. The induced relaxation is unaffected by hexamethonium and partially reduced by tetrodotoxin (TTX) and ATP-desensitization (Maggi *et al.*, 1986).

rCGRP not only inhibits the spontaneous and EFS-evoked [illegible]action of the guinea pig ileum and duodenum, but also decreases the frequency of the action potentials of the longitudinal smooth muscle cells without changing the resting membrane potential (Ohkawa, 1989). CGRP has a weak and inconsistent relaxant effect on human jejunum (Maggi *et al.*, 1988a).

b. Effects on innervated strips

Maggi *et al.* (198b) showed that in the guinea pig ileum rCGRP causes a small potentiation of the twitches followed by a depression of the twitches. This is almost abolished or markedly reduced by anti-CGRP serum. In the lm strips of the human ileum, CGRP inhibits the cholinergically mediated twitch response, whereas the effect of CGRP on the twitch response in human jejunal strips is inconstant (Maggi *et al.*, 1989b).

4. Actions on enteric reflex

Peristalsis of the guinea pig ileum is inhibited by CGRP (Holzer *et al.*, 1989). The inhibitory effect is seen as a rise in the intraluminal pressure threshold of the peristaltic waves in the guinea pig ileum. CGRP inhibits both the hexamethonium- and atropine-resistant pathways of the ascending enteric reflex while it leaves the main cholinergic pathway unaffected.

5. Actions on myenteric neurons

CGRP evokes a long-lasting depolarization of the cell membranes that is dose-dependent (ED_{50} = 50 nM). This depolarization is associated with an increase in the input resistance, suppression of post-spike hyperpolarizing potentials, and enhanced excitability in all AH/type 2 neurons tested. These results are consistent with a neurotransmitter or neuromodulator role for CGRP in the enteric nervous system, and suggest that CGRP may participate in local neurohumoral regulation of gastrointestinal effector systems (Palmer *et al.*, 1986).

IV. OBJECTIVES OF THIS THESIS

The presence of CGRP-IR cell bodies and nerve fibers in the small intestine suggests that this peptide may play some role in the functions of this tissue, including motility of both longitudinal and circular smooth muscle. The present study focusses on the effects of CGRP on the longitudinal smooth muscle so that the results may not apply to circular smooth muscle. Conflicting observations about effects of CGRP on the motility of longitudinal muscle have, as mentioned above, been made by other investigators in studies using the whole segment of the intestine. Actions of CGRP must be investigated in a simpler model. The lm-mp preparation is ideal for this kind of investigation. In order to study the direct effects of CGRP on smooth muscle it is preferable to use the plexus-free muscle. The objectives of this work were as follows:

1. To study the actions of CGRP on the lm-mp preparation and to determine

the sites of action.

2. To study the direct effects of the peptide on the lm.

3. To elucidate the subcellular mechanisms involved in the effect of the peptide on the smooth muscle.

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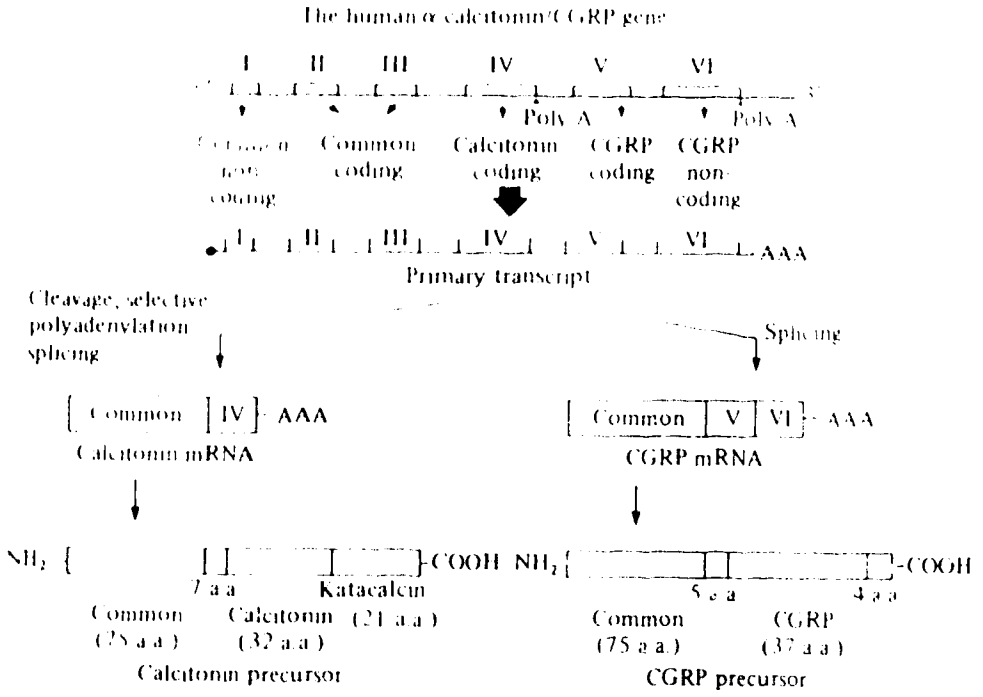

Figure 1-1. Organization of the human calcitonin/αcalcitonin gene-related peptide gene. (From Breimer LH, MacIntyre I and Zaidi M. 1988: Biochem J 255: 377. Reproduced by permission from Dr. M. Zaidi and the Physiological Society Administration and Publications Office.)

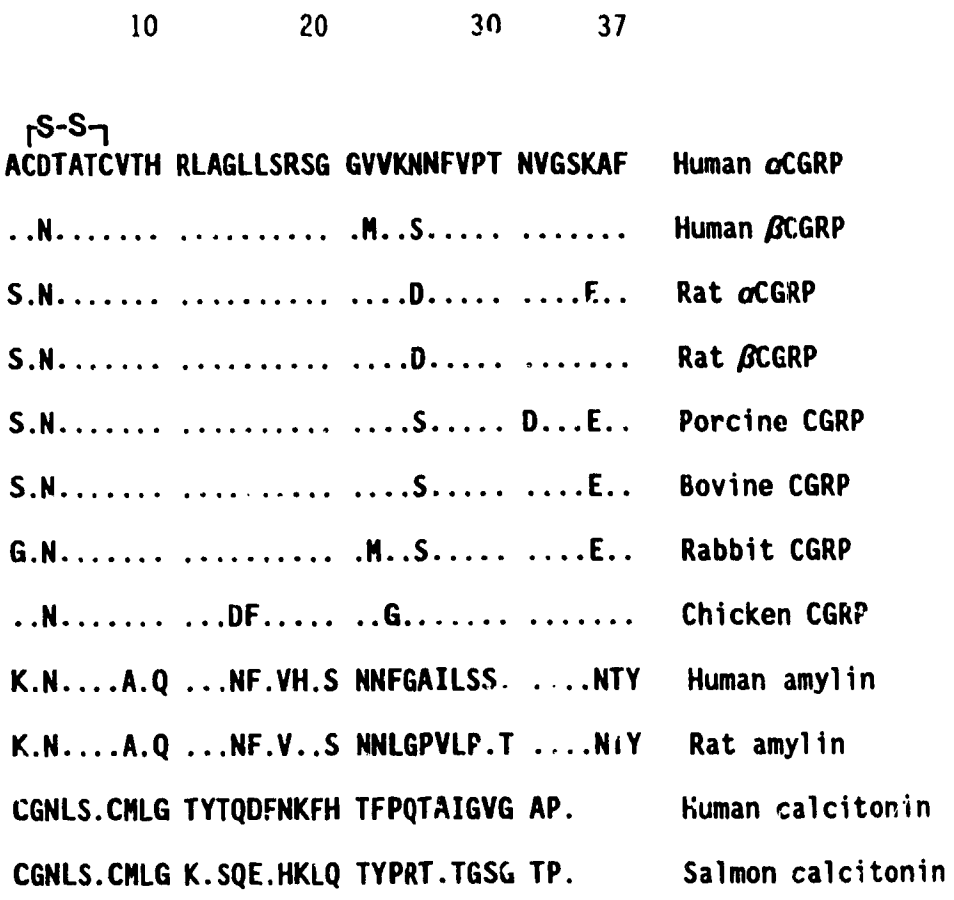

Figure 1-2. Amino acid sequences of calcitonin, calcitonin gene-related peptide and amylin of different species. Single letter designations of the amino acids are used.

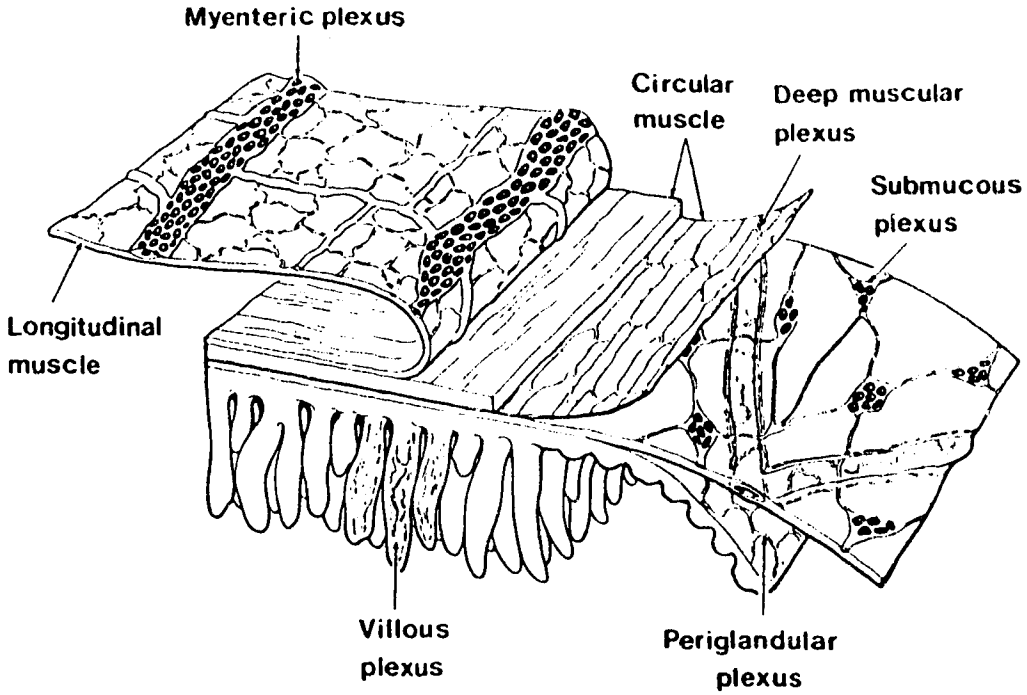

Figure 1-3. The wall of the small intestine (From Cost H, Furness JB and Llewellyn-Smith IJ. 1987: Histochemistry of the enteric nervous system. In: "Physiology of the Gastrointestinal Tract", 2nd Ed. Johnson L, ed. Raven Press, New York. pp. 1. Reproduced by permission from Raven Press Ltd.).

CHAPTER 2. ACTIONS AND ACTION SITES OF CGRP ON THE LONGITUDINAL MUSCLE-MYENTERIC PLEXUS

I. INTRODUCTION

Histochemical study has revealed the innervation of the small intestine by CGRP-IR fibers from both capsaicin-sensitive extrinsic sensory neurons and capsaicin-insensitive enteric neurons (Sternini *et al.*, 1987). The innervation of the small intestine and also the presence of specific CGRP binding sites on the enteric neurons indicate the possible physiological role of this peptide in the regulation of the functions of this organ. In fact, CGRP has been reported to modulate the motility of the intestine (Tippins *et al.*, 1984; Maggi *et al.*, 1986; Barthó *et al.*, 1987). Experimental data from the whole segment of the small intestine have been inconsistent. This may be due to the complex anatomical interactions between nerves and muscle in this preparation. In addition, the sites of action have yet to be clarified. Therefore a simpler preparation, the longitudinal muscle-myenteric plexus (lm-mp) of the guinea pig ileum, was used in the present study to characterize the actions and sites of actions of CGRP. Lm-mp strips can be made from the intestines of several common laboratory animals: guinea pig, rabbit, mouse, cat etc. Lm-mp strips from the guinea pig possess a high content of acetylcholine (ACh) and a good correlation between inhibition of evoked contraction and inhibition of evoked ACh release. For these reasons, guinea pig lm-mp strips have been used to a greater extent in studies

on cholinergic mechanisms than have preparations from other species.

II. MATERIALS AND METHODS

A. Tissue preparation

Male guinea pigs weighing from 300 to 500 g were anesthetized by pentobarbital (approximately 50 mg/kg, i.p.). Larger animals were used in experiments on the effects of rCGRP on the lm-mp because there was a greater variability in the response to rCGRP of the preparation from younger guinea pigs than those from older guinea pigs. The preparation was made according to the method described by Kilbinger (1982). A 4 cm segment of the ileum from a region about 15 cm proximal to the caecum was quickly removed and placed in preoxygenated Krebs solution (composition in millimolar: NaCl, 117; KCl, 4.7; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 11, bubbled with 95 O_2-5% CO_2). To isolate the lm-mp, the contents of the intestine were removed by rinsing the inside with Krebs solution and then the segment was drawn over a horizontal 1 ml glass or plastic pipette. Incisions were made gently along the length of the intestine, one on each side of the mesenteric attachment. With a damp wisp of cotton wool the longitudinal muscle with attached myenteric plexus was separated from the underlying circular muscle by stroking away either side from its mesenteric attachment. The tissue was kept moist throughout the procedure. The strip was suspended in a Sawyer-Bartlestone chamber filled with 10 ml of continuously oxygenated Krebs solution. One end of the strip was

fixed at the bottom of the chamber and the other end attached to a force displacement transducer (Grass FT .03) which was connected to a polygraph (Grass model 79D). Each strip was equilibrated under 0.7 g of initial tension for 1 hr before starting the experimental protocols. The solution in the chamber was replaced every 15 min. The total volume of the drugs added was no more than 0.2 ml, i.e. 2% of the chamber volume.

B. Tension study on non-precontracted tissue

When the lm-mp was challenged with a single dose of rCGRP, the responsiveness of the strip recovered only after a long period of incubation in rCGRP-free Krebs solution (Fig. 2-1). Therefore, the effect of single and cumulative doses of rCGRP on non-precontracted lm-mp, as well as the effects of TTX, atropine, 2-chloroadenosine and hexamethonium on rCGRP-induced responses were each observed in different pieces of lm-mp from the same segment of ileum. A concentration of 1 μM atropine was used, since this concentration was found to abolish the electrically induced contraction (Fig. 2-9). A concentration of 1 μM TTX was selected, because this concentration had been observed to block nerve conduction (data not shown; Narahashi, 1972). Concentrations of 1 μM clonidine and 2-chloroadenosine were selected because these had previously been shown to inhibit electrically induced contractions (Benishin *et al.*, 1986; Fig. 2-9).

C. Study on stimulated tissue

1. DMPP-stimulated tissue

Dimethylphenylpiperazinium (DMPP, 5 μM) produced a transient contraction: 0.88$\pm$0.13, 1.14$\pm$0.15 and 1.15$\pm$0.12 g, respectively, for the first, second and third applications (n=7). DMPP was washed out after each challenge and 20 min elapsed between challenges. The second and third challenges induced consistent contractions, but the first stimulation was consistently smaller. Therefore, the effects of rCGRP on the DMPP-evoked contractions were determined at the second (DMPP only) and third (DMPP and rCGRP) exposures to DMPP.

2. Electrically driven tissue

a) Constant contractions were elicited by maximal electrical field stimulation (EFS) (9 V/cm, 0.1 Hz and 1 msec) and then rCGRP was added cumulatively.

b) In some experiments, rCGRP was added to the bath 2 min before the onset of electrical stimulation (2.5 V/cm to 9 V/cm).

D. Materials

Drugs used were: 1,1-dimethyl-4-phenylpiprazinium iodide, hexamethonium, clonidine, 2-chloroadenosine and tetrodotoxin (Sigma Chemical Co., USA) and rat αCGRP (Bachem Inc., Essex UK). Instruments used were: Grass force-displacement transducer FT03 and Grass SD9B stimulator (Grass instrument Co., USA) and Gould

polygraph recorder (Gould Inc., USA).

E. Statistics

All the data in this chapter are presented as mean ± S.E.. Student's *t*-test was used for comparison of paired or unpaired values. Newman-Keuls test and Duncan Multiple Range test were used for those comparisons with a common control. The level of statistical significance was accepted as $P<0.05$.

III. RESULTS

A. Non-precontracted lm-mp

Following an initial challenge of the tissue with CGRP, a long period of incubation in rCGRP-free Krebs solution was needed for the recovery of the responsiveness of the lm-mp to a second challenge with rCGRP. In non-stimulated tissue, a single dose of rCGRP (263 nM) was applied twice to the same piece of lm-mp with different intervals. As seen in Fig. 2-1, the relaxing response of the tissue to rCGRP did not recover completely until 30 min, and the contractile response did not recover until 60 min after the first dose.

The myenteric plexus contains a high level of ACh which is the major stimulatory neurotransmitter of intestinal smooth muscles including the longitudinal muscle (Kilbinger, 1982). Other stimulatory and inhibitory neurotransmitters or modulators of smooth muscle are also present in this plexus (Dockray, 1988). Changes in tension

of the longitudinal muscle reflect the sum of the actions of CGRP on the smooth muscle and indirect actions of this peptide through neuronal pathways. rCGRP induced a transient contraction and then a long-lasting relaxation of non-stimulated lm-mp (Fig. 2-2a). The contractile phase lasted for approximately 1 min with a peak contraction at 10 sec, whereas the relaxing effect had a peak action at 3 min. A sustained relaxation then lasted throughout the duration of the experiment (20 min). Both the contraction and relaxation induced by rCGRP were dose dependent within the range of 2.6×10^{-10} to 7.9×10^{-6} M (Fig. 2-2b, n=5). The threshold concentration was 0.26 nM which produced an increase in tension of 47 ± 11 mg and a decrease in tension of 15 ± 12 mg, respectively. The contractile effect increased markedly as the concentration of rCGRP increased (to about 0.9 g at 7.9 μM of rCGRP). The magnitude of the relaxation was much less than that of the contraction (to about 0.2 g at 7.9 μM) because of the small basal tension. The maximal contractile effect was not determined due to the limited availability of peptide, and, therefore, the ED_{50} of this peptide could not be calculated. An ED_{50} for the contractile effect larger than 0.5 μM is expected according to this study.

rCGRP may exert its biphasic actions on the muscle through neuronal pathways, i.e. this peptide may excite cell bodies of enteric neurons, evoking the release of some stimulatory or inhibitory substances, and finally influencing the tension of the muscle. TTX was previously reported (Narahashi, 1972) to block nerve conduction. In order to test this possibility, the effect of TTX on the actions of rCGRP on

the non-stimulated tissue was examined. TTX (1 μM) abolished the contractile effect induced by this peptide at a concentration of 263 nM. The relaxing effect of rCGRP was also significantly reduced by around 50% (Fig. 2-3, n=7) and the time of maximal relaxation was shifted from 3 minutes in the control to 1 minute in TTX-treated strip. It appears that a neuronal mechanism is involved in the actions of rCGRP.

ACh, the major stimulatory neurotransmitter in the enteric nervous system, contracts the longitudinal muscle by activation of muscarinic receptors on the muscle (Birdsall *et al.*, 1986). Atropine is a non-selective muscarinic receptor antagonist and can block the action of ACh released from the neurons, as in Fig. 2-9. In the presence of 1 μM of atropine, the relaxing effect of rCGRP was unaffected. However, the contractile effect of the peptide was markedly reduced but not abolished (Fig. 2-4, n=7). In the atropine-treated tissue, rCGRP induced an initial relaxation followed by a return of the tension toward base line, then a long-lasting relaxation, as if a small contractile component were superposed on the relaxation phase. This small contractile component was not observed in the TTX-treated tissue. These results suggest that rCGRP may contract the muscle by stimulating the release of ACh and another stimulatory substance from enteric neurons.

Neurotransmitter release at the nerve ending can be regulated via presynaptic mechanisms. Activation of presynaptic receptors, e.g. muscarinic receptors, purinergic receptors and adrenergic receptors leads to inhibition of the release (Drew, 1978;

Andrejak *et al.*, 1980; Paton, 1981; Moody and Burnstock, 1982; Kilbinger, 1984; Schwartz *et al.*, 1990; Menkveld and Timmerman, 1990; Poli *et al.*, 1991). Clonidine, a presynaptic α_2 adrenoceptor agonist, and 2-chloroadenosine, a presynaptic P_1 purinergic receptor agonist, have been reported to inhibit EFS-induced contractions of the guinea pig ileum with IC_{50} values of 45 and 65 nM, respectively (Benishin *et al.*, 1986). To further confirm the possible ACh-releasing effect of rCGRP, we examined the influence of 2-chloroadenosine and clonidine on rCGRP-induced biphasic actions on the muscle.

2-Chloroadenosine at a concentration of 1 μM abolished the contractile effect and also significantly diminished the relaxing effect of rCGRP (Fig. 2-5, n=6). The peak relaxation was shifted from 3 min in the control strip, to 40 sec.

Clonidine (1 μM) showed a similar effect to 2-chloroadenosine on rCGRP-induced contraction and relaxation. In the presence of 1 μM of clonidine, rCGRP induced an immediate small contraction of 9 ± 29 mg, which was not significantly different from the basal tension, followed by significantly reduced relaxation (Fig. 2-6, n=7). There was no delayed small contractile component as in the atropine-treated strip in either the 2-chloroadenosine or the clonidine-treated tissues.

The rCGRP-induced relaxation was depressed by TTX, 2-chloroadenosine and clonidine. In order to determine whether the effects of TTX and presynaptic modulators were additive, the influence of a combination of TTX and clonidine or 2-chloroadenosine on the actions of rCGRP were examined. TTX did not significantly

modify the effect of 2-chloroadenosine; it, however, showed a tendency to reduce the effect of this compound on rCGRP-induced relaxation (Fig. 2-5). Although there was a trend toward further inhibition of the effects of the peptide in the presence of the combination of TTX and clonidine compared with that in the presence of TTX or clonidine alone, the difference was not significant (Fig. 2-6). There were no significant differences among rCGRP-induced relaxations in the presence of TTX and 2-chloroadenosine or clonidine.

TTX, clonidine, 2-chloroadenosine and atropine all decreased the basal tension of the non-stimulated lm-mp. After an initial drop, the tension returned to a constant level below the basal level. The lm-mp of the guinea pig ileum under resting condition releases ACh partially in response to the propagated neuronal activity which is sensitive to the action potential conduction blocker, TTX (Paton *et al.*, 1971). Therefore the decrease of basal tension of non-stimulated lm-mp produced by those compounds in the present study could be due to the inhibition of the release of ACh under resting conditions. Partial rebound of the tension after an initial drop could be caused by negative feedback regulation of the release at nerve endings in the case of TTX, or through a unknown myogenic mechanism.

To test the possibility that rCGRP may excite the motor neurons via the interneurons which use ACh as a neurotransmitter, the effect of blockade of the ganglionic nicotinic cholinergic receptor with hexamethonium (Trčka, 1980) on the action of rCGRP was determined. Hexamethonium (1 μM) had no effect on the rCGRP-

induced contraction or relaxation (Fig. 2-7).

B. DMPP-stimulated tissue

In order to determine whether rCGRP might affect the tension of the muscle by altering ganglionic transmission, the effects of rCGRP on the contraction induced by DMPP, a ganglionic nicotinic receptor agonist were examined (Volle, 1980). DMPP at a concentration of 5 μM induced a transient contraction lasting 6.7 ± 0.4 min (n=3). rCGRP had no significant effect on the DMPP-evoked contraction in a concentration range of 0.26 to 7.9 μM (Fig. 2-8).

C. Electrical field-stimulated tissue

EFS excites different types of neurons to release neurotransmitters, affecting the tension of muscle (Paton and Aboo Zar, 1968). This action is dependent on the field strength. Generation and conduction of action potentials seems essential because TTX inhibits the release of ACh from enteric neurons (Kilbinger and Wessler, 1980). The preparation responded to EFS starting from 2.5 V/cm. Maximal contractions were obtained at stimulation strengths equal to or greater than 9V/cm and the induced twitch was sensitive to inhibition by atropine (Fig. 2-9) and TTX (data not shown; see also Kilbinger and Wessler, 1980).

Maximal stimulation (9 V/cm) produced consistent twitch contractions. When rCGRP was added cumulatively to the stimulated preparation, a biphasic effect of

rCGRP was apparent: an initial potentiating and a subsequent dominant inhibitory effect (Fig. 2-10a,b). rCGRP reached its maximal inhibitory effect (to about 50% of the control values) at a concentration of 26.3 nM. No further inhibitory effect was observed at higher doses up to 263 nM. Single doses ranging from 10 nM to 132 nM showed inhibition to a similar extent (data not shown). The transient potentiating effect, however, increased with increasing doses up to 78.8 nM (120% of the control values) (Fig. 2-10b). Salmon calcitonin at a comparable concentration did not show significant effect on the twitch (Fig. 2-11).

The strength-response curve of EFS (2.5 V/cm to 9/cm) was depressed in a dose-dependent manner but the voltage needed to produce 50% of the maximal contraction was not changed by concentrations of rCGRP up to 263 nM (Fig. 2-12). Only the inhibitory effect of this peptide was observed because the stimulatory effect occurred during the first minute after the addition of the peptide and waned by the time of onset of EFS (Fig. 2-12). Similarly, rCGRP at dose of 26.3 maximally depressed the curves (about 50%) and no further inhibition was seen when higher doses of the peptide were added. This result indicates that there is no desensitization of the tissue to rCGRP occurring when this peptide is added cumulatively to the stimulated tissue.

IV. DISCUSSION

A. Non-precontracted tissue

1. Actions of rCGRP

The effects of CGRP on the motility of longitudinal muscle of intestine have been investigated by different laboratories using various regions of the intestine from the same or different species. Most of these studies were carried out with an intestinal segment of full thickness, i.e. the whole segment. The whole segment preparation contains both the longitudinal and circular muscle layers and all the plexuses are present. Simultaneous non-selective exposure of the neurons in these plexuses, as well as the endocrine cells, to an application of exogenous CGRP, could lead to much more complicated interactions among neurons and between neurons and muscle than would be seen in lm-mp preparations. The results obtained in the segment can be, therefore, more difficult to interpret. In the present study, the effects of rCGRP have been examined in a preparation free of influences extrinsic to myenteric plexus and longitudinal muscle in attempt to resolve some of the uncertainties about its mechanism and sites of action in the ileum.

There have been conflicting reports from different laboratories regarding the action of this peptide in the same type of preparation from the same species. CGRP seems to have different types of actions on the small intestine of different species.

The effect of CGRP on the guinea pig ileum was first reported by Tippins *et al.* (1984). By measuring the longitudinal contraction of the whole segment, they

found that both rαCGRP and hαCGRP produce a biphasic twitch which is characterized by an initial rapid contraction followed by some decay and then a slower contraction. The two peptides are about equipotent and the threshold for response of each peptide is about 1 nM, the ED_{50} value 3.5 to 5 nM, and the dose for maximal responses approximately 30 nM. The rαCGRP-induced contraction is partially inhibited by either an antihistaminergic compound, mepyramine or an anticholinergic compound, hyoscine, and is completely abolished by a combination of the two compounds. However, the hαCGRP-induced contractions could be only partially depressed by the combination. CGRP appears to act by releasing both ACh and histamine. In a similar preparation, Barthó *et al.* (1987) observed a biphasic effect on longitudinal tension induced by CGRP, i.e. an initial contraction followed by a long lasting relaxation, with a threshold concentration of 3 to 10 nM. The contraction is abolished by atropine. Therefore, results from these two groups are inconsistent, not only regarding the potency of the peptide required to induce contractions, but also in the types of actions of CGRP. The former reported only contractile effects and the latter showed both contractile and relaxing effects. The mediators for the contraction appear to be histamine and ACh in the former report and ACh in the latter report.

In the present study, the isolated lm-mp preparation was used to investigate the actions of CGRP. This preparation consists of only the outer longitudinal muscle and the attached myenteric plexus. rCGRP produced biphasic effects on the tension of this tissue, i.e. an initial transient contraction followed by a long lasting relaxation,

which is consistent with the results of Barthó *et al.* (1987).

rCGRP seems to be very potent at inducing contraction in lm-mp, with a threshold concentration of 0.26 nM (compare with 1 nM reported by Tippins *et al.*, 1984, and 3 to 10 nM reported by Barthó *et al.*, 1987). The difference in sensitivity to the peptide of these two types of preparations may reflect the differences in the fate of the peptide before it reaches the target cells. Myenteric neurons are directly exposed to the exogenous CGRP in the present experiment, while in the whole segment, the administered peptide must penetrate cell layers, e.g. muscle layers or epithelial cell layers, before it reaches the target tissues. Less of the peptide would reach the target tissue in whole segment preparation than in lm-mp. In addition, the possibility that CGRP could be degraded during its diffusion through the tissue can not be excluded.

On the other hand, the lm-mp seems to respond to the peptide over a wide range of concentrations. The ED_{50} value for the stimulatory effect of rCGRP in the present study is larger than 0.5 μM and at least 100-fold higher than that in the segment preparation. The maximal response is not reached at concentrations even as high as 7.9 μM, which is 260 times of that for the dose needed for maximal response in the whole segment preparation (Tippins *et al.*, 1984). Recently, Baidan *et al.* (1992) reported that the CGRP-induced increase in intraganglionic cAMP levels of isolated myenteric ganglia of guinea pig ileum was dose-dependent. The ED_{50} value was 0.7 μM, which is very close to the ED_{50} value in our tension study. The

mediator for the contractile phase seemed to be primarily ACh released by this peptide, since atropine greatly inhibited the contraction. Direct evidence for the ability of CGRP to release ACh has recently been obtained from primary culture of the myenteric plexus by Mulholand and Jaffer (1990). They found that CGRP (1 pM to 1 μM) produced a dose dependent increase in [^{3}H]-ACh release which may involve neuronal calcium channels. In the present study, rCGRP-induced contraction is completely abolished by TTX but not atropine. This suggests that at least two stimulatory substances, including ACh, are released from enteric neurons, and mediate rCGRP-induced contraction of lm-mp of GPI. The action of rCGRP on the present preparation, therefore, differs from that reported by Barthó and co-workers (1987) on the whole segment preparation, where ACh is the only mediator of rCGRP-induced contraction. This small atropine-insensitive contraction seems to be different from the contraction insensitive to hyoscine reported by Tippins *et al.* (1984). In our study, this component occurs after the initial relaxation phase, while in the investigation conducted by Tippins and co-workers, CGRP induced an immediate contraction after the administration of hyoscine.

The stimulatory effect of CGRP on force has also been observed on the lm-mp of guinea pig jejunum and duodenum, but not the intestine of other species (Schwörer *et al.*, 1991). They found that the stimulatory effect was abolished by TTX or scopolamine, a muscarinic receptor antagonist. However, a small relaxation was seen only in 50% (duodenum) and 64% (jejunum) of the strips tested. This may

reflect the regional differential actions of CGRP. CGRP, therefore, acts on non-stimulated lm-mp and whole segment preparations differently: a) CGRP shows both stimulatory and inhibitory mechanical effects in the lm-mp; b) the lm-mp preparation responds to a much wider range of doses of CGRP than does the segment; c) the lm-mp is more sensitive to the peptide than is the segment. In addition, there may be two stimulatory mediators involved in the contractile action of rCGRP on lm-mp.

2. *Action sites of the peptide*

CGRP-IR fibers emanating from the myenteric ganglia terminate on other myenteric ganglia as well as in the smooth muscle layers of the intestine (Furness *et al.*, 1985; Ekblad *et al.*, 1987; Lee *et al.*, 1987; Timmermans *et al.*, 1992). CGRP receptors have been found on the myenteric neurons (Gates *et al.*, 1989). Exogenous application of h- and rCGRP, but not calcitonin, excites the AH/type 2 neurons of the guinea pig ileum (Palmer *et al.*, 1986). All these findings suggest that CGRP may have actions on both neurons and muscle. TTX was reported to block nerve conduction (Narahashi, 1972). We have found that in the lm-mp preparation of the guinea pig ileum, the stimulatory effect and part of the inhibitory effect of rCGRP are blocked by 1 μM TTX. Therefore, the generation and conduction of action potentials appear to be fundamental for part of the action of rCGRP in this tissue. The actions of rCGRP on the lm-mp may, therefore, be divided into those on the enteric neurons and those on the longitudinal smooth muscle. TTX abolished the contractile effect

of rCGRP, suggesting that nerves are absolutely required for the contraction. Partial diminution of the inhibitory effect of CGRP by TTX indicates that rCGRP acts on both nerves and smooth muscle cells to exert the inhibitory effect. rCGRP induces a transient relaxation of rat duodenum segment preparation and this effect is reduced by 40% in the presence of 1 μM TTX (Maggi *et al.*, 1986). It appears that in both the guinea pig ileum and the rat duodenum, CGRP produces relaxation directly by acting on smooth muscle and indirectly by releasing inhibitory substances from enteric neurons.

The release of neurotransmitters from the nerve endings can also be regulated by presynaptic receptors. At least four types of presynaptic receptors have been found to be involved in the regulation in the guinea pig ileum: adrenergic α-2; purinergic P_1 or "R site"; muscarinic; and histaminergic H_3 receptors (Drew, 1978; Andrejak *et al.*, 1980; Paton, 1981; Kilbinger, 1984; Moody and Burnstock, 1982; Menkveld and Timmerman, 1990; Schwartz *et al.*, 1990; Poli *et al.*, 1991). Activation of these receptors inhibits the release of ACh from the nerve endings of enteric neurons. Clonidine, an agonist for the presynaptic α_2-adrenoceptor (Drew, 1978; Wikberg, 1978), and 2-chloroadenosine, a presynaptic adenosine receptor agonist have been reported to inhibit EFS-induced contractions (Benishin *et al.*, 1986) with IC_{50} values of 45 and 65 nM, respectively. The maximal EFS-induced twitch is abolished by atropine, indicating that ACh is the only stimulatory mediator involved (Fig. 2-9). 0.1 μM clonidine inhibits 87% of the induced twitches and no further inhibition is seen at concen-

trations up to 10 μM (Fig. 2-9). This result is consistent with those reported by Drew (1978) and Andrejak *et al.* (1980). There may exist two populations of cholinergic neurons in the myenteric nervous system: the clonidine-sensitive and the clonidine-insensitive neurons.

By using 2-chloroadenosine and clonidine, we further confirmed the involvement of the nerves in the actions of rCGRP on motility of the lm. rCGRP, at a dose of 263 nM, induces a much smaller contraction than does maximal EFS. Clonidine abolished the contractile effect of CGRP. It seems CGRP excites only the clonidine-sensitive cholinergic enteric neurons. This type of neuron is also sensitive to inhibition by 2-chloroadenosine. In addition, the atropine-insensitive contraction induced by rCGRP is blocked by both clonidine and 2-chloroadenosine.

Clonidine and 2-chloroadenosine, like TTX, significantly reduce the inhibitory action of CGRP, suggesting that CGRP also releases inhibitory transmitter(s) from clonidine-sensitive inhibitory myenteric neurons. ATP has been reported as a possible inhibitory neurotransmitter which may mediate the relaxing effect of CGRP on rat duodenum (Maggi *et al.*, 1986).

TTX would be expected to inhibit only part of the actions of rCGRP on the neuron; if rCGRP facilitates the release of substances from enteric neurons by direct actions on both nerve endings and sites above nerve endings of neurons innervating the lm. We found, however, that TTX, clonidine and 2-chloroadenosine all abolished rCGRP-induced contraction and there were no significant differences among the

rCGRP-induced relaxations in the presence of these three compound individually. The combination of clonidine and TTX does not show more inhibition than each of these two compounds alone. These results indicate that the populations of neurons affected by rCGRP are all sensitive to these three compounds and that rCGRP is unlikely to have a facilitating effect on release of substances from enteric neurons by acting at the nerve ending.

Hexamethonium blocks the nicotinic cholinergic receptors in the ganglion, preventing activation of the postsynaptic neuron by ACh (Trčka, 1980). Hexamethonium at concentration of 1 μM does not alter the actions of CGRP on the non-stimulated lm-mp, indicating that activation of interneurons using ACh as a neurotransmitter may not play a major role in the effect of CGRP.

B. Stimulated tissue

1. DMPP-stimulated tissue

DMPP is an agonist for nicotinic cholinergic receptors in the ganglia (Volle, 1980). DMPP activates the receptors and excites postsynaptic cholinergic neurons, resulting in release of ACh, and muscle contraction in the small intestine. The inability of CGRP to affect DMPP-induced contraction suggests that CGRP may not inhibit the motility of smooth muscle of GPI by interferring with the release of ACh from enteric neurons.

2. Electrical field stimulated preparation

Non-specific excitation of the neurons and generation of action potentials in the preparation by EFS results in release of ACh (Paton and Aboo Zar, 1968). TTX abolishes the nerve conduction, and, therefore, the ACh release induced by EFS and the resulting contraction. Atropine totally depressed the EFS-induced contraction, suggesting that, at the strength of the stimulation used in the present study, ACh is the primary stimulatory neurotransmitter released.

The effects of rCGRP on the electrically stimulated contraction and on the non-stimulated lm-mp have the same pattern of activity. CGRP causes a small and transient potentiation, followed by a predominant inhibition on EFS-induced contraction. This may result from a direct effect of the peptide on smooth muscle, as well as an effect on nerves innervating the muscle. At low concentrations of rCGRP, the stimulatory effect of this peptide is much smaller than that of the EFS-induced contraction, and may be masked by the latter. When the EFS-evoked contraction has been reduced dramatically by previous doses CGRP, the stimulatory effect of CGRP at the higher doses may be unmasked and it becomes additive to the effect of EFS.

Schwörer *et al.* (1991) examined the actions of CGRP on electrical field stimulated lm-mp of guinea pig and pig duodenum and jejunum and human duodenum and ileum. They found that CGRP exhibits both stimulatory and inhibitory effects on the strips of guinea pig intestine, while only the inhibitory effects are seen in strips

from other species. rCGRP induces a transient relaxation of rat duodenum segment (Maggi *et al.*, 1986). CGRP produces a small and inconsistent inhibition of human jejunum (Maggi *et al.*, 1989). Results from the EFS-evoked preparation together with the those from non-stimulated tissues suggest that the relaxing action of CGRP on intestinal muscle is universal while the stimulatory effect of this peptide is specific to the guinea pig intestinal preparation.

Calcitonin usually does not cross-react with the CGRP receptors in the vasculature tree, or vice versa (Kubota *et al.*, 1985; Sigrist *et al.*, 1986). Human calcitonin, up to a concentration 10 μM, is ineffective in relaxing carbachol-induced contraction, in displacing ^{125}I-CGRP binding and in promoting the production of cAMP in smooth muscle cells of the guinea pig stomach. However, rat and human CGRP have IC_{50} values for ^{125}I-CGRP displacement and relaxation, or EC_{50} values for the production of cAMP, which are in the nanomolar range (Maton *et al.*, 1988). Similarly, in the present study, salmon calcitonin, up to concentrations 260 nM, has no significant inhibitory effect on electrical field stimulated lm-mp whereas rCGRP has a threshold dose of 0.23 nM for the inhibitory effect. These results suggest that rCGRP exerts its actions on smooth muscle through the receptors specific to CGRP.

C. Summary

In summary, we may make the following conclusions: a) the responses of the lm-mp to rCGRP differ from those of the whole segment preparation; b) CGRP may

influence the mechanical activity of the longitudinal muscle through actions on both neurons and smooth muscle; c) CGRP exerts its stimulatory effect through a neuronal mechanism, and the inhibitory effect is probably mediated through both direct actions on the muscle and neuronal pathways; d) the major excitatory mediator is ACh in myenteric neurons. Unknown excitatory and inhibitory mediators are also released from the neurons; and e) CGRP may not have either facilitatory or inhibitory effects on transmitter release by acting on prejunctional nerve endings. The reason why CGRP has opposite pharmacological actions on the muscle is not known. Studies *in vitro* may not reflect the situation *in vivo* in terms of timing and regional sequence., i.e. *in vivo*, the stimulatory and inhibitory effects of CGRP may occur at different times or different regions under physiological conditions. Further study is needed to provide direct evidence of the actions of this peptide on the smooth muscle.

Based on the results obtained in the present investigation, we propose a model to describe the actions and sites of actions of rCGRP on the lm-mp of the guinea pig ileum (Fig. 2-13). rCGRP excites three populations of enteric neurons at sites above nerve endings in the myenteric plexus of guinea pig ileum. Two populations are stimulatory neurons: type I ACh-containing neuron and type II neurons which release the unknown muscle-contracting substance(s). The third population of neurons is inhibitory, which releases unknown muscle-relaxing substances. CGRP induces excitation of all these populations of neurons at sites above the nerve endings, and leads to gen-

eration and conduction of action potentials. TTX blocks the conduction of action potentials generated by rCGRP, and therefore blocks the actions of this peptide on neurons. Both 2-chloroadenosine and clonidine inhibit the release of neurotransmitters at nerve endings of these three populations of neurons. ACh and the unknown substances released from the enteric neurons act on their own receptors on smooth muscle and mediate the contractions. Atropine blocks the action of ACh at the muscarinic receptors on smooth muscle. Stimulation of the inhibitory neuron together with direct actions on the smooth muscle underlie the muscle-relaxing action of rCGRP.

Cholinergic interneurons may not play a key role in the action of rCGRP. It is still not known whether any other populations of interneurons are involved. The timing sequence of the actions of rCGRP on different populations of neurons and muscle, and regional differential distribution of those neuron populations, have not been described in this model, but may explain the apparent conflicting actions observed.

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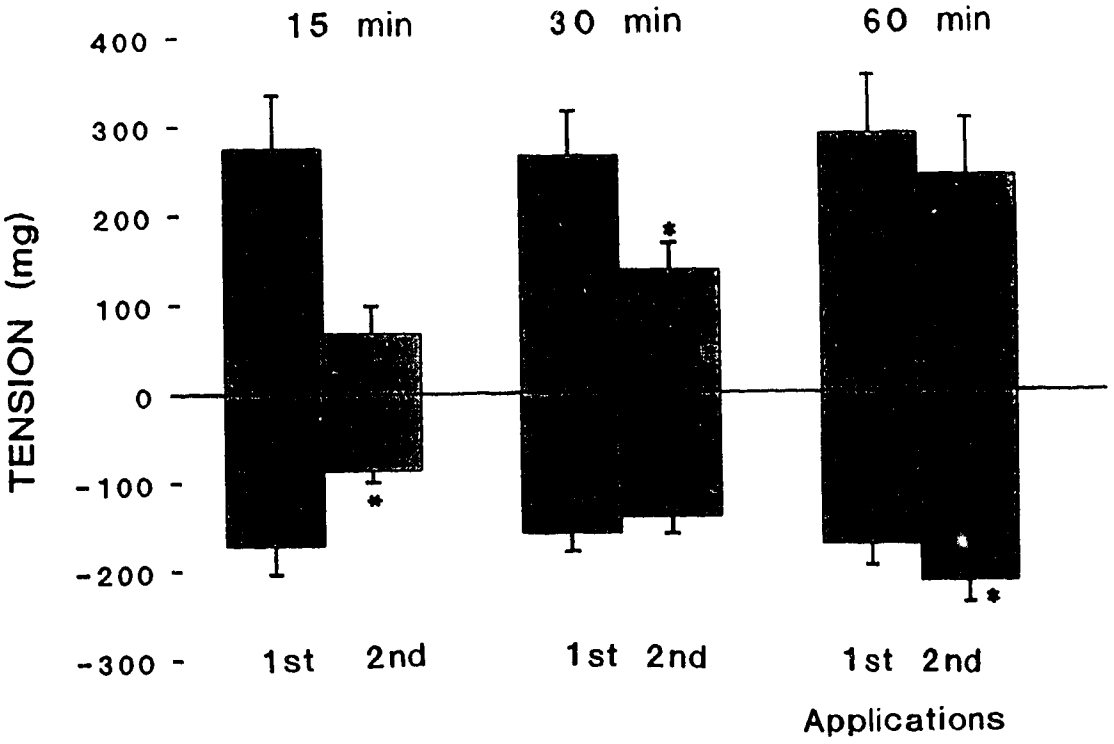

Figure 2-1. Responsiveness of lm-mp to repetitive single dose of rCGRP (263 nM) given at different time intervals. rCGRP was applied to non-stimulated lm-mp to elicit the biphasic response and then washed away. Subsequent applications of the same dose of rCGRP were added at the time intervals indicated. Data are expressed as mean $\pm$ S.E., n=8. *Significantly different from control (Student *t*-test).

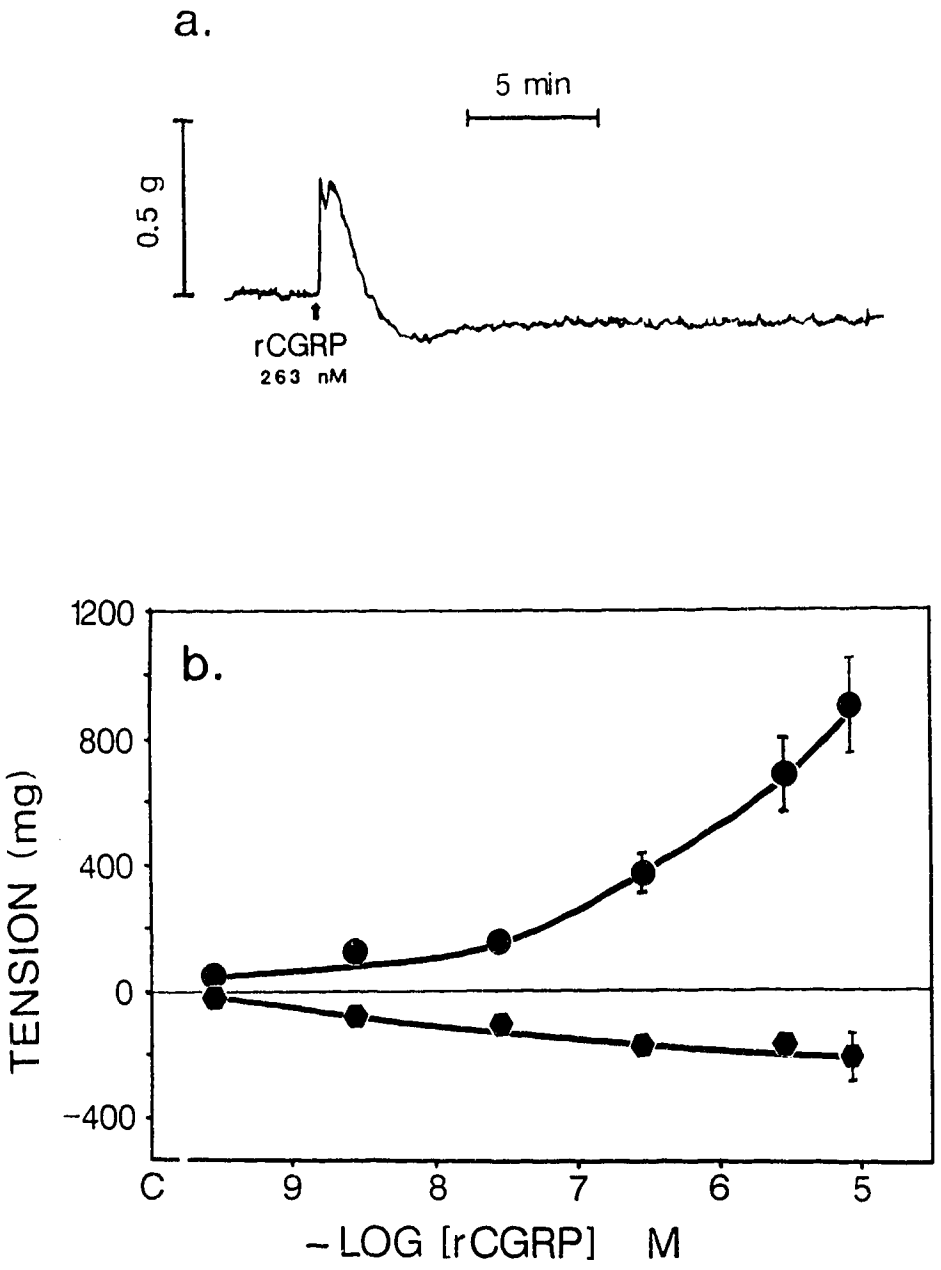

Figure 2-2. Effects of rCGRP on non-stimulated lm-mp of guinea pig ileum.
(a) Tracing of the tension in the presence of a single dose of rCGRP (263 nM). (b) Dose-response curve of rCGRP for peak contraction (•) and relaxation (◆) within the concentration range from 2.63×10^{-10} to 1×10^{-5} M. Peak contraction and relaxation induced by rCGRP were measured at 10 and 180 sec after the addition of the peptide. Data are represented as mean ± S.E., n=5.

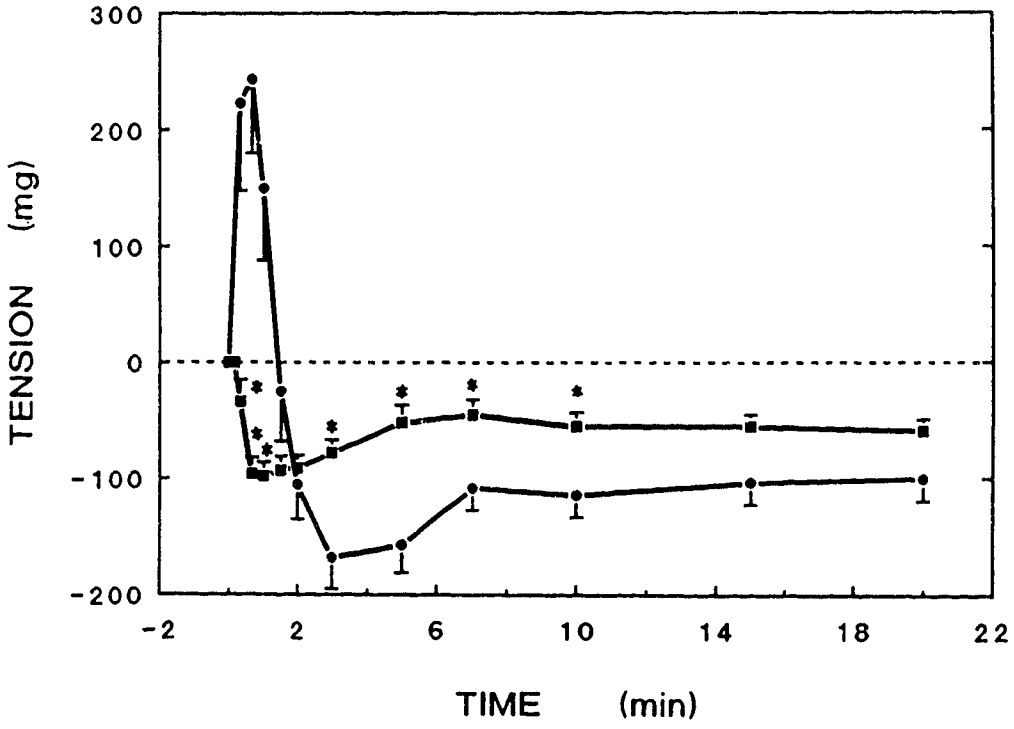

Figure 2-3. Influence of TTX on rCGRP-induced biphasic actions on non-stimulated lm-mp. Time course of rCGRP-induced tension change in the absence (•) or presence (■) of 1 μM TTX. *Significantly different from the tension in TTX-untreated tissue at the same time point (Duncan Multiple Range test). Data are expressed as mean $\pm$ S.E., n=7.

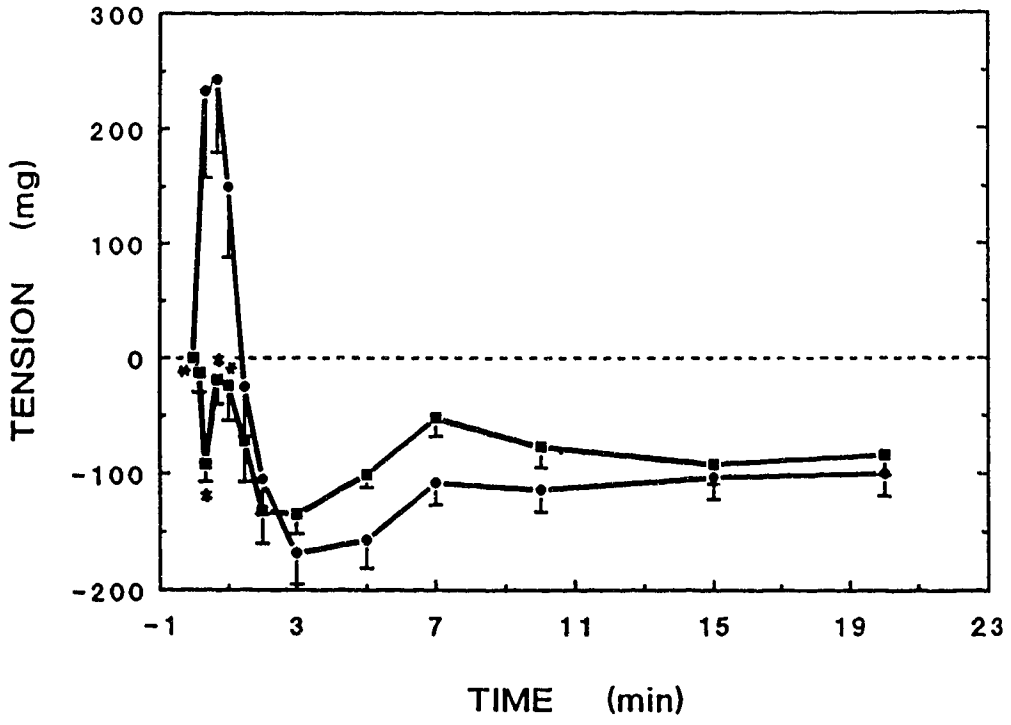

Figure 2-4. Influence of atropine on rCGRP-induced biphasic actions on non-stimulated lm-mp. Time course of rCGRP-induced tension change in the absence (•) or presence (■) of 1 μM atropine. *Significantly different from the tension in atropine-untreated tissue at the same time point (Duncan Multiple Range test). Each point represents mean ± S.E., n=7.

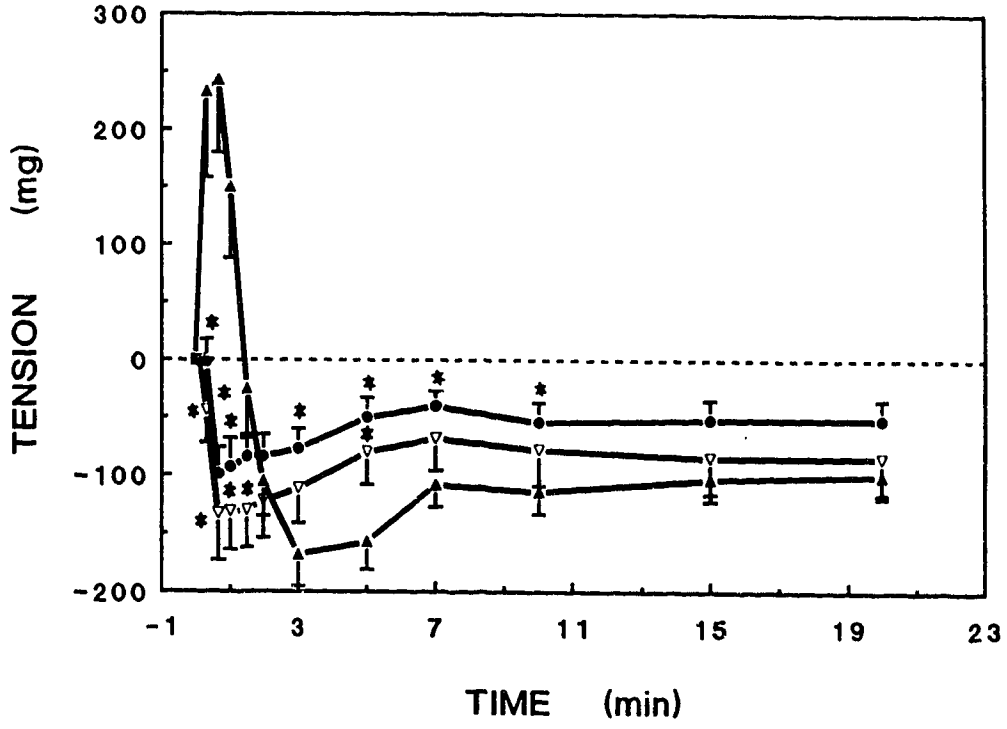

Figure 2-5. Influence of 2-chloroadenosine on rCGRP-induced biphasic actions on non-stimulated lm-mp. Time course of rCGRP-induced tension change in the absence (▲) or presence of 1 μM 2-chloroadenosine (•), or presence of 1 μM 2-chloroadenosine plus 1 μM TTX (▽). *Significantly different from the tension in 2-chloroadenosine-untreated tissue at the same time point (Duncan Multiple Range test). Data represent mean ± S.E., n=6.

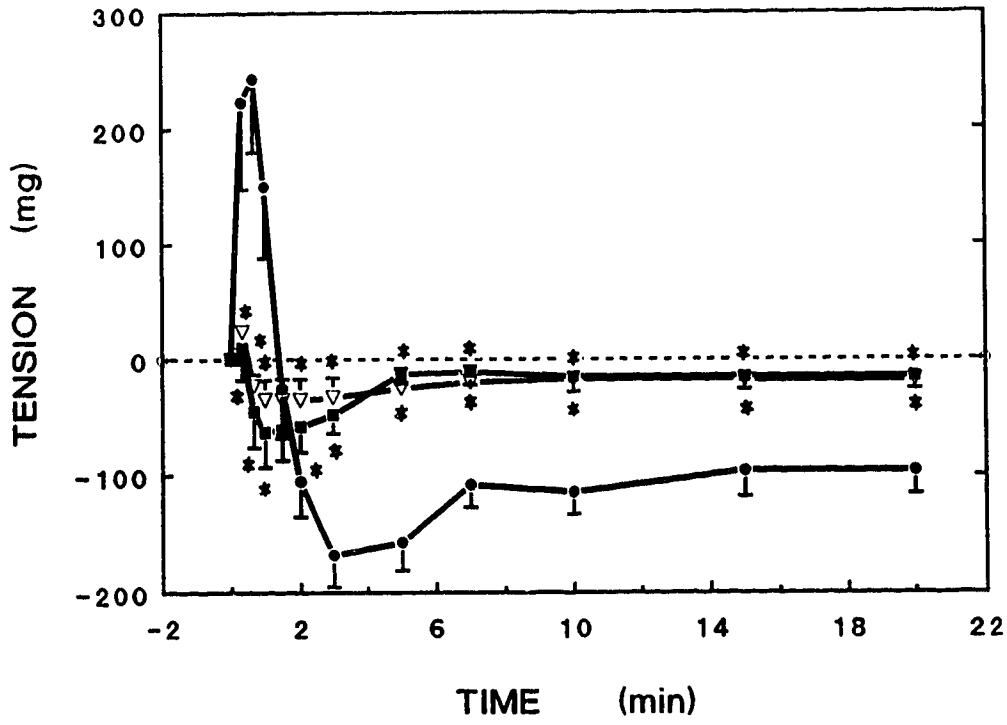

Figure 2-6. Influence of clonidine on rCGRP-induced biphasic actions on non-stimulated lm-mp. Time course of rCGRP-induced tension change in the absence (•), or presence of 1 μM clonidine (■) or in the presence of 1 μM clonidine plus 1 μM TTX (▿). *Significantly different from the tension in clonidine-untreated tissue at the same time point (Duncan Multiple Range test). Each point represents mean ± S.E., n=7.

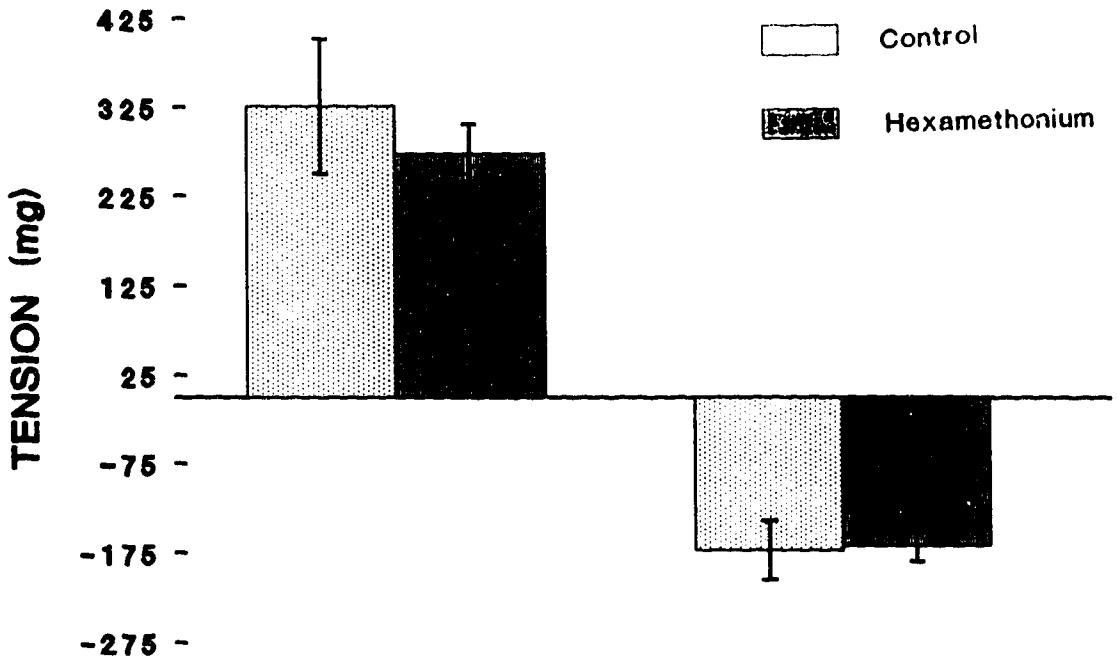

Figure 2-7. Effect of hexamethonium (1 μM) on rCGRP-induced biphasic actions on lm-mp. Data are expressed as mean $\pm$ S.E., n=8.

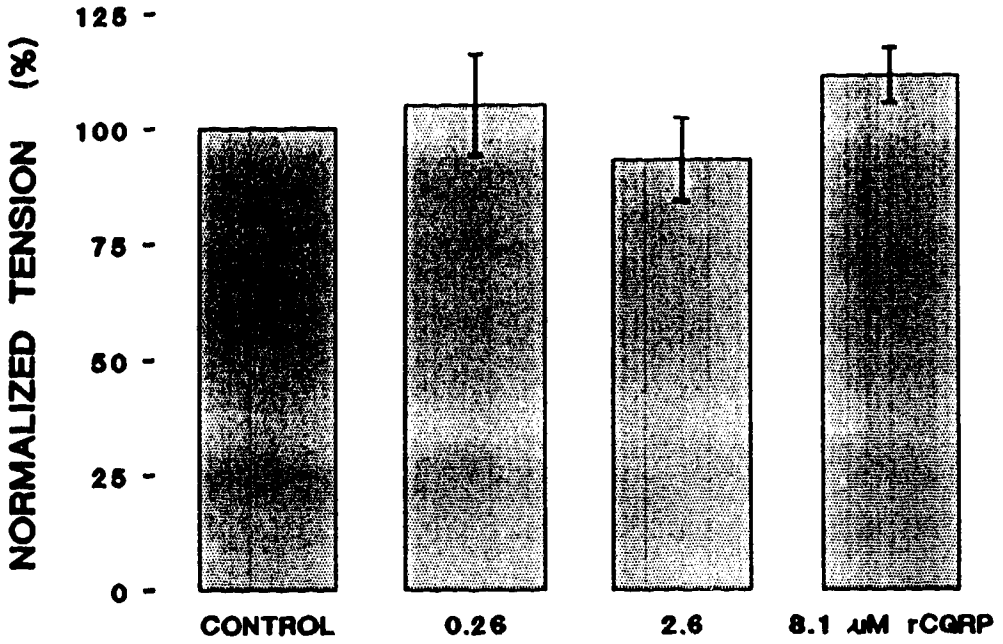

Figure 2-8. Effect of rCGRP on dimethylphenylpiperazinium (DMPP)-induced contraction of lm-mp. Contraction was produced by 5 μM DMPP in the absence or presence of a single dose of rCGRP raging from 0.26 μM to 7.9 μM. Each bar represents mean $\pm$ S.E., n=4.

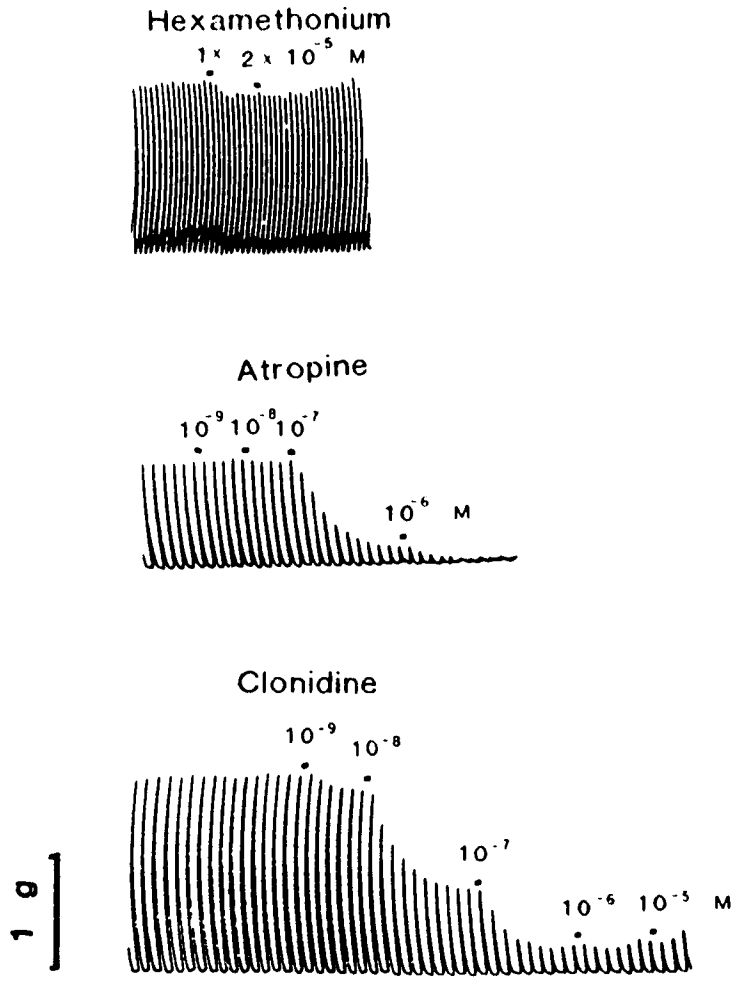

Figure 2-9. Effects of hexamethonium, atropine and clonidine on the electrical field stimulated twitch contractions of lm-mp of guinea pig ileum.The contractions were evoked by maximal electrical field stimulation (9 V/cm, 0.1 Hz and 1 ms).

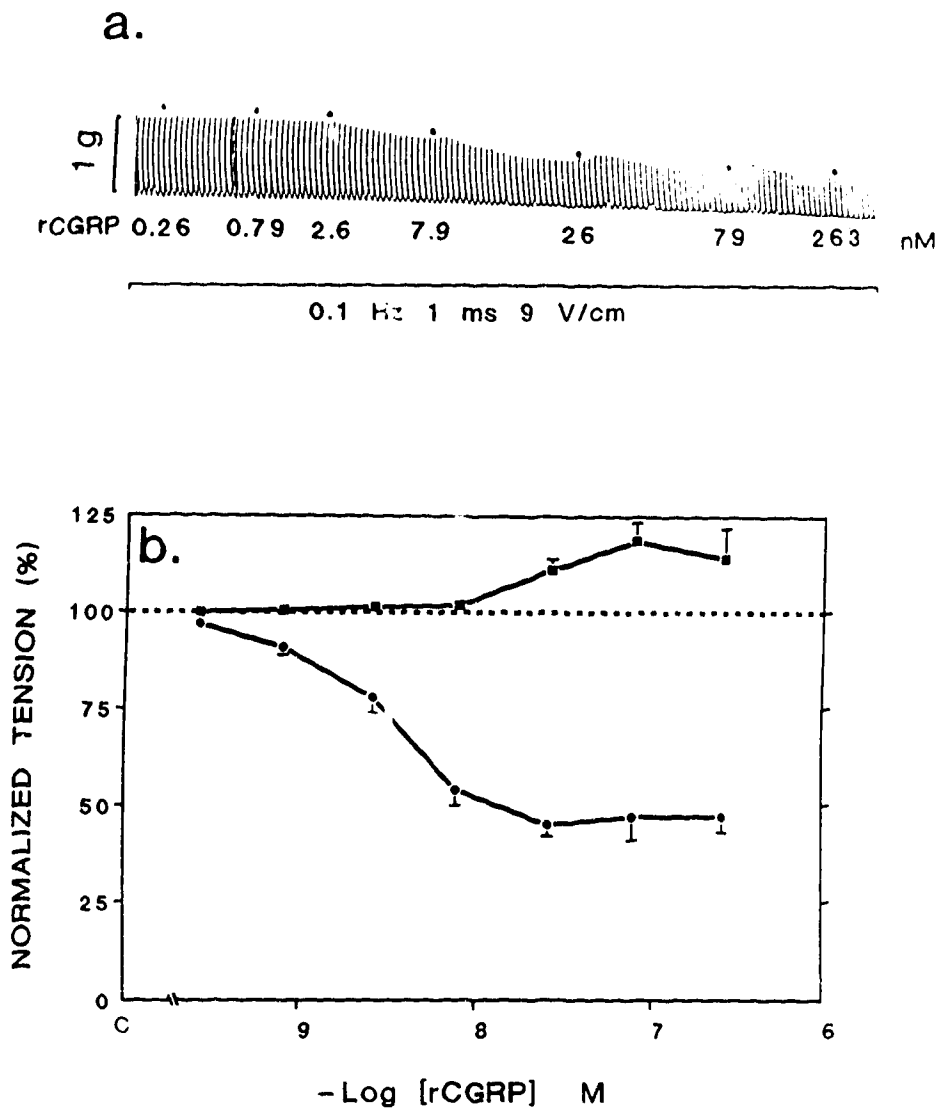

Figure 2-10. Biphasic effects of rCGRP on contractions evoked by maximal EFS (9 V/cm, 0.1 Hz and 1 msec). (a) Tracing of tension electrically evoked in the presence of cumulative doses of rCGRP (2.63×10^{-10} to 2.63×10^{-7} M). (b) Summary of results from nine experiments. Each point represents mean ± S.E.

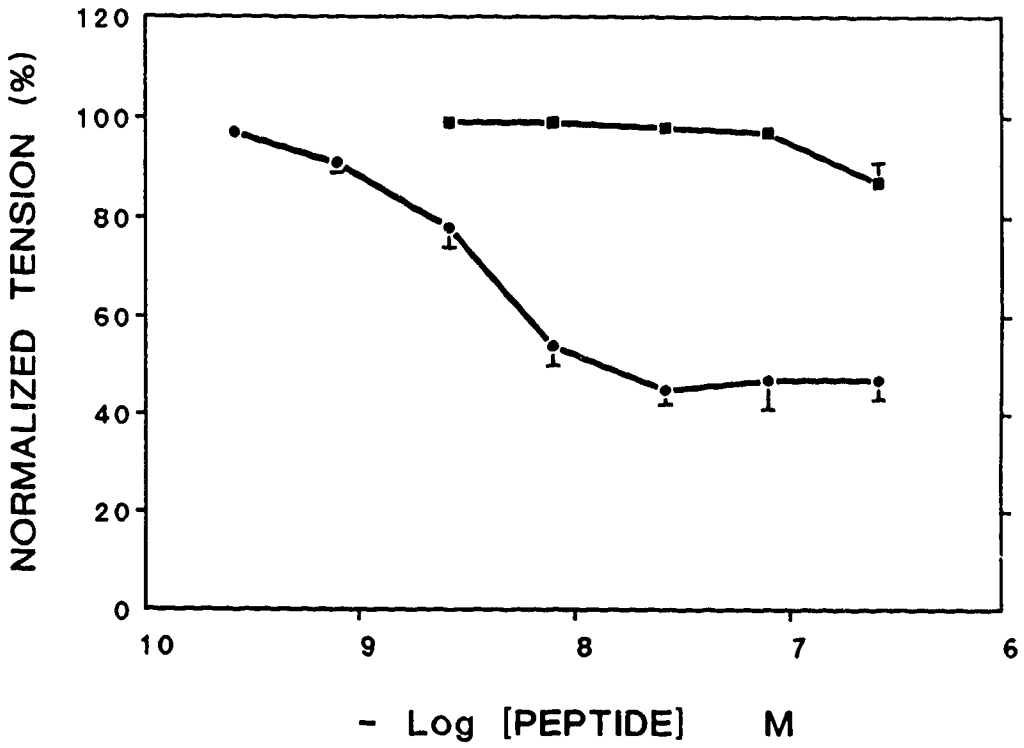

Figure 2-11. Inhibitory effects of rCGRP (•) and calcitonin (■) on contractions evoked by maximal EFS (9 V/cm, 0.1 Hz and 1 msec). Each point represents mean ± S.E., n=9 for rCGRP and n=4 for salmon calcitonin.

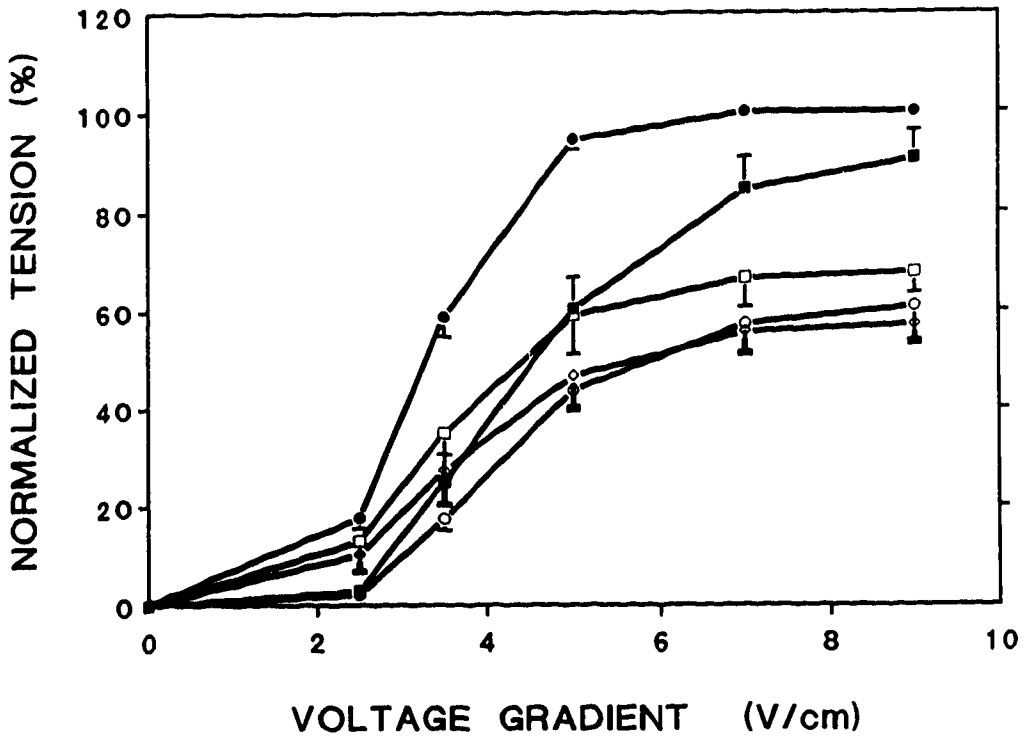

Figure 2-12. Inhibitory effect of rCGRP on voltage-contraction curve of lm-mp. Contractions were elicited by EFS at increasing strengths. A single dose of rCGRP (control, ●; 2.63×10^{-9}, ■; 7.9×10^{-9}, □; 2.63×10^{-8}, ◇; and 2.63×10^{-7} M, ○) was applied 3 min before the onset of the stimulation sequence. Data are expressed as mean ± S.E., n=19 for control, n=3, 3, 4, 6 for each dose of rCGRP (from low to high), respectively.

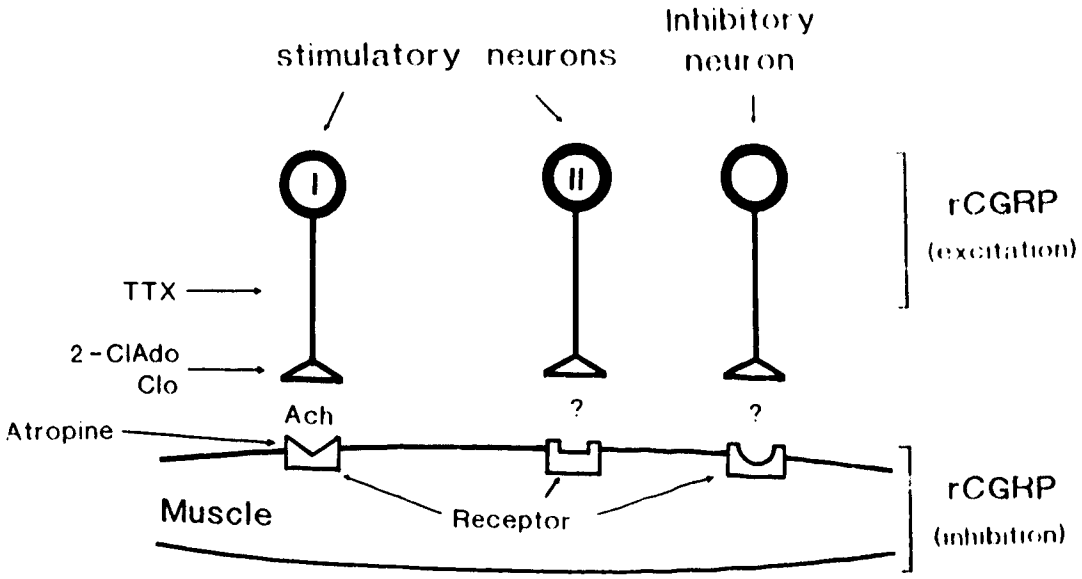

Figure 2-13. A model for the action sites of rCGRP in lm-mp of guinea pig ileum. The longitudinal muscle cells are innervated by three populations of enteric neurons. rCGRP has excitatory effects on those neurons and direct inhibitory effects on smooth muscle. rCGRP releases stimulatory substances, including ACh, and also an inhibitory substance, regulating the muscle tension. TTX affects all three populations of neurons by blocking the propagated action potentials to the nerve endings, inhibiting the release of neurotransmitters. Clonidine (Clo) and 2-chloroadenosine (2-ClAdo) activate the α_2-adrenoceptors and purinergic receptors, respectively, on the nerve endings, thereby inhibiting the release of neurotransmitters. Atropine blocks the muscarinic receptors on lm, therefore inhibiting the action of ACh released by rCGRP.

CHAPTER 3. DIRECT ACTIONS OF CGRP ON SMOOTH MUSCLE

I. INTRODUCTION

CGRP has been found to have direct effects on different types of muscle including skeletal muscle (Jennings and Mudge, 1989), cardiac muscle (Ishikawa *et al.*, 1988), vascular smooth muscle (Mejia *et al.*, 1988; Beny *et al.*, 1989; Foulkes *et al.*, 1991) and gastric smooth muscle (Maton *et al.*, 1988; Katsoulis and Conlon, 1989). This peptide has stimulatory effects on the skeletal and cardiac muscle while it displays inhibitory effects on the vascular and gastric smooth muscle. In the previous chapter, we used pharmacological methods to show that, in addition to its actions on the enteric neurons, rCGRP might have a direct effect on the longitudinal smooth muscle of the guinea pig ileum. However, an effect observed in a plexus-free muscle preparation could produce unambiguous evidence for the direct action of the peptide. Paton and Zar (1968) described a preparation of longitudinal smooth muscle proven by morphological and pharmacological criteria to be denervated or plexus-free. This type of preparation was used in the following study.

II. MATERIALS AND METHODS

A. Tissue preparation

A segment of ileum (similar to that used in Chapter 2) was cut and transferred to oxygenated Krebs solution (composition as described in Chapter 2). A

plexus-free longitudinal muscle (lm) preparation was made according to procedures described by Paton and Zar (1968). To isolate the plexus-free lm, gentle incisions were made along the length, and the lm with attached myenteric muscle was dissected free. The free end was held by a fine forceps, and a long piece of strip (20-30 cm) was detached by pulling. As the pull progressed, an increasing amount of nerve plexus remained behind on the circular muscle. A dissecting microscope wa used to confirm that at the distal end, the preparation had no plexus. This distal portion, 16-20 cm in length, was used in the experiments (Fig. 3-1a). The strip was hung and equilibrated as described in Chapter 2. To ensure that the plexus had, indeed, been removed, the responses of the tissue to EFS or addition of DMPP were also examined. These stimulations produced no contractile responses.

B. Actions of rCGRP in normal Krebs solution

The smooth muscle was contracted by the following stimulants: KCl, histamine, oxotremorine or sodium fluoride (NaF). These stimulants induced an initial contraction followed by tonic contraction (or tension oscillation in the case of NaF).

For initial contractions, a single dose of stimulant was repeated twice with a fixed interval (10 min for KCl, 5 min for histamine and 40 min for NaF). A single dose of rCGRP was added 2 min before the second stimulant challenge. To obtain control values, nothing was added between the two challenges of stimulant. All the stimulants used were dissolved in Krebs solution. The tension developed by the

second challenge was calculated as a percentage of the tension developed by the first challenge.

For tonic tension studies, rCGRP was added cumulatively (KCl and histamine-precontracted muscle) during the tonic phase of the contractions. The tension developed after each dose of rCGRP was calculated as a percentage of the tension just before the addition of the peptide. Comparisons were made between rCGRP-treated and -untreated tissues. In studies of antagonism by $hCGRP_{8-37}$, this antagonist was added 2 min before the cumulative doses of rCGRP to histamine-precontracted muscle. In some experiments, a cumulative dose-response curve of the stimulant was first made in the absence of rCGRP (control); a second curve was then made in the same strip of tissue in the presence of a single dose of rCGRP.

C. Extracellular calcium-dependent contraction

The tissue was first equilibrated for 1 hr in normal Krebs solution containing 2.5 mM $CaCl_2$. After challenge by the stimulant, the solution was replaced by calcium-free Krebs solution, and 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N'-N'-tetraacetic acid (EGTA) was added to the solution to chelate the membrane-bound calcium. The bath solution was changed, 5 min later, to a solution with a low concentration of EGTA (20 μM). About 10 min later, a single dose of stimulant (1 μM histamine or 30 mM KCl) and 2 min after that the cumulative doses of $CaCl_2$ were added. The tissue was then washed twice with normal Krebs solution

(2.5 mM $CaCl_2$) during the 20 min incubation period. min. The process was repeated as mentioned above with the same piece of tissue except that rCGRP was added together with the stimulant. The tension developed by each dose of $CaCl_2$ after treatment of rCGRP was calculated as a percentage of the tension developed by the same dose of $CaCl_2$ before addition of rCGRP. Comparison was made between the tensions of rCGRP-treated and -untreated tissue.

D. Materials

Drugs used were: histamine, oxotremorine and sodium fluoride (Sigma Chemical Co., U.S.A.), rat αCGRP, human $\alpha CGRP_{8-37}$ (Bachem Inc., Essex, U.K.), rat amylin (a gift from Dr. Sunil J. Wimalawansa, University of London, U.K.) and glibenclamide (Research Biochemicals Inc., U.S.A.). Pinacidil, incorandil and cromakalim were generously donated by Leo Pharmaceutical Products (Copenhagen), Zenyaku Kogyo Co., Ltd. (Japan) and Beecham Pharmaceuticals (Sussex, U.K.).

E. Statistics

The data are expressed as mean ± S.E.. Significance was tested using Newman-Keuls or Duncan Multiple Range tests (comparison with a common control) or Student's *t*-test (paired or unpaired data). *P* values smaller than 0.05 are considered significant.

III. RESULTS

A. Effects of rCGRP on non-precontracted tissue

As mentioned in the previous chapter, both EFS and DMPP contract the lm-mp by releasing ACh from enteric neurons. The lm-mp responded to both types of stimulation, while the lm was not contracted by these two stimuli (Fig. 3-1a,b). These results were consistent with those of Paton and Zar (1968). In addition, no myenteric plexus was observed under the dissection microscope (Fig. 3-1c). These results indicate that the preparation used is plexus-free smooth muscle.

Studies described in the previous chapter suggest that rCGRP has both contractile and relaxing effects on the lm-mp, and both the contractile effect and part of the relaxing effect are mediated by enteric neurons. In contrast to the biphasic effects on the lm-mp preparation, rCGRP (263 nM) showed only a relaxing effect on the plexus-free muscle. The basal tension was decreased by 49.3±10 mg (Fig. 3-2, n=10) and was much smaller than the relaxing effect seen in the lm-mp (168±27 mg).

B. Effects of CGRP on stimulated tissue

The four stimulants evoked different types of contractions (Fig. 3-9 and 3-13). Histamine, oxotremorine and KCl induced a fast initial contraction followed by a slow decreasing tonic contraction. NaF, only at the high dose, caused a profound effect on the lm. The initial contraction was characterized by a a fast phase followed

by a slow phase. The initial contraction was followed by a tension oscillation. The histamine-, KCl- and oxotremorine-induced contractions are reproducible in the same piece of tissue within a short period of time. However, contractions stimulated with NaF required a longer interval between the two challenges (40 min). The influence of rCGRP on these induced contractions was examined to study the mechanism of the inhibitory effects of rCGRP on the smooth muscle.

1. Effect on KCl-induced contraction

KCl depolarizes the plasma membrane of smooth muscle cells and increases permeability to calcium ions, resulting in an increase in $[Ca^{2+}]_i$ and contraction of the muscle (Bolton, 1986). The plexus-free lm was very sensitive to stimulation by KCl. The ED_{50} for the cumulative dose-response curve of KCl was 8.1 mM (Fig. 3-3), and a concentration of 30 mM KCl produced maximal contraction. rCGRP at concentrations up to 263 nM inhibited the tonic, but not the initial, contractions induced by maximal KCl stimulation (Fig. 3-4). Significant inhibition was observed at doses of 0.788 to 78.8 nM rCGRP. The tonic tension returned to the control value when the dose of the peptide was increased to 263 nM. The supermaximal stimulation (50 mM KCl) -induced contractions were not affected by rCGRP, even up to a concentration of 263 nM.

2. Effect on histamine-induced contraction

Histamine activates the H_1 subtype of histamine receptors on longitudinal smooth muscle of the guinea pig ileum, resulting in the contraction of this muscle (Hill, 1990). rCGRP inhibited the phasic, and relaxed the tonic, contractions induced by histamine in a concentration-dependent manner (0.26 to 263 nM). rCGRP was more effective in inhibiting the histamine-induced tonic than the phasic contraction (Fig. 3-5). The maximal inhibition of histamine (1 μM) -induced tonic contraction by rCGRP at 79 nM was 89±2%, whereas the maximal inhibition of the phasic contraction was 35±3%. rCGRP relaxed the tonic contractions induced by histamine at doses of 0.1, 1, 10, but not 100 μM (Fig. 3-6). Rat amylin at concentrations up to 510 nM had no effect on the histamine (0.5 μM)-induced tonic contraction (Fig. 3-7).

3. Effect on oxotremorine-induced contraction

ACh, the major neurotransmitter in the enteric neurons, contracts the lm by interaction with the muscarinic receptors on the muscle cells. However, ACh is easily inactivated by acetylcholinesterase hydrolysis and, therefore, oxotremorine instead of ACh was used to determine the effect of rCGRP on contractions induced by activation of lm muscarinic receptors. rCGRP, at a dose of 263 nM, showed minimal effects on the dose-response curve of oxotremorine, an agonist for muscarinic receptors (Fig. 3-8b). rCGRP, at concentrations up to 263 nM, also displayed no significant influence on the tonic contraction produced by oxotremorine at a concentration of

0.1 μM (Fig. 3-8a).

4. Effect on NaF-induced contraction

NaF is a G-protein stimulator (Sternweiss and Gilman, 1982; Bigay *et al.*, 1985). It contracts both vascular and intestinal longitudinal smooth muscle (Watson *et al.*, 1988; Zeng *et al.*, 1989). The induced contraction of intestinal lm can be abolished by nifedipine, a voltage-dependent calcium channel blocker (Watson *et al.*, 1988). NaF (10 mM) induced a phasic and an irregular tonic contraction in the plexus-free lm. The tissue recovered 20 min after washout of the first dose. In three of the four experiments conducted , rCGRP inhibited the phasic contraction by approximately 90%, but showed no inhibition of the irregular contraction which occurred afterwards (Fig. 3-9).

5. Effect on extracellular calcium-induced contraction

In order to ascertain whether rCGRP might affect extracellular calcium utilization by the plexus-free lm during the contractions produced by KCl and histamine, the effects of the peptide on extracellular calcium-dependent contractions were examined. The calcium dose-response curve was shifted to the right for KCl-induced contraction and the maximal contraction was significantly depressed (Fig. 3-10). The histamine (1 μM)-induced extracellular calcium-dependent contraction was not significantly affected by rCGRP (Fig. 3-11) while nifedipine (1 μM) totally inhibited the

calcium-induced contraction.

6. Antagonizing effect of hαCGRP$_{8-37}$ on the action of rCGRP

hαCGRP$_{8-37}$, a fragment of hαCGRP, has been reported to be an antagonist of CGRP in several tissues (Morita *et al.*, 1989; Dennis *et al.*, 1990; Mimeault *et al.*, 1991). The purpose of this experiment was to determine whether rCGRP exerts its action on smooth muscle through CGRP receptors. The histamine (50 nM) -induced tonic contraction was inhibited by rCGRP in a dose-dependent manner, with an ED_{50} of 1.9±0.3 nM. hCGRP$_{8-37}$ (1 and 10 μM) shifted the inhibitory curve to the right and increased the ED_{50} values to 10±3 nM and 192±33 nM, respectively (Fig. 3-12). The pA_2 was calculated to be 6.48.

7. Effects of K channel openers on the smooth muscle

Opening of ATP-sensitive potassium channels (K_{ATP}) has been reported to be one of the mechanisms underlying the vasodilation effect of CGRP on rabbit mesenteric artery. Nelson *et al.* (1990) showed that the relaxing effect of CGRP on this muscle is reversed by the K_{ATP} inhibitor, glibenclamide (Ashford *et al.*, 1988; Quast and Cook, 1989a; Standen *et al.*, 1989), and barium ion (Standen *et al.*, 1989). This mechanism may also be involved in the action of rCGRP on the lm. It is, however, still not known whether there are K_{ATP} on the longitudinal smooth muscle of guinea pig ileum. We examined the effects of three putative K_{ATP} openers on the lm to

determine if they would relax the stimulant-pre-contracted muscle as in the vasculature (Nelson *et al.*, 1990). The effect of three potassium channel openers, nicorandil, pinacidil and cromakalim, were observed on individual strips of plexus-free lm precontracted with oxotremorine (5 μM), histamine (5 μM) or KCl (30 mM) (Fig. 3-13). The effects of the three putative openers were not consistent. Only KCl-induced tonic contraction was inhibited by pinacinil and cromakalim (50 μM). These agents exhibited no effect on the oxotremorine-induced tonic contraction, or had a potentiating effect on the tonic contractions induced by histamine. Among the three openers, cromakalim abolished and pinacidil relaxed (47%), the KCl-induced tonic contraction while nicorandil was ineffective. Barium ion at a concentration of 100 μM did not reverse the inhibitory effect of cromakalim on KCl-induced tonic contraction (Fig. 3-13d). Interestingly, the histamine-evoked tonic tension was potentiated by nicorandil and cromakalim by 33% and 23% respectively, while pinacidil was ineffective. Relaxation of histamine (0.5 μM) pre-contracted muscle produced by rCGRP (2.63 nM) was not reversed by glibenclamide at concentrations of 0.1 to 1 μM (Fig. 3-14).

IV. DISCUSSION

A. Tissue preparation

The following characteristics have been reported for the plexus-free lm (Paton and Zar, 1968): a) it is unresponsive to EFS, which is known to excite neurons and

release neurotransmitters; b) it fails to respond to nicotine or dimethylphenylpiperazinium (DMPP), which excite neurons through the activation of ganglionic nicotinic receptors and release neurotransmitters; c) it does not exhibit spasms in response to eserine, which inhibits acetylcholinesterase and results in accumulation of ACh released from nerve endings; d) it does not contain detectable ACh; and e) it is shown, under the dissecting microscope, to have no nerve plexus. In the present study, the preparation was shown to be unresponsive to EFS and DMPP, and, upon visual examination, was also found to have no nerve plexus. The preparation we used in the studies, therefore, was effectively a plexus-free muscle preparation.

B. Action of rCGRP on non-precontracted tissue

In the previous chapter, we reported that rCGRP-induced contraction of the lm-mp of GPI could be blocked by atropine, suggesting that rCGRP might release ACh. Using the plexus-free lm in the present study, we found that rCGRP did not cause any contraction in the plexus-free lm. This provided further evidence that the preparation we used is free from nerve innervation. It is, therefore, an ideal preparation to study the direct actions of drugs and other bioactive substances on ileal smooth muscle. On the other hand, this result supports the conclusions drawn in the previous chapter that enteric neurons mediate the contractile effect and part of the relaxing effect of rCGRP and that rCGRP also has direct inhibitory actions on the smooth muscle. There have been reports that CGRP can hyperpolarize the

smooth muscle cells of large cerebral arteries of cats (Saito *et al.*, 1989) and muscle cells in the intact mesenteric artery of rabbit (Nelson *et al.*, 1990). However, Ohkawa (1989) found that the resting membrane potential of longitudinal muscle cells of the guinea pig ileum was not affected by CGRP at concentrations up to 104 nM. Therefore, the mechanism underlying the decrease of basal tension by rCGRP is not clear.

C. Effect of rCGRP on depolarization-induced contraction

A high concentration of potassium depolarizes the membrane of smooth muscle (Burnstock and Straub, 1958; Holman, 1958; Sperelakis, 1962). The consequence of the depolarization is an opening of voltage-gated calcium channels in the smooth muscle cell membrane (Bolton, 1986). This event leads to an inward movement of calcium ions, elevation of $[Ca^{2+}]_i$ and ultimately to smooth muscle contraction. The plexus-free lm was very sensitive to the stimulation by KCl (ED_{50}, 8.1 mM). rCGRP inhibited the KCl-induced contraction, indicating that this peptide might interfere with the calcium movement caused by KCl. After the tissue is depolarized by KCl in calcium-free Krebs solution, addition of calcium produces contraction, indicating the entry of calcium from outside. rCGRP shifts the calcium dose-response curve. The maximal contraction is slightly but significantly reduced, suggesting that this peptide may interfere with the calcium entry into longitudinal smooth muscle cells induced by depolarization.

D. Effects of rCGRP on histamine-induced contractions

Recent evidence suggests that histamine is a putative neurotransmitter or modulator in the GI tract and ENS (Costa *et al.,* 1987). Mast cells are found in close association with the myenteric ganglia (Dale and Zilletti, 1970). Using an antiserum to the enzyme histidine decarboxylase purified from rat liver, Ekblad *et al.* (1985) described immunoreactive nerve cell bodies but did not find immunoreactive nerve fibers in the myenteric ganglia of the proximate regions of the rat small intestine. Panula *et al.* (1985) reported the presence of histamine immunoreactive nerve fibers in the enteric plexus of the guinea pig stomach and nerve cell bodies in the rat submucous ganglia. Recently, a population of histamine-containing neurons and nerve fibers were found in the rat peripheral nervous system including neurons in the myenteric ganglia and fibers in the intestinal wall (Häppölä *et al.,* 1991). The presence, after exposure of the intestine to radio-labelled histamine, of a releasable neuronal pool of tritiated histamine in the gut wall suggests that histamine may be used as a transmitter by a population of enteric neurons (Hakanson *et al.,* 1983). Application of histamine to AH/type 2 myenteric neurons mimics slow synaptic excitation in these cells (Nemeth *et al.,* 1984). Therefore, histamine may play some physiological role in the regulation of GI tract functio

Three subtypes of histaminergic receptors, H_1, H_2, and H_3, have now been identified. H_1, H_3 and, perhaps, also H_2, subtypes are present in the small intestine. The effect of histamine on smooth muscle varies according to the source and the

species. However, the amine typically induces a contraction of non-vascular smooth muscle through activation of H_1 receptors. The guinea pig ileum has H_1 receptors on the longitudinal muscle cells and circular muscle cells (Yamanaka and Kitamura, 1987). The presence of the H_2 subtype on the circular smooth muscle is still questionable (Leurs *et al.*, 1991a). H_3 receptors are located on myenteric neurons (Schwartz *et al.*, 1990). Activation of the H_3 receptor on the myenteric neurons prevents the release of ACh (Menkveld and Timmerman, 1990; Poli *et al.*, 1991).

Activation of H_1 receptors on longitudinal smooth muscle cells of the small intestine leads to an increase in the frequency of discharge and membrane depolarization due to the increase in the membrane permeability to cations (Bülbring, 1957; Bolton *et al.*, 1981a; Morel *et al.*, 1987; Yamanaka and Kitamura, 1987). The depolarization is induced by histamine at concentrations as low as 30 nM (Yamanaka and Kitamura, 1987). Bolton *et al.* (1981a) have reported that 0.1 mM histamine can produce a depolarization as great as 30 mV. A parallel increase in tension accompanies the depolarization of the membrane (Yamanaka and Kitamura, 1987). It seems likely that the histamine-induced depolarization can activate the voltage-gated calcium channels and contribute to the increase in $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ by histamine may also be partially caused by mobilizing calcium from intracellular stores. Histamine has been shown to elevate $[Ca^{2+}]_i$ in smooth muscle from different sources (Matsumoto *et al.*, 1986; Murray and Kotlikoff, 1991).

Contractions produced by histamine depend upon extracellular calcium to

different degrees in various tissues. The contraction of the longitudinal smooth muscle depends largely on extracellular calcium. Both the phasic and tonic contraction evoked by histamine are inhibited by nifedipine, an organic blocker of voltage dependent calcium channels (Morel *et al.*, 1987). We have demonstrated that histamine increases $[Ca^{2+}]_i$ in plexus-free lm of the guinea pig ileum while nifedipine diminishes the elevated $[Ca^{2+}]_i$ (for details see Chapter 4). Tonic contractions induced by high concentrations of histamine cannot be totally inhibited by nifedipine, indicating that a mechanism other than extracellular calcium influx is involved (Morel *et al.*, 1987). One possibility is that histamine stimulates the IP_3 (1,4,5-InsP) production, which releases calcium from intracellular stores. Histamine does promote IP_3 production in longitudinal smooth muscle cells (Bielkiewica-Vollrath *et al.*, 1987; Murthy *et al.*, 1992). The situation, however, is complicated. Donaldson and Hill (1985) have observed a dissociation of contraction and the production of inositol phosphates; i.e. H_1 receptor antagonists potently inhibit the histamine-induced contraction but are very weak or ineffective in inhibiting the histamine-stimulated production of inositol phosphates. In addition, muscarinic receptor agonists and fluoride produce only a small amount of IP_3 in the longitudinal smooth muscle cells relative to the circular muscle cells (Murthy *et al.*, 1992). Furthermore, only low affinity IP_3 receptors which are activated by high concentrations of IP_3 are present in longitudinal smooth muscle cells (Murthy *et al.*, 1991; Murthy and Makhlouf, 1991). The effect of histamine on intracellular calcium mobilization may play a

minimal role in the induced contraction of the lm.

Activation of protein kinase C by phorbol ester has been reported to contract smooth muscles from different sources without changing the $[Ca^{2+}]_i$ (Jiang and Morgan, 1987, 1989; Karaki *et al.*, 1989; Morgan *et al.*, 1989; Sato *et al.*, 1989; Mori *et al.*, 1990; Ozaki *et al.*, 1990). Activation of protein kinase C, therefore, could be the pathway by which stimulants caused contraction of smooth muscle at low $[Ca^{2+}]_i$.

Barthó *et al.* (1987) have reported that rCGRP has a potent inhibitory effect on the histamine-induced tonic contraction in the ileal segment. In the present study, we observed that rCGRP inhibits not only the histamine-induced tonic contraction of plexus-free lm, which is consistent with the results obtained by Barthó *et al.* in the whole segment GPI, but also inhibits the induced phasic contraction. Again, CGRP inhibited tonic contraction to a larger degree than the phasic contraction.

It is interesting that rCGRP had no effect on the histamine (1 μM) -induced extracellular calcium dependent contractions while it had inhibitory actions on histamine-induced contractions in normal Krebs solution. The dose-response curve of extracellular calcium was neither shifted nor depressed. Degradation of the peptide does not seem likely, because under similar experimental conditions it shows an inhibitory effect on depolarization-induced extracellular calcium-dependent contractions. A similar phenomenon was observed by Kline and Pang (1988). They found that in rat tail artery helical strips, CGRP inhibits the contraction induced by NE in normal or calcium free Krebs solution, but displays no significant effect on the NE-induced

extracellular calcium-dependent contraction. We found, however, that both the KCl-evoked contraction in normal physiological solution and the KCl-evoked extracellular calcium-dependent contraction were inhibited by CGRP. It is likely that either 1) rCGRP can distinguish between calcium influx evoked by histamine and that elicited by KCl, or 2) this peptide affects mechanisms other than histamine-induced calcium influx. For example, KCl-depolarization opens the voltage-gated calcium channels (Bolton *et al.*, 1989) while histamine increases the permeability of the membrane to other cations in addition to calcium (Bolton and Clark, 1981b). Moreover, it has been reported that time-dependence is a characteristic of the interaction of drugs (such as nifedipine) with the L-type voltage-operated calcium channels (Godfraind and Dieu, 1981; Bolton *et al.*, 1983; Godfraind, 1983; Morel *et al.*, 1987). Morel *et al.* (1987) found that the blocking of histamine-induced tonic contractions by nifedipine was not time-dependent. These results suggest that histamine opens channels in the membrane that are sensitive to dihydropyridines but may differ from classic voltage-operated channels.

E. Effect of CGRP on the muscarinic receptor pathway

ACh is a classical and major stimulatory neurotransmitter in the ENS (Walsh, 1987). The M_2 subtype of muscarinic receptors (Birdsall *et al.*, 1986) is present on the smooth muscle cells of the guinea pig ileum lm. Activation of these receptors results in membrane depolarization, increases in membrane conductance and, finally,

contraction of the muscle (Bolton and Clark, 1981a,b).

Barthó *et al.* (1987) reported that CGRP inhibits the longitudinal contractions of whole segment guinea pig ileum caused by bethanechol, a muscarinic receptor agonist, to the same extent as those on the contraction caused by histamine. However, our results showed that rCGRP at doses as high as 263 nM do not relax the oxotremorine (0.1 μM) -induced tonic contraction or shift the cumulative dose-response curve of oxotremorine at doses ranging from 0.01 to 1 μM. One of the possible causes for this discrepancy might be that the preparation used here differed from that used by Barthó and co-workers. The possibility that CGRP may release some inhibitory substance(s) from enteric neurons which then affect the contraction induced by bethanechol can not be excluded. This discrepancy could also be due to the difference in the methods used for tension recording. Schowörer *et al.* (1991) recently found that in the guinea pig jejunum lm-mp, the relaxant effect of CGRP was not very pronounced under isometric recording conditions. By using an isotonic recording method, CGRP was shown to inhibit the tonic contraction induced by carbachol (3 μM), a muscarinic receptor agonist. These investigators speculated that the inability of CGRP to relax the carbachol-induced tonic contraction using isometric recording methods might be due to a fast decline in the tonic plateau contraction phase. Barthó *et al.* (1987) also used isotonic recording methods. We noticed that after the phasic portion of the oxotremorine-induced contraction of the lm, the tension dropped until a tonic plateau was reached. In our experiments, rCGRP was

applied during the plateau. Why there is a discrepancy in the effects of the peptide observed with these two different recording methods is not clear.

Activation of muscarinic receptors opens voltage-sensitive channels which are permeable to most mono- and divalent cations via a G-protein pathway in smooth muscle cells of GPI and rabbit jejunum (Benham et al., 1985; Inoue and Isenberg, 1990a,b). This type of channel is facilitated by $[Ca^{2+}]_i$ (Inoue and Isenberg, 1990c), which may result in a positive feedback mechanism to prolong the depolarizing effect of muscarinic receptor activation. Influx of cations leads to an increase in the frequency of discharge and membrane depolarization which may in turn open voltage-dependent calcium channels (Triggle, 1979; Bolton and Clark, 1981a,b). Calcium then enters the cell through non-selective, perhaps as well as voltage-gated calcium channels, resulting in an increase in $[Ca^{2+}]_i$ and development of tension (Inoue and Isenberg, 1990a). Release of calcium from intracellular stores is another event which may be related to receptor activation with IP_3 as the mediator (Bielkiewica-Vollrath *et al.,* 1987). Increases in $[Ca^{2+}]_i$ result in contraction of the muscle. Voltage-gated calcium channel blockers inhibit the contraction produced by activation of muscarinic receptors (Rosenberg *et al.,* 1979; Braading and Sneddon, 1980). Again, the extent of contribution of calcium release from intracellular stores to the contraction is still not clear. Konno and Takayanagi (1989) found that oxotremorine acts as a full agonist with a potency equal to that of carbachol in inducing contraction in normal bathing solution or in inducing extracellular calcium-

dependent contraction. Oxotremorine is, however, a partial agonist in contractions occurring in calcium-free medium and in the production of inositol phosphates. Contraction in calcium-free medium and formation of inositol phosphates are seen only at concentrations of agonists much higher than those required for normal contractions. This indicates that contractions under normal conditions are mainly associated with the influx of extracellular calcium. It seems that the muscarinic receptor pathway uses a signalling pathway similar to that of the the H_1 receptor. rCGRP selectively inhibits histamine-induced contraction while it leaves oxotremorine-induced contraction unaffected, showing that a difference exists in the mechanisms for the contractions produced through these two receptor pathways.

F. Effect of CGRP on fluoride-induced contraction

Guanine nucleotide regulatory proteins (G-proteins) are transducers that convert extracellular signals to intracellular signals by coupling receptor-activation to different effector systems in the plasma membrane (Gilman, 1987). Fluoride (F^-) and aluminum activate G-proteins, including G_s, G_i and transducin (Haslam and Vanderwal, 1982; Blackmore *et al.*, 1985; Kanaho *et al.*, 1985; MacDonald and Martin, 1988). It has been suggested that F^-, in the presence of Al^{3+} (which can come from contamination of disposable glass test tubes; Sternweis and Gilman, 1982) forms a complex, AlF_4^-, which has a similar structure to PO^{3-}. This complex interacts with guanosine 5'-diphosphate situated on the α-subunit of the G-protein, resulting in activa-

tion by mimicking GTP at its binding site (Bigay *et al.*, 1985). Fluoride has been reported to produce contraction of smooth muscles including the vasculature (Casteels *et al.*, 1981; Nguyen-Duong, 1985; Zeng *et al.*, 1989; Cushing *et al.*, 1990; Ratz and Blackmore, 1990; Kawase and Van Breemen, 1992), airway smooth muscle (Leurs *et al.*, 1991b) and intestinal smooth muscle (Watson *et al.*, 1988; Himpens *et al.*, 1991; Murthy *et al.*, 1992).

Fluoride ion may stimulate phospholipase C through a G-protein pathway in smooth muscle cells. Activation of phospholipase C leads to production of two important intracellular second messengers: IP_3 and diacylglycerol. IP_3 releases calcium from intracellular stores, and diglycerol activates protein kinase C (Berridge and Irvine, 1989). Fluoride-induced contraction of porcine coronary artery is almost completely abolished by pertussis toxin, but is less affected by cholera toxin (Cushing *et al.*, 1990), indicating the involvement of G-proteins. Fluoride has been shown to produce contraction of smooth muscle of rabbit ear artery, airway and circular smooth muscle of GPI in calcium-free medium or in the presence of calcium channel blockers (Casteels *et al.*, 1981; Nguyen-Duong, 1985; Leurs *et al.*, 1991b; Murthy *et al.*, 1992), suggesting an IP_3-induced release of calcium from an intracellular store. Both the levels of inositol phosphates (Watson *et al.*, 1988; Ratz and Blackmore, 1990; Murthy *et al.*, 1992) and the activity of protein kinase C (Murthy *et al.*, 1992) are elevated by fluoride as the result of activation of phospholipase C. H-7, a protein kinase C inhibitor, relaxes fluoride-induced contraction in permeabilized rabbit

mesenteric artery. No additive effect is found between fluoride and PDBu, a protein kinase C stimulator, indicating the involvement of protein kinase C in the action of fluoride on smooth muscle (Kawase and Van Breemen, 1992). Both extracellular calcium and calcium released from intracellular stores are needed for the fluoride-induced contraction. In the induced contractions, the dependence on extracellular calcium differs from tissue to tissue. While bovine facial vein and artery and longitudinal muscle cells of guinea pig intestine largely depend on the extracellular calcium (Nguyen-Duong, 1985; Murthy *et al.*, 1992), the circular muscle cells of intestine and airway smooth muscle use mainly intracellular calcium sources (Leurs *et al.*, 1991b; Murthy *et al.*, 1992).

The responses of circular and longitudinal smooth muscle cells to fluoride differ greatly (Murthy *et al.*, 1992). In circular muscle cells, there are high affinity IP_3 receptors. Fluoride preferentially produces IP_3, and also activates protein kinase C (Murthy *et al.*, 1991). In addition, fluoride mobilizes calcium and leads to calcium channel blocker-insensitive contractions. On the other hand, longitudinal smooth muscle cells have only a few IP_3 receptors and these are low affinity (Murthy *et al.*, 1991; Murthy and Makhlouf, 1991) and IP_3 evokes release of calcium from the microsomes of smooth muscle cells of this tissue (Murthy *et al.*, 1992). Fluoride produces a small amount of IP_3 but stimulates protein kinase C to the same extent as in the circular muscle cells. The fluoride-induced increase in $[Ca^{2+}]_i$ and contraction are abolished by organic calcium channel blockers (Murthy *et al.*, 1992; Watson *et al.*,

1988). Therefore, fluoride-induced contraction of longitudinal smooth muscle is due to the influx of calcium through nifedipine or verapamil sensitive calcium channels and intracellular calcium mobilization plays little role in this action. Fluoride seems to selectively stimulate certain type(s) of G-protein while it leaves others unaffected. In intestinal muscle, fluoride increases IP_3 levels and protein kinase C activity but does not influence cAMP levels, while isoprenaline elevates cAMP levels markedly (Watson *et al.*, 1988). The exact relationship between fluoride-induced activation of phospholipase C and calcium influx through the plasma membrane is not known. It appears that G-proteins are involved in stimulating phospholipase C and inducing membrane depolarization by fluoride. Introduction of GTPγS into t. 'ongitudinal muscle cells elicits the membrane depolarization and calcium influx, and both the events are blocked by GDPβS (Inoue and Isenberg, 1990).

In the plexus-free preparation, fluoride produced an initial contraction and an irregular oscillation of tension. This is in contrast to the fluoride-induced contractions in the vasculature where a 5 to 10 min delay is observed, and also in contrast to lm-mp of guinea pig ileum where no fast phasic contraction is seen (Watson *et al.*, 1988). In three of four experiments here, rCGRP showed a more than 87% inhibition of NaF induced phasic contraction, but no effect on the irregular tension oscillation. Calcium channel blockers inhibit the contractions induced by KCl, fluoride and activation of both muscarinic receptors and histamine H_1 receptors. rCGRP selectively inhibits the contractions-induced by KCl, fluoride and histamine therefore acts differ-

ently from calcium channel blockers.

G. Antagonism of the action of CGRP by $hCGRP_{8-37}$

It has been shown that $hCGRP_{8-37}$ exhibits a nanomolar affinity to the CGRP binding sites in different tissues (Morita *et al.*, 1989). Also it displays no intrinsic activity but is antagonistic both in *in vivo* (Donoso *et al.*, 1990; Gardiner *et al.*, 1990; Han *et al.*, 1990; Chen *et al.*, 1992), and in *in vitro* bioassays (Chiba *et al.*, 1989; Dennis *et al.*, 1990).

$CGRP_{8-37}$ behaves as a specific CGRP receptor antagonist since it blocks CGRP-induced hypotension without affecting the hypotensive responses induced by bradykinin, histamine, substance P (Donoso *et al.*, 1990), or isoproterenol (Gardiner *et al.*, 1990; Han *et al.*, 1990); it attenuates the CGRP- but not ACh- or adenosine-induced vasodilation of rat gastric submucosal arterioles (Chen *et al.*, 1992).

The antagonistic property of this fragment of hCGRP to CGRP has been studied in the segment (Barthó *et al.*, 1991) and the lm-mp (Dennis *et al.*, 1990) preparations of guinea pig ileum. In the segment preparation, this fragment has been reported to antagonize the relaxing effect of rCGRP, but not adrenaline, on the lm contraction induced by histamine and circular muscle spontaneous twitches. In the lm-mp preparation, Dennis *et al.* (1990) found that 1 μM $hCGRP_{8-37}$ causes a blockade which increased the ED_{50} value of hαCGRP twelve-fold. Our study on the pure muscle preparation confirmed that this fragment antagonizes the direct effect of

rCGRP on the muscle cells. We observed a 5.5-fold increase in the ED_{50} value of rCGRP in the presence of 1 μM $hCGRP_{8-37}$. This is about two to three times less potent than that reported by Dennis *et al.* (1990). There are two possible explanations for the discrepancy. One is that we used the pure muscle preparation and, therefore, can exclude the nerve factors which could be involved in the lm-mp preparation. The second is that we used rat CGRP instead of human CGRP, as in experiment carried out by Dennis *et al.* (1990). The two peptides may have different potencies on the preparation used as was seen in the guinea pig ileum and basilar artery (Tippins *et al.*, 1984; Jansen *et al.*, 1992).

Receptors for CGRP have been reported in both the central nervous system and peripheral tissues (Goltzmann and Mitchell, 1985). Smooth muscles reported to possess CGRP specific binding sites are: the vascular musculature (Kubota *et al.*, 1985; Sigrist *et al.*, 1986) and gastric muscle cells (Maton *et al.*, 1988). Gates *et al.* (1989), using autoradiographic techniques to study the distribution of CGRP binding sites in canine gastrointestinal tract, found that myenteric neurons, rather than muscle cells, possess CGRP receptors in the small intestine. There has not been any direct evidence of the existence of CGRP receptors on smooth muscle cells of the small intestine. However, studies of the actions of CGRP and its fragment, $hCGRP_{8-37}$, on guinea pig ileum preparations (especially the results which we obtained on pure muscle preparation) suggest the presence of CGRP receptors. The fact that Gates *et al.* (1989) could not detect CGRP receptors on canine intestine lm may be due to

a low density or absence of receptors on canine intestine smooth muscle.

H. Effects of K channel openers on the smooth muscle

A common effect of many synthetic vasodilator agents as well as some endogenous compounds is their ability to hyperpolarize vascular smooth muscle (Cook, 1988; Brayden, 1990; Brayden *et al.*, 1991). Membrane hyperpolarization can in turn reduce voltage-gated calcium channel activity and lead to vasodilation. Opening of potassium channels or increasing the membrane permeability to potassium is one mechanism underlying the hyperpolarization. The ATP-sensitive potassium channel (K_{ATP}) is one type of potassium channel present in some smooth muscles. This channel may be important for the relaxing effect of many synthetic and endogenous compounds, such as the synthetic potassium channel openers, ACh and vasoactive intestinal peptide (Quast and Cook, 1989; Brayden, 1990). The K_{ATP} channel has been identified on the cardiac muscle (Noma, 1983), skeletal muscle (Spruce *et al.*, 1985), pancreatic beta cells (Cook and Hales, 1984), neuronal cells (Ashford *et al.*, 1988), and vascular smooth muscle cells (Standen *et al.*, 1989). A primary characteristic of this ion channel is its inhibition by intracellular ATP, as well as by sulfonylurea compounds such as glibenclamide, tolbutamide, and by low concentrations of Ba^{2+}. Nelson *et al.* (1990) found that a) the relaxing effect of CGRP on NE pre-contracted rabbit mesenteric artery strips is partially reversed, and b) CGRP-produced hyperpolarization of the artery cell membrane, is totally prevented by, glibenclamide, a

relatively selective inhibitor of K_{ATP}. Finally, this peptide opens single potassium channels in the muscle cells.

Cromakalim has been reported to inhibit the guinea pig ileum peristaltic reflex *in vitro*, and the guinea pig small intestine transit *in vivo* (Buchheit and Bertholet, 1988). Pinacidil inhibits spontaneous and KCl-induced contractions in the guinea pig taenia coli with an IC_{50} of 3-5 μM, while nicorandil is less potent (Matsui *et al.*, 1986). Both high and low affinity binding sites for ^{3}H-glibenclamide in the guinea pig lm-mp preparation have been recently reported (Gopalakrishnan *et al.*, 1991; Zini *et al.*, 1991). More and more evidence shows that synthetic K channel openers activate K_{ATP} (Cavero *et al.*, 1989; Quast and Cook, 1989b; Winquist *et al.*, 1989; Brayden *et al.*, 1991). To examine whether rCGRP has an K_{ATP} opening effect, K_{ATP} must be shown to exist in the muscle. If K_{ATP} is present on the lm, these potassium channel openers should show inhibitory effects on the lm as is the case in vascular smooth muscle.

We have demonstrated that both pinacidil and cromakalim relax the KCl-precontracted plexus-free lm of GPI. This result is consistent with the actions of these two compounds on other smooth muscles (Allen *et al.*, 1986; Matsui *et al.*, 1986; Weir and Weston, 1986a; Bray *et al.*, 1987; Mellemkjaer *et al.*, 1989). Leurs *et al.* (1991a) recently reported that pinacidil relaxed a KCl (40 mM) -precontracted guinea pig jejunal preparation (lm-mp) with an IC_{50} value of 120 μM. Nicorandil relaxes smooth muscle similarly precontracted by KCl (Holzman, 1983; Weir and

Weston, 1986b). This compound, however, did not show any inhibitory effect on lm of GPI in the present study.

None of these three compounds shows inhibitory effects on oxotremorine (5 μM) -precontracted lm in the present study. Zini *et al.* (1991) found that cromakalim, at concentrations up to 5 μM, does not inhibit the contraction produced by carbachol (at concentrations of 3 nM and more) while cromakalim, lemakalim, diazoxide and two other potassium channel openers dose-dependently inhibit the electrical field stimulated guinea pig ileum lm-mp preparation. They concluded that in the ileal preparation, these potassium channel openers may act presynaptically, inhibiting ACh release. These findings are consistent with our results from the plexus-free lm. However, Leurs *et al.* (1991a) found that in guinea pig jejunal muscle, pinacidil inhibits the contraction produced by methacholine (1 μM), an agonist of muscarinic receptors. The IC_{50} value is about 8 μM (Leurs *et al.*, 1991a). Different actions of these K_{ATP} openers on different regions of the intestine may reflect differences in tissue sensitivity to these compounds.

Pinacidil inhibits histamine (1 μM)-induced contraction of guinea pig trachea and pulmonary artery with a IC_{50} value of 1-3 μM (Mellemkjaer *et al.*, 1989). This compound does not display an inhibitory effect on histamine-induced contractions in lm of GPI. Nicorandil and cromakalim have been shown to inhibit the contractions induced by various agonists on vascular smooth muscle: i.e. by norepinephrine, angiotensin II and endothelin (Hamilton *et al.*, 1986; Weir and Weston, 1986a; Bray *et al.*,

1987; Quast and Cook, 1989; McPherson and Angus, 1990; Erne and Hermasmeyer, 1991). It is interesting that, in lm of GPI, both nicorandil and cromakalim, each at a concentration of 50 μM, have a potentiating effect, rather than an inhibitory effect, on the contractions produced by histamine (5 μM).

One of the results of the activation of both muscarinic and histamine H_1 receptors is depolarization of the cell membrane of guinea pig lm (Bolton and Clark, 1981a,b). It is, therefore, reasonable to deduce that potassium channel openers could antagonize the contractions induced by activation of these two types of receptors if K_{ATP} is present on the lm of GPI. Inability of these compounds to inhibit the oxotremorine-induced contraction, as well as inability of pinacidil to inhibit the histamine-induced contraction, may be due to the low potencies of these compounds and the relatively high concentrations of the agonists used. However, these agents, at the same concentrations, inhibit KCl-induced contractions. Moreover, it does not seem reasonable that the potentiating effect of nicorandil and cromakalim on histamine-induced contractions is a result of their potassium channel opening effect. It appears unlikely that there are the potassium channels sensitive to nicorandil, pinacidil and cromakalim on the longitudinal smooth muscle of the guinea pig ileum. Our primary results show that the relaxing effect of rCGRP on histamine-pre-contracted plexus-free longitudinal muscle is not reversed by glibenclamide as in the report by Nelson *et al.* (1990). These potassium channel openers may, in fact, have actions on the smooth muscle in addition to their channel opening effects.

Yanagisawa *et al.* (1990), simultaneously recording [illegible] and $[Ca^{2+}]_i$ in the canine coronary artery strip, found that nicorandil and pinacidil shift the $[Ca^{2+}]_i$-force curve for KCl to the right while cromakalim does not affect the curve. In other words, nicorandil and pinacidil depress the KCl-induced tension more than the $[Ca^{2+}]_i$, indicating that an additional mechanism is involved in the muscle relaxing actions of these compounds. Stimulation of guanylate cyclase may be involved in the action of nicorandil on blood vessels (Borg *et al.*, 1991). Thus, the mechanisms of actions of potassium channel openers on the lm need further investigation.

I. Summary

This study provides the first direct evidence for effects of rCGRP on the longitudinal smooth muscle of the small intestine. rCGRP selectively inhibits the contractions produced by some stimulants and agonists. The inhibitory effect of rCGRP on histamine-induced contraction can be antagonized competitively by the CGRP receptor blocker, $hCGRP_{8-37}$.

Thus far, this work has investigated the action sites of CGRP on the lm-mp preparation and deduced that part of inhibitory effect of rCGRP on the lm-mp occurs through a direct action on the smooth muscle. This hypothesis is substantiated by the results from studies on the plexus-free longitudinal smooth muscle described in this chapter. However, the exact mechanisms of action of this peptide on the muscle remain unclear. At least two possible mechanisms might exist. rCGRP might

stimulate the production of cAMP which relaxes the elevated tension. It has, in fact, been reported that rCGRP activates adenylate cyclase in several types of tissues (Kubota *et al.,* 1985; Sigrist *et al.,* 1986; Maton *et al.,* 1988). On the other hand, rCGRP may interfere with the calcium balance in muscle cells. These possibilities will be examined in the following chapter.

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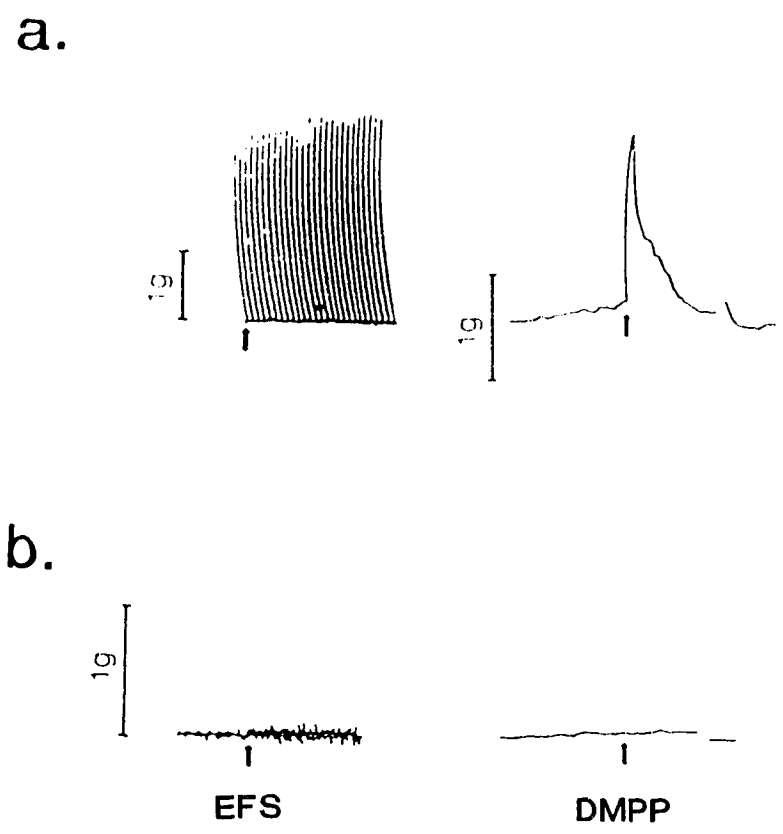


Figure 3-1. Responsiveness of lm-mp and lm to EFS and DMPP. 5 μM DMPP and maximal electrical field stimulation (EFS, 0.1 Hz, 1 msec and 9 V/cm) were used. (a) Response of the lm-mp to EFS (left) and DMPP (right). (b) Response of the plexus-free lm to EFS (left) and DMPP stimulation (right).

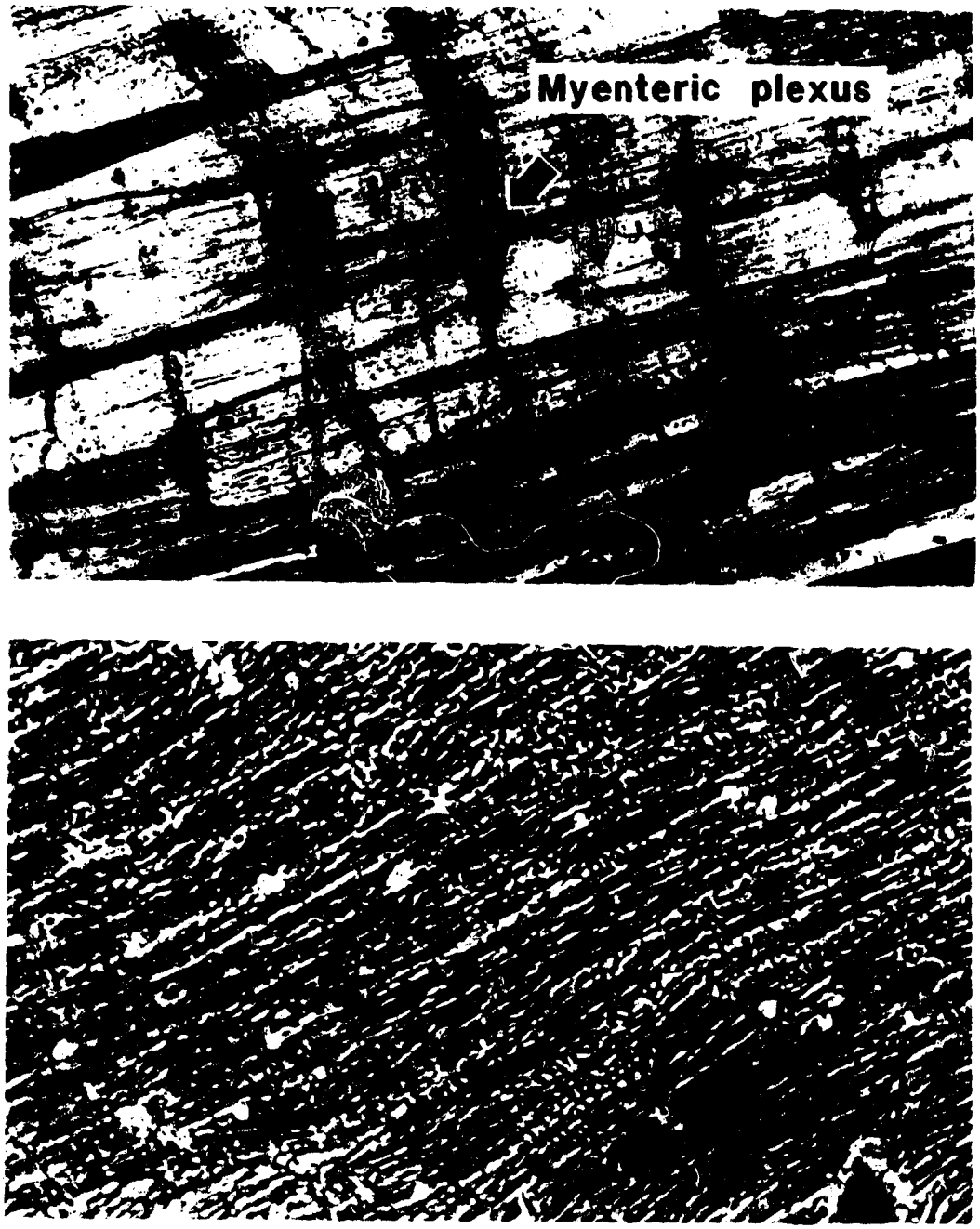


Figure 3-1(c). Upper: Morphological appearance of lm-mp, 400X magnification. Lower: Morphological appearance of lm, 400X magnification.

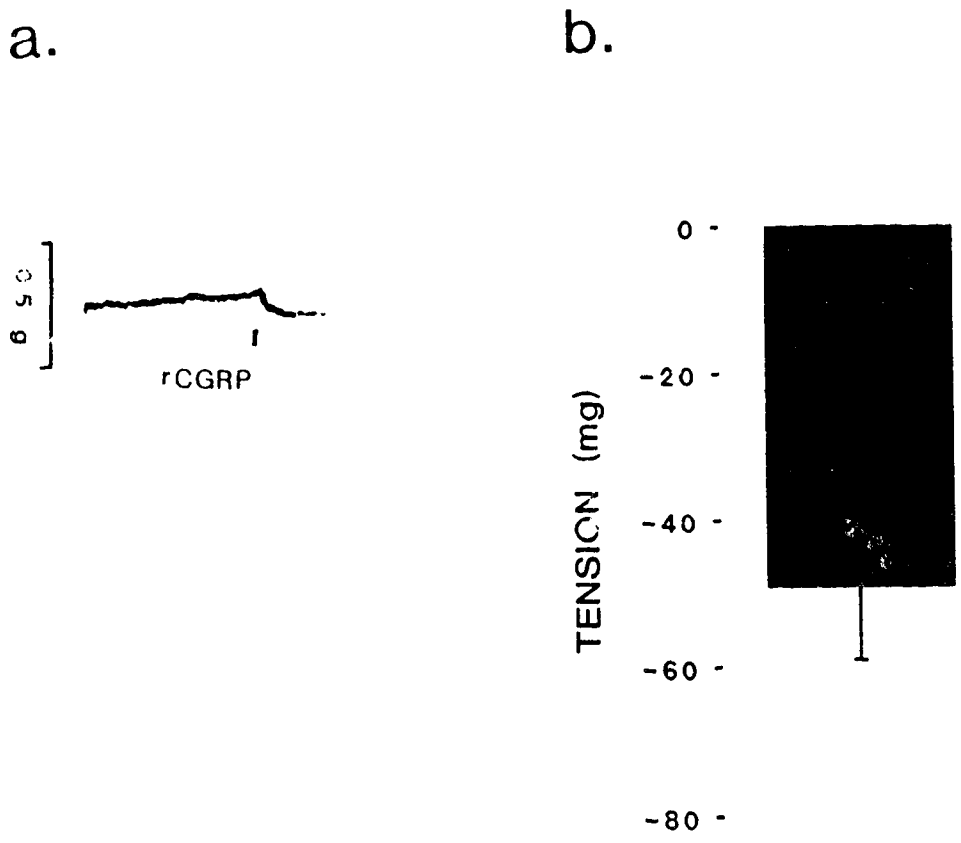


Figure 3-2. Effect of rCGRP on non-stimulated plexus-free lm. (a) Tracing of tension in the presence of 263 nM of rCGRP. (b) Summary of results from ten experiments. The value is expressed as mean ± S.E.

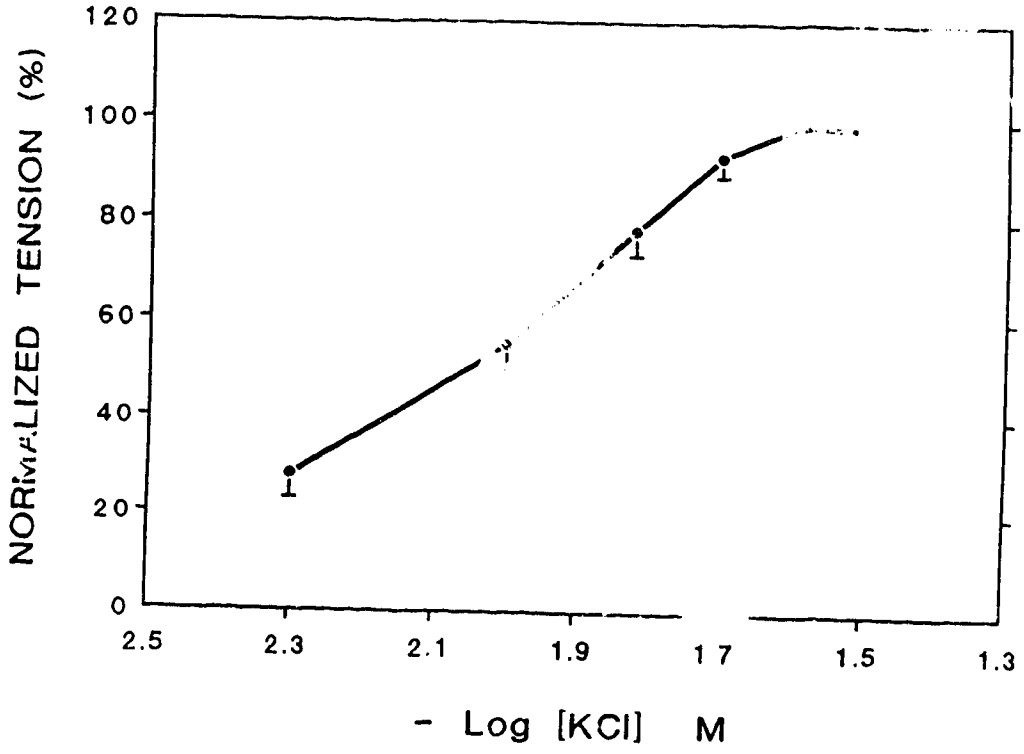


Figure 3-3. Dose-response relationship of KCl. Each point is mean ± S.E. from seven experiments.

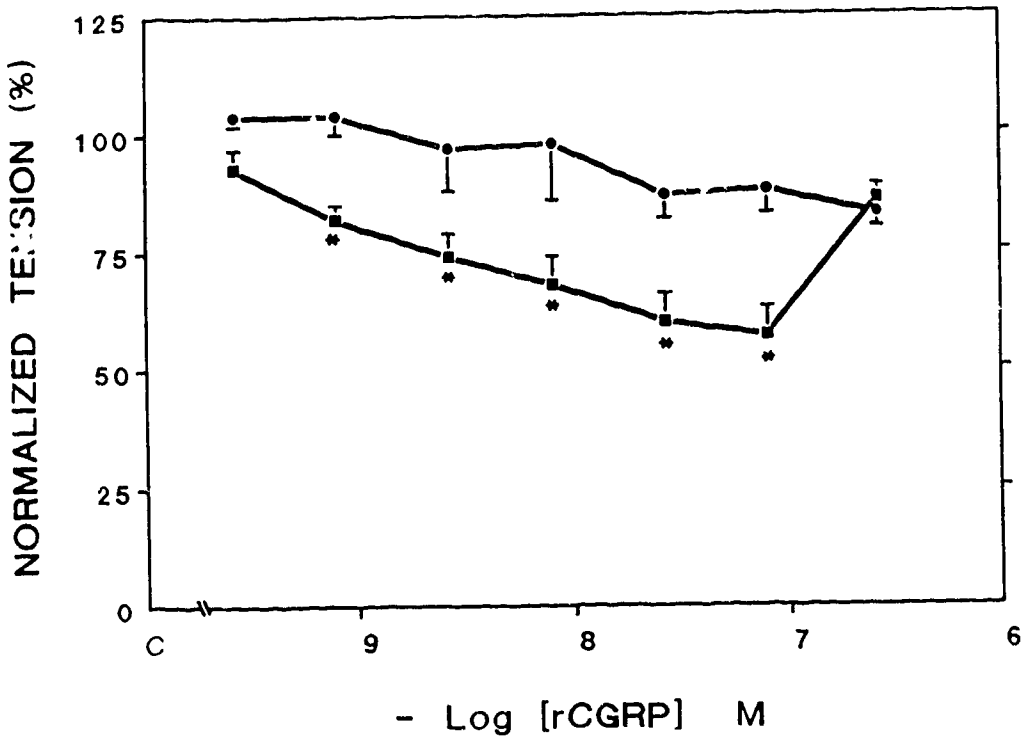


Figure 3-4. Effect of rCGRP on KCl (30 mM)-induced initial (•) and tonic (■) contraction of plexus-free lm. Data are expressed as mean ± S.E. from five experiments. *Significantly from the control (Newman Keuls test).

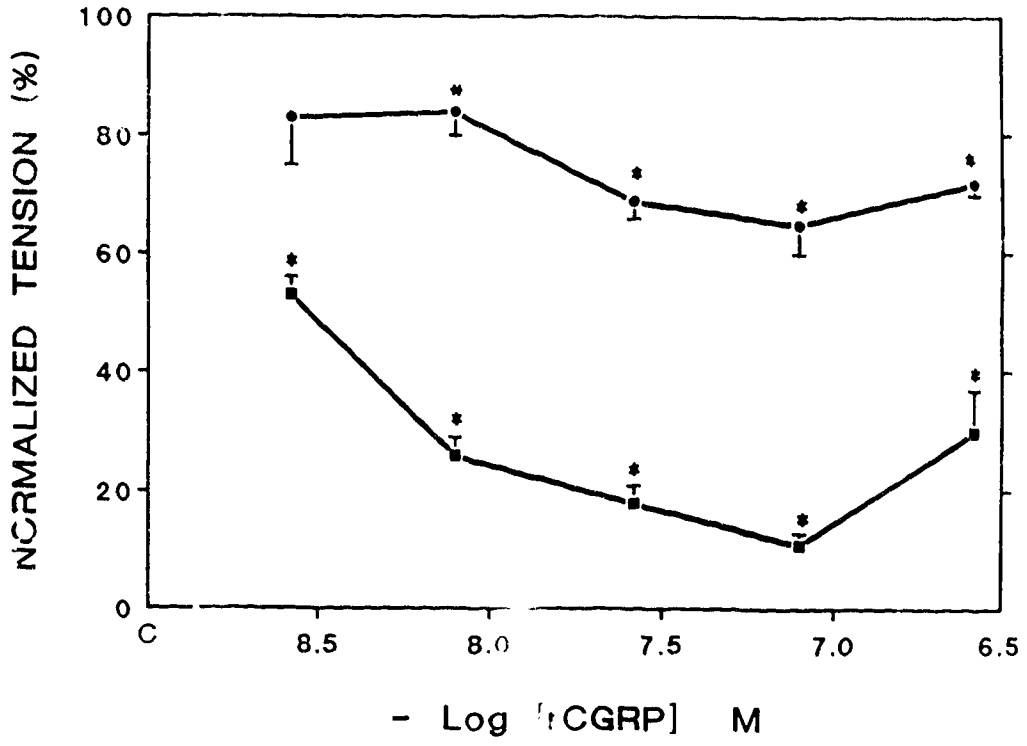


Figure 3-5. Effect of rCGRP on the histamine (1 μM) -induced initial (•) and tonic (■) tension of plexus-free lm. Data are represented as mean ± S.E. of four (initial) and five (tonic) experiments. *Significantly different from the control (Newman Keuls test).

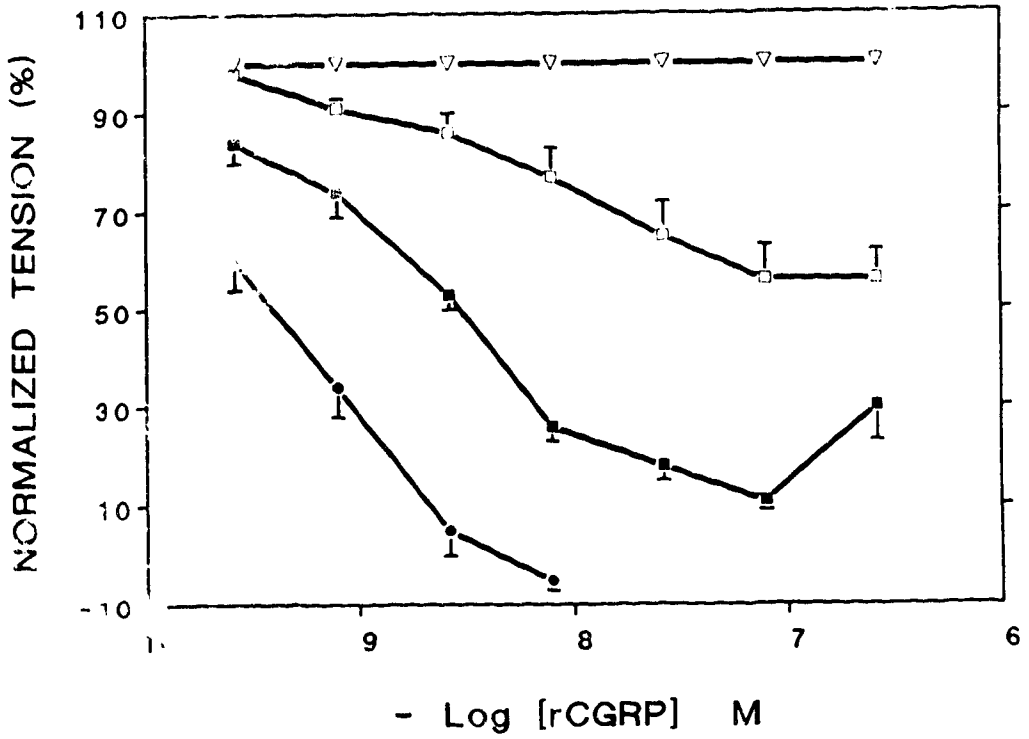


Figure 3-6. Effect of rCGRP on the tonic contraction induced by histamine. Contractions were produced by histamine at concentrations of 0.1 μM (•), 1 μM (■), 10 μM (▫) and 100 μM (▽) and rCGRP (from 0.26 nM to 263 nM) was applied cumulatively. Data are mean ± S.E., n=5.

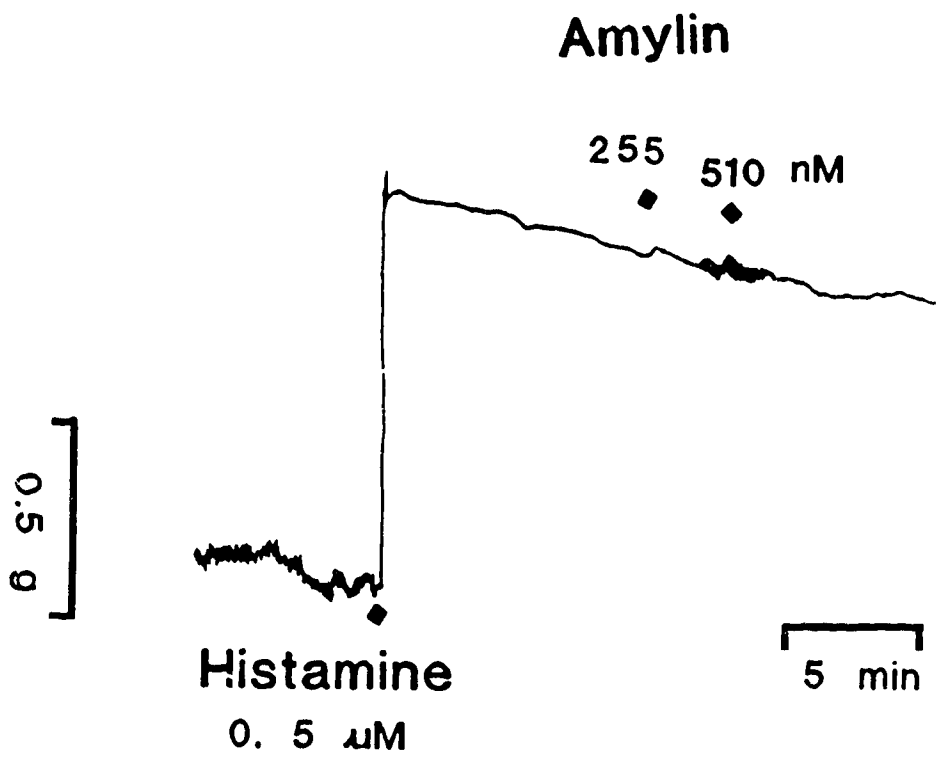
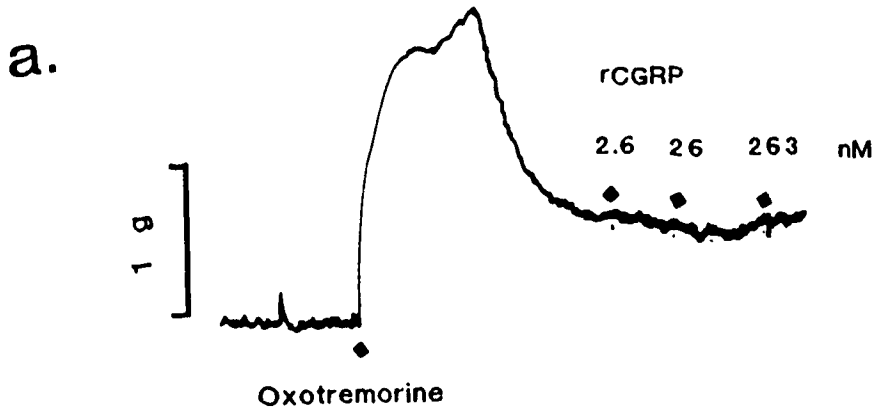


Figure 3-7. Effect of rat amylin on histamine-induced contraction.



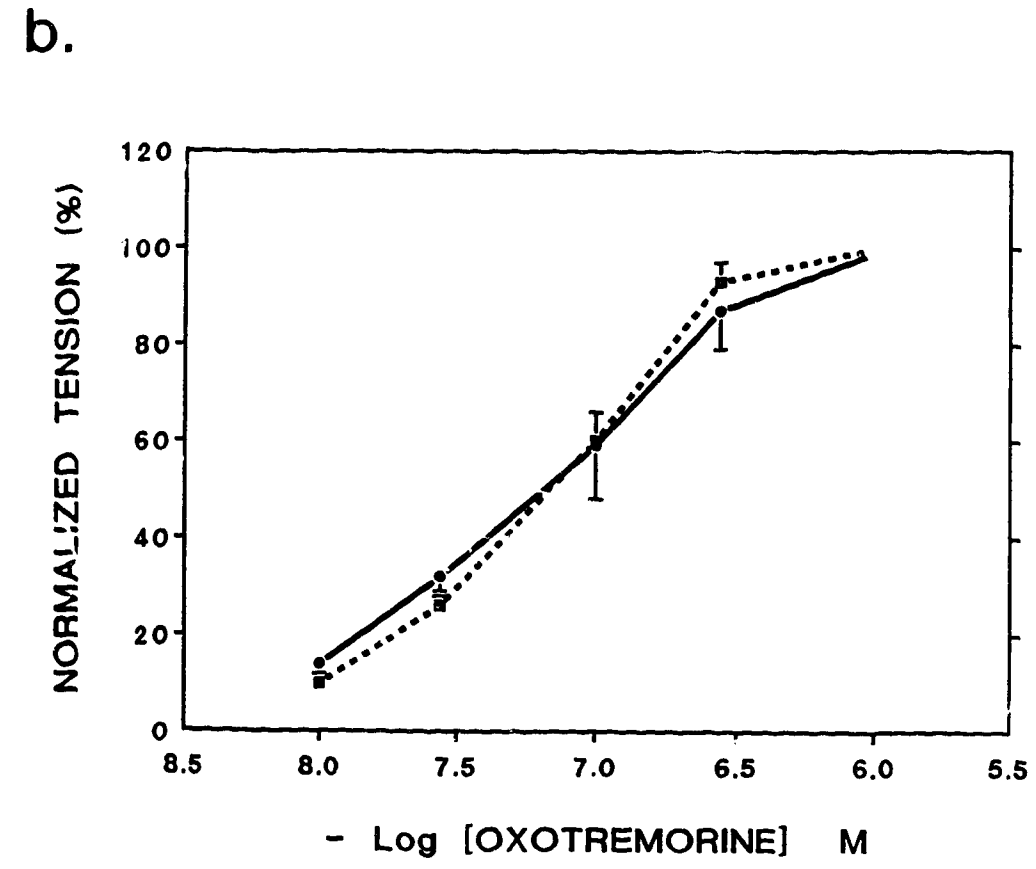


Figure 3-8. Influence of rCGRP (263 nM) on the contraction induced by oxotremorine in plexus-free lm. (a) Effect of rCGRP on the tonic tension. (b) Contractions were induced by cumulatively applied oxotremorine (from 10 to 1 μM)in the absence (•) or presence (■) of rCGRP. Results are mean ± S.E. from four experiments.

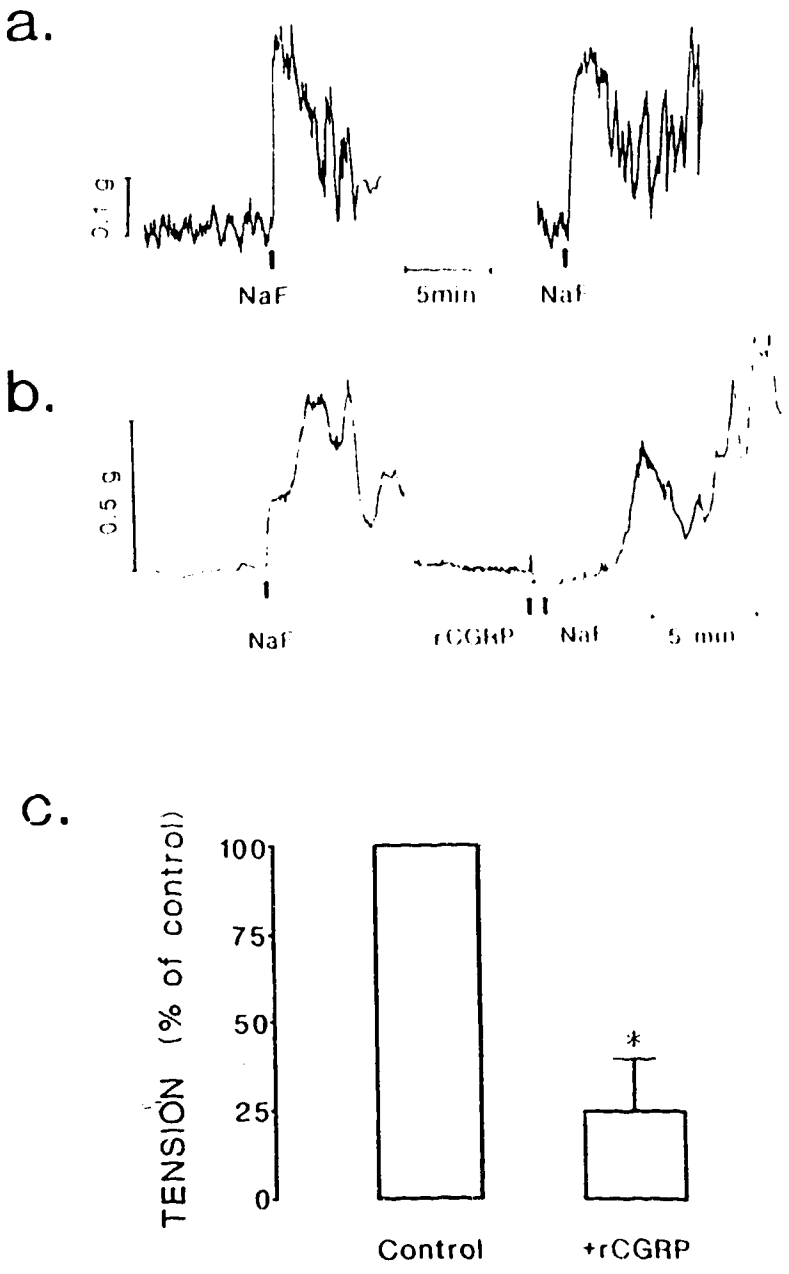


Figure 3-9. Effect of rCGRP (263 nM) on the contraction evoked by NaF (10 mM) in plexus-free lm. a) Tracing of tension induced by NaF; b) tracing of tension induced by NaF in the presence of rCGRP; c) summary of results from b. Data are expressed as mean ± S.E., n=4. *Significantly different from the control (Student *t*-test).

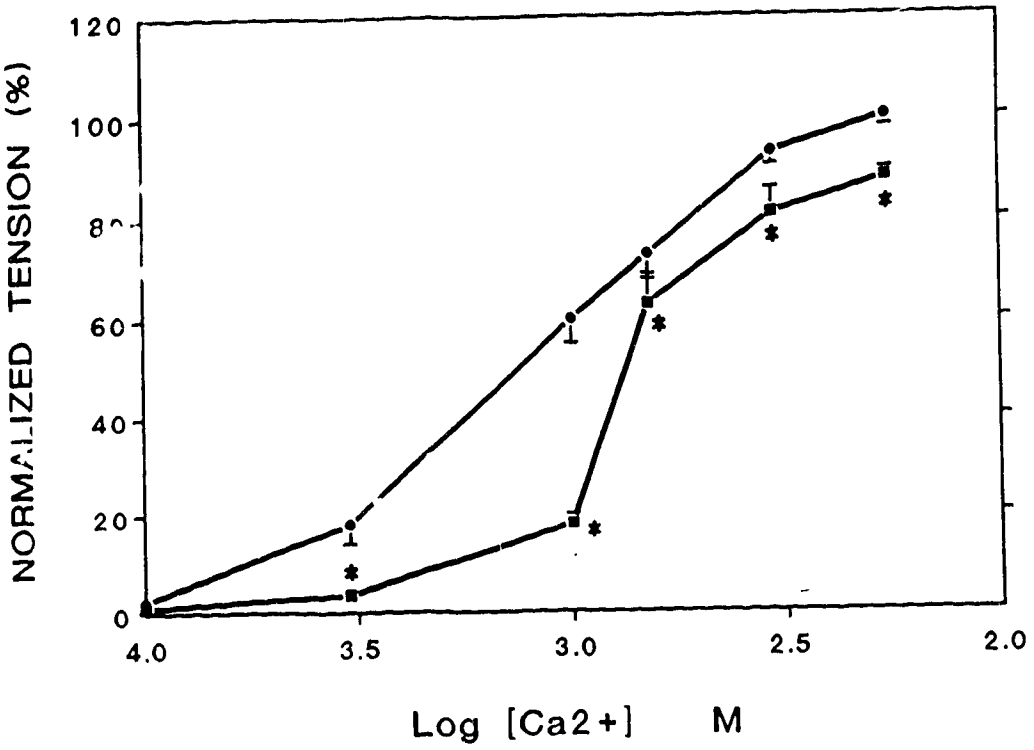


Figure 3-10. Inhibitory effect of rCGRP on KCl-induced extracellular calcium-dependent contraction of plexus-free Im. In calcium-fr physiological solution and the presence of 30 ...M of KCl, contractions were produced by cumulative dose of calcium. rCGRP (263 nM; ■) was added 2 min before the calcium stimulation. Each value is mean ± S.E., n=5. *Significantly different from the control (Student *t*-test).

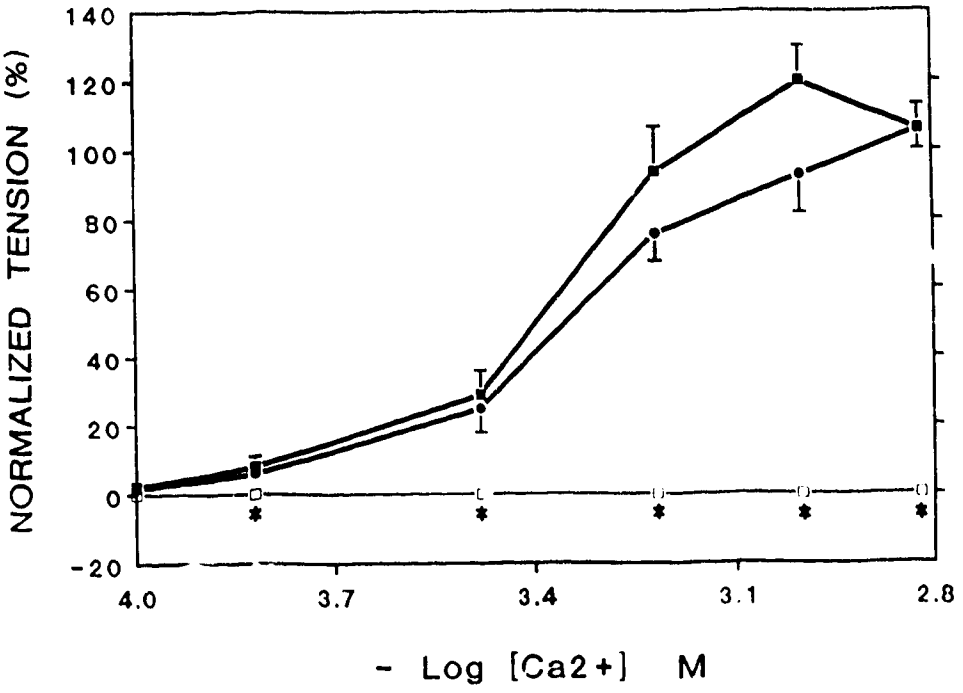


Figure 3-11. Influence of rCGRP (263 nM) and nifedipine (1 μM) on the histamine (1 μM)-induced extracellular calcium-dependent contraction of plexus-free lm. In the presence of histamine in the calcium free physiological solution contractions were produced by addition of $CaCl_2$ in the absence (•) or presence of rCGRP (■) or nifedipine (▫). Data are expressed as mean ± S.E., n=6. *Significantly from the control (Newman-Keuls test).

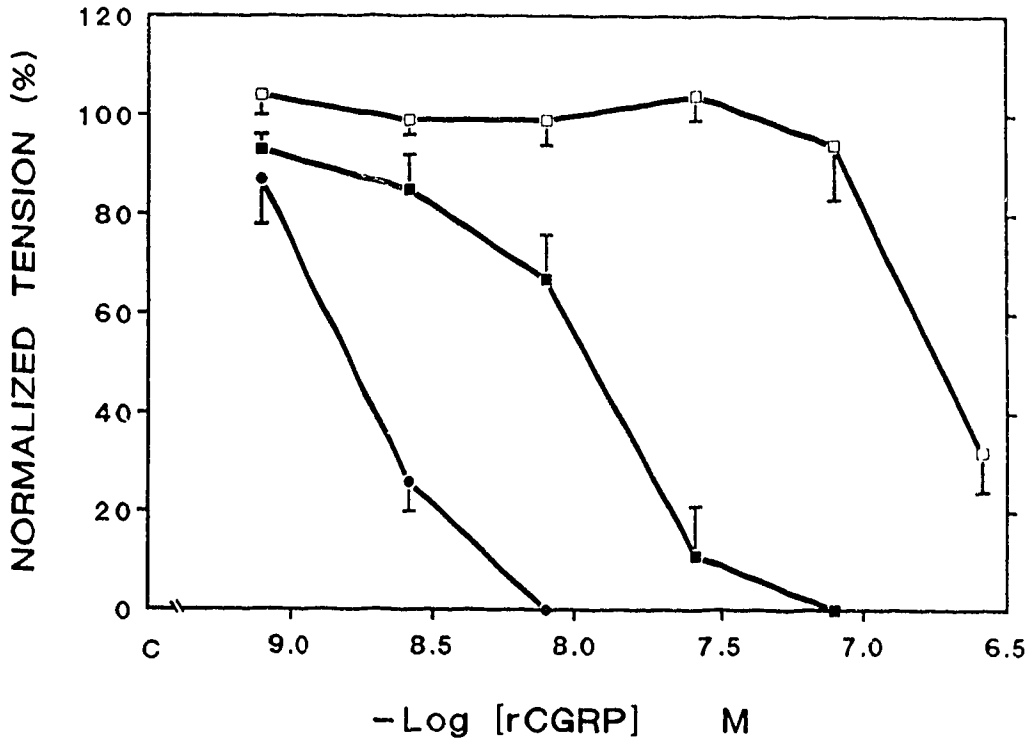


Figure 3-12. Effect of haCGRP$_{8-37}$ on rCGRP-produced relaxation of precontracted plexus-free lm by histamine. Tonic contractions were induced by 50 nM histamine and rCGRP was applied cumulatively (from 0.79 nM to 263 nM) in the absence (•) or presence (1 μM,■; and 10 μM □) of hCGRP$_{8-37}$. Each point is mean ± S.E., n=4.

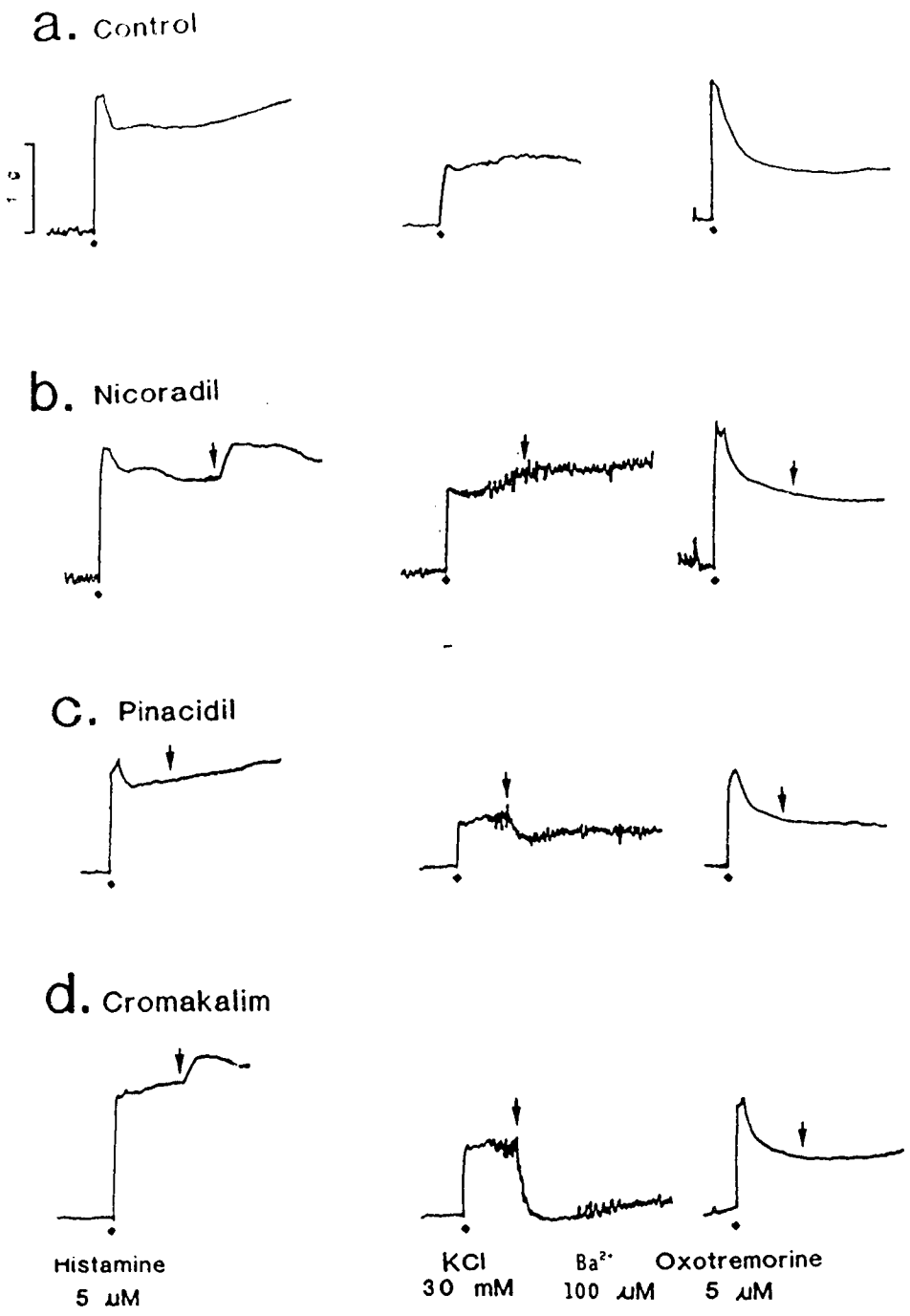


Figure 3-13. Effect of K channel openers on the tonic contractions produced by histamine, KCl and oxotremorine. The same stimulant was used in each column and the same K channel opener in each row of tracings.

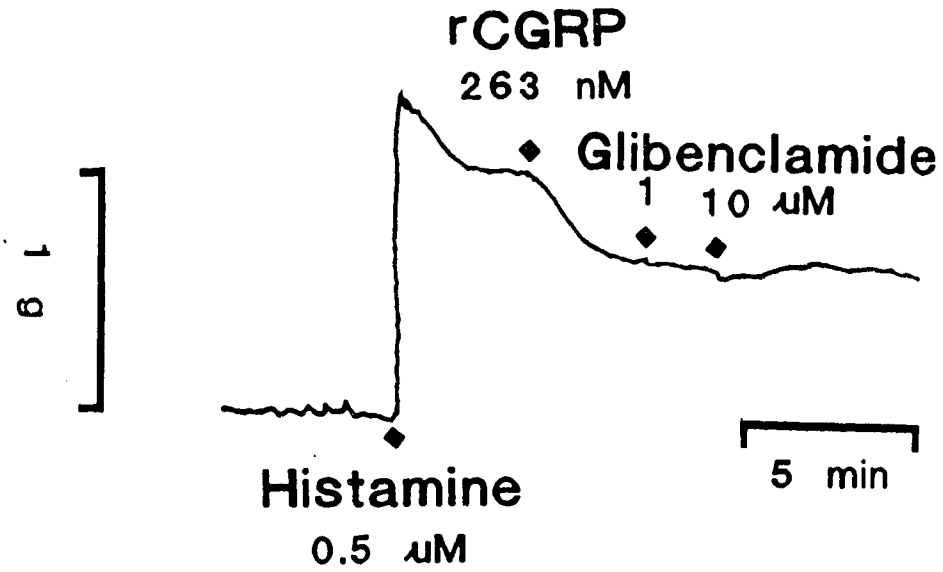


Figure 3-14. Effect of glibenclamide on the relaxing actions of rCGRP in plexus-free lm.

CHAPTER 4. MECHANISMS OF ACTIONS OF rCGRP ON SMOOTH MUSCLE

I. INTRODUCTION

It is generally accepted that Ca^{2+} is the primary regulator of contraction in smooth, as well as striated muscle (Bolton, 1979; Johansson and Sömlyo, 1980). Stimulants mobilize calcium at both the plasma membrane level and the intracellular level through different mechanisms and finally increase the $[Ca^{2+}]_i$ and contract the muscle (Triggle *et al.*, 1989). Smooth muscle relaxing agents could affect these mechanisms to decrease the $[Ca^{2+}]_i$ and relax the muscle (Triggle *et al.*, 1989; Consigny, 1991).

The recent development of fluorescent calcium indicators (Tsien, 1980, 1981; Grynkiewicz *et al.*, 1985) has made the measurement of $[Ca^{2+}]_i$ at both resting and activated conditions a widely used method. Cultured muscle cells or cell suspensions are the preparations typically used for the measurement of $[Ca^{2+}]_i$ in studying muscle constrictors and relaxants. However, techniques have recently been developed for the simultaneous recording of $[Ca^{2+}]_i$ and tension. These techniques make it possible to correlate directly the change in $[Ca^{2+}]_i$ and the change in tension (Neering and Morgan, 1980; Morgan and Morgan, 1982, 1984a,b; Patchard and Shley, 1986; Williams and Fay, 1986; Himpens and Casteels 1987; Ishii *et al.*, 1987, Ozaki *et al.*, 1987; Himpens and Sömlyo, 1988).

The previous studies (Chapters 2 and 3) suggest that CGRP may relax the

contracted lm by acting on some events related to cell calcium regulation. Therefore, the effects of this peptide may be reflected in altered responses in $[Ca^{2+}]_i$. The purpose of the study described in this chapter is to determine the effects of CGRP on the $[Ca^{2+}]_i$ response in muscle precontracted by histamine and other stimulants. Simultaneous recording techniques were used in these investigations. Furthermore, these studies will examine the possible role of cyclic nucleotides in the smooth muscle cell response to CGRP.

II. METHODS

Guinea pig plexus-free lm was prepared as described in Chapter 3. The prepared sheets were thin and about 7 mm wide by 20 mm long.

A. Tension-$[Ca^{2+}]_i$ simultaneous recording

1. Loading with Fura-2

The tissue strip was loaded with Fura-2 according to the method of Himpens and Sömlyo (1988) with slight modifications. To load the strips, three strips were incubated in the dark in 3 ml of HEPES-buffered Krebs solution containing 1.2 mM Ca^{2+}, 5 μM Fura-2/AM and 0.01% Pluronic F127. The HEPES-buffered modified Krebs solution contained (mM): NaCl, 135.5; KCl, 5.9; $CaCl_2$, 1.2; $MgSO_4$, 1.2; HEPES, 11.6; and glucose 11.6; pH 7.4. Pluronic F127 is a high molecular weight surfactant that helps to solubilize large molecules of dye in physiological media

(Poenie *et al.*, 1986). It was used to facilitate the tissue uptake of Fura-2/AM. Loading was performed at room temperature for 3 hr with a stirring bar rotating at low speed to facilitate the loading. The loading solution was continuously oxygenated with 95% O_2:5% CO_2. The strips were then removed from the loading solution and transferred to a large volume (about 100 ml) of fresh Krebs solution for at least 1 hr at room temperature to insure the complete conversion of Fura-2/AM to Fura-2 by the cells, and to remove the dye bound to the outside of the tissue. One strip was then transferred to, and fixed in, the measurement cuvette. The remaining strips were stored in the oxygenated solution in the dark for subsequent experiments.

The lm strip was fixed vertically in a cuvette containing 3 ml Krebs solution. One end of the strip was attached to an isometric force transducer (Grass FT.03), while the other end was fixed to the stainless steel wire near the bottom of the cuvette. Oxygen was introduced into the solution through stainless steel tubing. The buffer was stirred throughout the experiment to insure rapid mixing of the added compounds with the physiological medium. The position of the muscle strip was adjusted to lie exactly in the path of the excitation beam. In order to diminish the loss of dye from inside the cells by leakage at high temperature, the experiment was carried out at room temperature (23-25°C). The physiological medium was replaced every 15 min during the 1 hr equilibration period to provide adequate nutrients.

The tension-$[Ca^{2+}]_i$ measurement system was a combination of a polygraph (Grass model 79D) and a FluoroPlex III (Tracor Northern) spectrofluorometer. Tension could be recorded continuously for the duration of the experiment while the

fluorescence signals could only be recorded at specific intervals. The fluorescence signal sample rate was reduced to 1.0/sec to increase the measurement period (sampling rate 1/sec, 200 sec for each measurement). Because the FluoroPlex III spectrofluorometer was not initially designed for simultaneous recording, the tension signal and fluorescence intensity signals were recorded separately. Timing, therefore, was very critical. On the other hand, because of the limitation in the measurement period of the FluoroPlex III spectrofluorometer, it was difficult to obtain the data needed for analysis of the initial change of $[Ca^{2+}]_i$. Therefore, it was not possible to analyze the kinetics of the change in $[Ca^{2+}]_i$ or the detailed relationship between the change in $[Ca^{2+}]_i$ and the initial change of the tension after the addition of different agents. Those data for the initial changes would have been very useful in analyzing the role of $[Ca^{2+}]_i$ in initiating contractile events of smooth muscle cells.

The loaded tissue was exposed to an excitation beam altering between wavelengths of 340 and 380 nm. The fluorescence was measured at 510 nm. The autofluorescence was measured in an unloaded strip ot lm from the same guinea pig and was subtracted from the fluorescence signals of the loaded tissue. The drugs tested induced little change in autofluorescence compared to the change in fluorescence evoked by the same agents in the Fura-2-loaded strip.

The ratio of the fluorescence excited at 340 nm (F_{340}) divided by that excited at 380 nm (F_{380}) was calculated. The fluorescence ratio was converted to $[Ca^{2+}]_i$ using the equation $K_D[(R-R_{min})/(R_{max}-R)]\beta=[Ca^{2+}]_i$, as described by Grynkiewicz *et al.* (1985). R is the measured F_{340}/F_{380} ratio (corrected for autofluorescence) of the

loaded tissue. R_{max} is the ratio obtained when the tissue was exposed to digitonin, and R_{min} is the ratio obtained when the tissue was exposed to 10 mM EGTA. β is the ratio of F_{380} for Fura-2 loaded tissue in the presence of minimal free calcium (EGTA) divided by that for the loaded tissue in saturating free calcium (digitonin). One hundred μl of a saturated aqueous solution of digitonin added to 3 ml of the modified Krebs solution evoked maximal contraction of the lm. Therefore, this concentration of digitonin was considered to be high enough to permeabilize the cells and to saturate the Fura-2 with a high calcium concentration. K_D was calculated as described by Grynkiewicz *et al.* (1985).

2. *Tension-$[Ca^{2+}]_i$ simultaneous recording*

The tissue was stimulated with 0.5 μM histamine (final concentration) and the muscle relaxing agents (rCGRP, forskolin, nifedipine and verapamil) were added 2.5 min after histamine challenge. A saturated aqueous solution of digitonin (100 μl) was added to permeabilize the cells and obtain the R_{max}. After the tension reached the maximal response to digitonin, EGTA was introduced to the solution to a final concentration of 10 mM to obtain the R_{min}. In the control experiment, no relaxing agent was added. Recording of both mechanical and fluorescence signals started before the addition of histamine. KCl and oxotremorine were also used as stimulants.

The values of both the mechanical and the fluorescence signals at 2.5 min after histamine challenge were defined as 100%. Responses after that time point were then calculated as a percentage of that control response.

B. Intracellular cyclic nucleotides measurement

1. Tension-[cAMP] simultaneous recording

Each plexus-free muscle strip was fixed between a force displacement transducer and a stainless steel wire with an initial tension load of 0.7 g. The whole set was then slid into the chamber filled with oxygenated Krebs solution. After equilibration for 1 hr, the tissue was challenged by different agents. At designated time points, the tissue was slid up and out of the chamber and frozen rapidly in a cup of liquid nitrogen.

The strips were challenged with histamine (0.5 μM) and the induced tension development was measured continuously. The tissue pieces were immediately frozen with liquid nitrogen at times of 0, 2.5, 3.5, 5.5, 7.5 or 12.5 min after the histamine challenge (control). rCGRP was added 2.5 min after histamine administration and each tissue was then individually frozen at 3.5, 5.5, 7.5 or 12.5 min. Comparisons were made between the cyclic nucleotide content at 2.5 min after histamine administration and that at later time points in control or rCGRP-treated tissues, and also between control and rCGRP-treated strips at the same time point.

Protein content assay was carried out according to Lowry *et al.* (1951).

2. Tissue extraction

Tissue was extracted according to method described by Honma *et al.* (1977). The frozen piece of tissue was transferred into 100 μl of a 0.1 N HCl-10 mM EDTA solution and homogenized with a 0.5 ml glass homogenizer. The homogenizer was

rinsed with 500 μl of acetate buffer (50 mM sodium acetate, and 0.01 sodium azide, pH 5.8). The mixture was boiled for 3 min and was then centrifuged at 1,500×g at 4°C for 10 min. The supernatant was collected for measurement of both cyclic AMP and cyclic GMP and the pellet was dissolved in 0.5 ml of 1 N NaOH for protein assay.

3. Measurement of cyclic nucleotides

Cyclic AMP and cyclic GMP were measured by radioimmunoassay using a scintillation proximity assay (SPA) kit from Amersham. Sample supernatant (500 μl), 500 μl of acetate buffer (for nonspecific binding and total binding) or 500 μl of cAMP or cGMP standard solutions were each mixed with 25 μl of acetylation agent (acetic anhydride:triethylamine = 1:2) for at least 3 min. Aliquots (100 μl) of each sample (cAMP or cGMP standard solution, or buffer solution) were mixed with 100 μl of tracer (adenosine- or guanosine-3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester), 100 μl of rabbit anti-succinyl-cAMP or -cGMP serum and donkey anti-rabbit IgG coupled to SPA fluomicrospheres. 100 μl buffer, rather than 100 μl antiserum, was added in non-specific binding tubes. The mixture was incubated on a shaker for 15 to 20 hr at room temperature. The amount of [^{125}I]cAMP or [^{125}I]cGMP bound to the fluomicrospheres was determined by counting the incubation vials in a β-scintillation counter (LKB 1209/1215 Rack Beta with the window open from 5 to 999) for 2 min.

C. Materials

The FluoroPlex III spectrofluorometer was purchased from Tracor Northern and the Polygraph from Grass Instruments Co. Fura-2 salt, Fura-2/acetoxymethyl ester (Fura-2/AM) and Pluronic F127 were obtained from Molecular Probes, and were dissolved in dimethylsulfoxide/pure ethanol (1:1 in vol.) to a concentration of 1 mg/ml. Rat αCGRP was purchased from Biochem Inc., histamine, forskolin, nifedipine and EGTA from Sigma, digitonin from Behring Diagnostics, and the cyclic AMP and cyclic GMP assay systems from Amersham.

D. Statistics

Student *t*-test, Newman-Keuls test and Duncan Multiple Range test were used as indicated in each figure. *P* values less than 0.05 are considered as significant. All the data are expressed as mean ± S.E.

III. RESULTS

A. Tension-$[Ca^{2+}]_i$ simultaneous recording

1. Methodology

When exposed to excitation at 340 and 380 nm (data not shown), rCGRP, forskolin, nifedipine and verapamil did not exhibit fluorescence at the concentrations used in this study.

In dye-unloaded tissue, the autofluorescence was 33±4% (F_{340}, n=24) and 34±4% (F_{380}, n=24) of the signals of the dye in Fura-2-loaded tissue. Histamine

induced a slight decrease in both F_{340} and F_{380} by 200 to 300 counts. The basal levels of fluorescence of the dye inside the cells were 17746±2296 and 14854±1434 (fluorescence counts corrected for autofluorescence) for F_{340} and F_{380} (n=24), respectively. Histamine (0.5 μM) stimulation resulted in an increase of 24±4% in F_{340} (21635±2738), a decrease of 8±3% in F_{380} (13883±1422, n=24) and an average elevation of 0.40±0.05 in the ratio (n=18). Both the basal levels of fluorescence and histamine-stimulated changes in fluorescence in loaded tissue were much higher than the histamine-induced changes in the autofluorescence of the unloaded tissue. The induced changes in autofluorescence were 1.4 to 2.2% of the fluorescence of Fura-2-loaded tissue (corrected for autofluorescence). Therefore, histamine-induced autofluorescence changes should not significantly affect the accuracy of the measurement of intracellular free calcium concentration. The F_{340} and F_{380}, especially in the late period of the experiments, showed a slow and steady decrease which may be a result of a slow leakage of dye out of the muscle cells. The fluorescence ratio, however, remained at a constant level.

In initial experiments, we found all the stimulants (KCl, histamine and oxotremorine) increased the $[Ca^{2+}]_i$. Addition of 0.5 μM histamine, 15 mM K^+ or 0.1 μM oxotremorine induced an increase in F_{340} and a decrease in the F_{380}. Therefore, there was an increase in the ratio of F_{340}/F_{380} and $[Ca^{2+}]_i$ (Figs. 4-1 and 4-2). Atropine abolished the oxotremorine-induced concentration increase (Fig. 4-2). rCGRP (263 nM) did not affect the tonic increase in $[Ca^{2+}]_i$ produced by KCl (Fig. 4-1). Oxotremorine was not used as a stimulant in the simultaneous recording study

because rCGRP showed no effect on the oxotremorine-induced contraction in the tension study.

2. Histamine-induced change in tension and $[Ca^{2+}]_i$

Histamine (0.5 μM) evoked a parallel change in tension development and $[Ca^{2+}]_i$ in the phasic phase, but not in the tonic phase. While the $[Ca^{2+}]_i$ level in the tonic phase remained constant, the histamine-induced tension decreased with time; at 20 min after the addition of histamine, tension was 43±6% (n=8) of the value at 2.5 min after histamine challenge (Fig. 4-3). Digitonin elicited an increase in both the fluorescence ratio and tension development. 10 mM EGTA decreased both the ratio and tension, and brought them both back to and then below the basal level (Fig. 4-3).

3. Effects of smooth muscle relaxing agents on histamine-stimulated tension and $[Ca^{2+}]_i$

a. rCGRP

rCGRP (263 nM) produced a time dependent relaxation of the lm. The histamine-induced tonic tension was relaxed by 79±7% (n=8) compared to the control tissue. However, the effect of the peptide on the $[Ca^{2+}]_i$ has two phases. The initial decrease in $[Ca^{2+}]_i$ paralleled the change in tension. In the second phase, $[Ca^{2+}]_i$ returned to the control level. During the first 5 min, rCGRP caused a maximal reduction in $[Ca^{2+}]_i$ of 25±9% and the tension was depressed by 50%. After 5 min, $[Ca^{2+}]_i$ gradually returned to the control level, while the tension was reduced further

to 21±7% of control. The effect of this peptide on the $[Ca^{2+}]_i$ was weak and brief while it exhibited a more consistent and profound inhibitory action on the tension (Fig. 4-4).

b. Forskolin

Forskolin (0.5 μM) depressed both the tension and the increase in $[Ca^{2+}]_i$ induced by histamine. The inhibition was time-dependent. It caused a 92±13% and 57±6% (n=6) inhibition of tension and $[Ca^{2+}]_i$, respectively. As may be seen, it inhibited tension to a greater extent than it did $[Ca2+]_i$. The muscle-relaxing action of this agent, unlike rCGRP, had a slow onset (Fig. 4-5).

c. Nifedipine

Nifedipine, on the other hand, inhibited tension and $[Ca^{2+}]_i$ to almost the same degree. At a concentration of 10 nM, this calcium channel blocker exhibited a fast and potent inhibition of both the elevated force and $[Ca^{2+}]_i$. The histamine-induced tonic tension and elevation of $[Ca^{2+}]_i$ were abolished 13 min after the addition of this agent (Fig. 4-6).

By plotting the time-related change in tension versus the change in $[Ca^{2+}]_i$, we analyzed the relationship between the two parameters in the experiments mentioned above (Fig. 4-7). We found two types of relationship. The first type is shown by the line from the nifedipine experiment, where the slope was close to 1 (tension = 1.16× $[Ca^{2+}]_i$-20.84). The second type of relationship exhibited a large slope (around 2), and

was seen in lines from both rCGRP (tension = $2.31 \times [Ca^{2+}]_i$-139.5) and forskolin (tension = $1.89 \times [Ca^{2+}]_i$-81.5) experiments. There is a statistically significant difference ($P < 0.05$) between the lines for nifedipine and rCGRP or forskolin.

B. Tension-intracellular cyclic nucleotides simultaneous measurement

1. Tension and $[cAMP]_i$

In a preliminary experiment, the tissue was equilibrated for 1 hr at 0.7 g tension, and both rCGRP (263 nM) and forskolin (3 μM) were found to increase the cAMP content in the non-stimulated tissues. The basal level was 1521 fmol/mg protein. rCGRP (263 nM) and forskolin (3 μM) increase the cAMP level by 60% (2426 fmol/mg protein) and 96% (2943 fmol/mg protein), respectively.

Histamine at a concentration of 0.5 μM decreased the basal level of cAMP in lm of GPI. The decrease, however, was not significant. The level of cAMP was 1553$\pm$55 fmol/mg protein in non-stimulated tissue, 1476$\pm$58 fmol/mg protein 2.5 min after histamine challenge and 1189$\pm$176 fmol/mg protein 12.5 min after histamine challenge (n=4, $P > 0.05$, Fig. 4-8). A single dose of rCGRP (262 nM) was added 2.5 mins after histamine challenge. cAMP level after rCGRP did not significantly increase compared with those before rCGRP or before histamine. However, at time points of 3, 5 and 10 minutes after the addition of CGRP, the cAMP content in the rCGRP-treated tissues was significantly higher than that in rCGRP-untreated tissue at the same time points (Fig. 4-9). The time course for changes of tension and $[cAMP]_i$ are presented in Fig. 4-10.

2. Tension and $[cGMP]_i$

rCGRP (262 nM) did not change cGMP content (Fig. 4-11) although it relaxed the tension developed by histamine at the same time (Fig. 4-12).

IV. DISCUSSION

A. Methodology of tension-$[Ca^{2+}]_i$ simultaneous recording

One of the earliest measurements of $[Ca^{2+}]_i$ in smooth muscle cells was made in isolated amphibian cells using the bioluminescent calcium indicator aequorin (Fay *et al.*, 1979). Then in 1980, Neering and Morgan made the first simultaneous recording of tension and $[Ca^{2+}]_i$ in smooth muscle using this photoprotein. The photoprotein is introduced into the cell by microinjection through high resistance electrodes. This limits the use of this indicator in cells of relatively small size, such as smooth muscle cells. An alternative loading method is a reversible permeabilization method which is of low efficiency. The introduction of the fluorescent Ca^{2+} indicators Quin-2 (Tsien, 1980, 1981), Fura-2 and Indo-1 (Grynkiewicz *et al.*, 1985) and Fluo-3 (Minta *et al.*, 1989) has established the basis for the most widely used methods for measuring $[Ca^{2+}]_i$ in smooth muscle cells. Their popularity stems from the ease with which they can be loaded into cells by hydrolysis of membrane-permeant esters, as well as their sensitivity.

Measurement of $[Ca^{2+}]_i$ in isolated and/or cultured smooth muscle cells, studies of calcium influx and efflux using $^{45}Ca^{2+}$, and calcium current determinations by patch clamp techniques have all added valuable information to our understanding

of calcium as an intracellular messenger. The results of such studies can lead to inferences or predictions about the muscle contractile function under certain conditions. However, it is difficult to be sure that the functions of isolated cells or the responses of the cells, in terms of $[Ca^{2+}]_i$, to the stimulants have not been altered by the procedures used to isolate and culture the cells. Thus, the relationship between $[Ca^{2+}]_i$ and contractile function predicted from studies on isolated cells may not reflect the situation in the intact tissue.

Simultaneous recording of $[Ca^{2+}]_i$ and tension of smooth muscle began with the use of the Ca^{2+}-sensitive photoprotein aequorin (Neering and Morgan, 1980; Morgan and Morgan, 1982, 1984a,b) followed by the use of the fluorescent dye Quin-2 (Patcha ..nd Shley, 1986; Williams and Fay, 1986; Himpens and Casteels, 1987), and Fura-2 (Ishii *et al.*, 1987; Ozaki *et al.*, 1987; Himpens and Sömlyo, 1988). Instead of separate measurement of $[Ca^{2+}]_i$ in isolated cells and tension in the tissue strip, the tension-$[Ca^{2+}]_i$ simultaneous recording technique was chosen to study the effects of rCGRP on the $[Ca^{2+}]_i$ regulation in the present study. The unique advantages of this type of experiment are that a) the detailed role of the $[Ca^{2+}]_i$ in contraction events can be studied, as well as the relationship between $[Ca^{2+}]_i$ and contraction evoked by different stimulations; b) it represents more physiological conditions than those attainable in dissociated or cultured cells; c) it can be used to investigate both Ca^{2+}-dependent and -independent contraction by analyzing the relationship between the $[Ca^{2+}]_i$ and tension development. This type of study greatly advances our understanding of the role of calcium in smooth muscle contractile regulation. By using this

technique, Morgan (1990) found three patterns of relationships between vascular tone and $[Ca^{2+}]_i$ in response to stimulants: a) parallel changes in $[Ca^{2+}]_i$ and force; b) a sustained tension accompanied by a transient increase in $[Ca^{2+}]_i$; and c) a contraction with no detectable change in $[Ca^{2+}]_i$. The results from simultaneous measurement of tension, $[Ca^{2+}]_i$ and level of myosin light chain phosphorylation also lead Hai and Murphy (1989) to propose the "latchbridge hypothesis".

Fura-2 possesses several advantages over Quin-2 as a calcium indicator (Grynkiewicz *et al.*, 1985). It has a much stronger fluorescence, better selectivity for calcium over magnesium and heavy metal ions, somewhat weaker affinity for Ca^{2+}, and its excitation wavelength shifts upon Ca^{2+} binding. These properties make Fura-2 a Ca^{2+} indicator with less $[Ca^{2+}]_i$ buffering effects, a wider calcium concentration measurement range, and a higher specificity relative to Quin-2. Because the ratio is used in the calculation of free calcium concentration, the systemic variations which increase the error of measurements, such as dye concentration, and cell/tissue thickness (especially in contracting muscle cells) are eliminated. Fura-2, therefore, was selected for the present study.

The FluoroPlex III spectrofluorometer used was originally designed for the measurement of $[Ca^{2+}]_i$ in cultured cells by microscopy and in cell suspensions in the cuvette. In order to record simultaneously, some modifications were made to the cuvette by introducing a tissue holder and an oxygen supply tubing. Taking advantage of the mixing system built in the instrument, drug solutions were mixed rapidly after addition. In an instrument without such a mixing system, a concentration gradient of

the added drug might be formed. It would then take considerable time to attain a uniform distribution. Great care should be taken to avoid this type of artifact, which would provide misleading results in the analysis of the relationship between $[Ca^{2+}]_i$ and tension development. Alternatively, this problem could be solved by using a perfusion system.

One disadvantage in using a muscle strip for the measurement of $[Ca^{2+}]_i$ could be the heterogeneity of cell types in such a strip. The fluorescent signal from cell types other than smooth muscle cells could interfere with the measurement and the interpretation of the results (Sato *et al.*, 1990). Fortunately, this is not a serious problem in the preparation used in this study. We used the plexus-free lm strip instead of a preparation consisting of longitudinal muscle and myenteric plexus used by other investigators (Himpens and Sömlyo, 1988). Visual examination of the tissue under the microscope has revealed that the non-muscle cell type(s) are not present in significant amounts.

Another potential disadvantage in the use of fluorescent calcium indicators is that an agent to be used in the experiment may interfere with the Fura-2 fluorescence. Some compounds may themselves fluoresce, or extinguish Fura-2 fluorescence under the experimental conditions, and, therefore, interfere with the measurement. None of the agents used in this study was shown to produce interfering fluorescence or extinguish the Fura-2 fluorescence.

The loading procedures described by Himpens and Sömlyo (1988) were modi-

fied slightly for use in these experiments. The incubation time was shortened from 4 hr to 3 hr Both the loading solution and post-loading incubation solution were continually supplied with oxygen. These modifications keep the tissue responsive to even low concentrations of agonist.

B. Effects of rCGRP and other agents on the tension and $[Ca^{2+}]_i$

1. Histamine-induced change in tension and $[Ca^{2+}]_i$

The effect of histamine on smooth muscle varies according to the source and the species. It is one of the spasmogenic substances in the longitudinal muscle of small and large intestine of guinea pig (see Chapter 3; Leurs *et al.*, 1991a), and coronary arteries of pigs and humans (Smith *et al.*, 1951; Ginsburg *et al.*, 1984; Yamamoto *et al.*, 1987). Its contractile effect is mediated by H_1-receptor activation (Ginsburg *et al.*, 1984; Yamamoto *et al.*, 1987; Leurs *et al.*, 1991a). Histamine has been reported to induce a sustained increase in both force and $[Ca^{2+}]_i$ in porcine left circumflex coronary artery (Abe *et al.*, 1990; Hirano *et al.*, 1991). In our study, 0.5 μM histamine induced a rapid increase in both $[Ca_{2+}]_i$ and force. The tension then declined gradually with time. However, the $[Ca^{2+}]_i$ reached a plateau and remained at this level. A similar time course of histamine action was seen in porcine coronary artery smooth muscle (Hirano *et al.*, 1991).

2. *Effects of forskolin*

Forskolin directly activates the adenylate cyclase catalytic subunit, resulting in an increased production of cAMP (Seamon and Daly, 1981). Although there are exceptions (Vegesna and Diamond, 1983, 1984), it relaxes smooth muscle from different sources (Abe and Karaki, 1989; Ozaki *et al.,* 1990). The exact mechanism of the inhibitory effect is not fully understood. Forskolin inhibits the effects of some stimulants on $[Ca^{2+}]_i$. At concentrations of 1 μM and 10 μM, forskolin has no inhibitory effect on the $[Ca^{2+}]_i$ induced by high K^+ while it decreases the induced tension in canine tracheal smooth muscle. In the same tissue, this agent decreases both $[Ca^{2+}]_i$ and tension elevated by carbachol, although it depresses the tension to a larger extent than the $[Ca^{2+}]_i$ (Ozaki *et al.,* 1990). In the rat aorta, the action of forskolin on the two parameters are dose-dependent. At a low concentration, forskolin slightly depresses both $[Ca^{2+}]_i$ and tension induced by NE and high K^+, but did not change the tension-$[Ca^{2+}]_i$ relationship. At a higher dose, it produces a dissociation between $[Ca^{2+}]_i$ and tension, i.e. the elevated tension is more reduced than the $[Ca^{2+}]_i$ (Abe and Karaki, 1989). Experiments using radioactive $^{45}Ca^{2+}$ indicate that forskolin selectively inhibits the Ca^{2+} influx due to NE but not that due to high K^+ in rabbit aorta (Abe and Karaki, 1988). These results suggest that forskolin may only slightly affect voltage-dependent calcium channels. It seems that forskolin acts more selectively on the tension than on $[Ca^{2+}]_i$ stimulated by either KCl depolarization or receptor agonists. A similar action of forskolin on $[Ca^{2+}]_i$ was

observed in the present study. All these results indicate that the relaxant effects of forskolin cannot be solely explained by the reduction in $[Ca^{2+}]_i$. There may also be a change in the calcium sensitivity of the contractile proteins.

3. Effect of nifedipine

In contrast to forskolin, the mechanism of action of nifedipine seems much clearer. Nifedipine is known to inhibit smooth muscle contraction by blocking calcium entry through L-type voltage-dependent calcium channels (Godfraind, 1983; Triggle *et al.*, 1989). Morel *et al.* (1987) reported that both the phasic and tonic contractions induced by histamine are inhibited by nifedipine, and are potentiated by Bay K 8644. This latter agent has an effect opposite to nifedipine on calcium channels and prolongs their open state during activation (Schramm *et al.*, 1983). Bolton and Clark (1981) measured a depolarization of about 35 mV in the longitudinal smooth muscle of guinea-pig ileum in the presence of 10 μM histamine. They noted that this effect was quite similar to the depolarization produced by 120 mM K^+. Such a depolarization could lead to the opening of dihydropyridine-sensitive, voltage-operated Ca channels, which could be, to a certain degree, responsible for the increased $[Ca^{2+}]_i$. Therefore, it is expected to be that nifedipine would bring about a parallel change between $[Ca^{2+}]_i$ and the force induced by histamine. Actually, in the present study, nifedipine reduces both tension and $[Ca^{2+}]_i$ to almost the same extent. The effect of nifedipine on the $[Ca^{2+}]_i$ of smooth muscles in the presence of agonists has been

studied primarily in cultured cells.

4. Effect of rCGRP

There have been no previous reports on the effects of CGRP on the $[Ca^{2+}]_i$ of longitudinal smooth muscle. rCGRP greatly depresses the histamine-induced tonic contraction, and only slightly and briefly reduces the $[Ca^{2+}]_i$ of lm, resulting in a dissociation of tension and $[Ca^{2+}]_i$. After a brief reduction, $[Ca^{2+}]_i$ returns to control values while the tension is still greatly depressed. This result indicates that the $[Ca^{2+}]_i$ lowering effect may play some role in the initial relaxation induced by rCGRP. This peptide, therefore, appears to act in a manner similar to forskolin. This indicates that reduction of $[Ca^{2+}]_i$ plays a minor role in the relaxing effect of rCGRP on the lm.

Two types of relationship between time-related change in force and $[Ca^{2+}]_i$ are found: 1) changes between force and $[Ca^{2+}]_i$ were almost parallel as in the case of nifedipine and b) the decrease in force was greater than the decrease in $[Ca^{2+}]_i$ as in the case of forskolin. The effect of CGRP was similar to that of forskolin. The second type of force-$[Ca^{2}]_i$ relationship may reflect the change induced by some agonists in Ca^{2+}-sensitivity of cellular contractile proteins.

5. Influence of rCGRP on KCl-induced change in $[Ca^{2+}]_i$

KCl (15 mM) induced a biphasic increase in $[Ca^{2+}]_i$ as well as tension. In the tension studies, rCGRP inhibits the tension induced by KCl at this concentration (see

results in Fig. 3-4). However, it does not influence the KCl-stimulated elevation of $[Ca^{2+}]_i$. This is similar to the effect of this peptide on tension and $[Ca^{2+}]_i$ stimulated by histamine, suggesting a common mechanism underlying the relaxing effect of this peptide on smooth muscle pre-contracted by either histamine or KCl. We have demonstrated in the preceding experiments that rCGRP significantly depresses KCl-induced extracellular calcium-dependent contraction. The results from the two types of experiments then appear contradictory. Inhibition of extracellular calcium-dependent contraction by a relaxant can be a result of interfering with the entry of calcium into smooth muscle cells. The $[Ca^{2+}]_i$ will be subsequently decreased. Actually, relaxation can occur as a result of a decrease in either $[Ca^{2+}]_i$ or sensitivity of contractile elements to calcium, or both. Pfitzer and co-workers (1985) found that cAMP-dependent kinase relaxes skinned porcine coronary arteries and shifts the ED_{50} of calcium to a high value. In a chemically skinned smooth muscle preparation, the $[Ca^{2+}]_i$ lowering mechanism has been destroyed since calcium ion can freely penetrate the cell plasma membrane and the $[Ca^{2+}]$ can be maintained constant by EGTA buffers. cAMP-dependent kinase, therefore, relaxes the muscle by an action on contractile elements instead of lowering $[Ca^{2+}]_i$. Nitroglycerin has been reported to relax KCl-induced contraction of canine coronary artery smooth muscle in normal physiological solution and also KCl-induced extracellular calcium-dependent contraction. The $[Ca^{2+}]_i$ increased by extracellular calcium remains unaffected by this compound (Yanagisawa *et al.*, 1989). The results on the effects of rCGRP on KCl-

induced extracellular calcium-dependent contraction and $[Ca^{2+}]_i$ in the present study indicate that $[Ca^{2+}]_i$ lowering effect may not be the primary mechanism underlying the muscle-relaxing action of rCGRP.

6. *$[Ca^{2+}]_i$ and contraction of smooth muscle*

The exact sequence of events between an increase in $[Ca^{2+}]_i$ and the development of force is still subject to some debate. According to the phosphorylation theory (Cheung, 1980; Ito and Martshorne, 1990), several steps exist between the increase in calcium concentration and the final contraction. These steps are 1) an increase in $[Ca^{2+}]_i$ leads to Ca^{2+} binding to calmodulin; 2) activation of MLCK by the calcium/calmodulin complex; 3) phosphorylation of the myosin light chain which increases activity of actomyosin ATPase and is thought to initiate contraction. Dephosphorylation of the myosin light chain by myosin light chain phosphatase leads to relaxation of the smooth muscle. These steps are subject to regulation which may result in the modulation of contraction.

The exact regulatory mechanisms are unknown. The concept of altered calcium sensitivity of contractile elements is now being used to describe the phenomenon of non-parallel changes between $[Ca^{2+}]_i$ and tension development (Karaki *et al.*, 1989; Sõmlyo and Himpens, 1989; Gerthoffer, 1991). Contractile element sensitivity to Ca^{2+} is variable and there is not a simple relationship between $[Ca^{2+}]_i$ and force according to experimental results thus far reported.

a. Calcium sensitization

Calyculin-A, a marine toxin from *Discodermia calys*, induces muscle contraction in guinea pig taenia caeci and rat aorta without changing $[Ca^{2+}]_i$ and in the absence of extracellular calcium. Moreover, this compound induces a calcium-independent contraction in the permeabilized taenia in the presence of 2 mM EGTA (Ishihara *et al.*, 1989). It may act by inhibiting myosin phosphatase and then regulating the tension in a calcium-independent manner.

The calcium sensitivity of contractile elements may be increased by stimulants. By using a fluorescent indicator to monitor the $[Ca^{2+}]_i$, agonists have been shown to induce a greater contraction than depolarization at a given $[Ca^{2+}]_i$, as in the case of norepinephrine in vascular smooth muscle (Sato *et al.*, 1988), carbachol in tracheal muscle (Ozaki *et al.*, 1990) and histamine in porcine coronary arteries (Abe *et al.*, 1990; Mori *et al.*, 1990). The sensitizing effects of agonists have also been confirmed in permeabilized smooth muscle strips. When skinned muscle was incubated in fixed calcium concentration solution and the SR was depleted, NE and GTPγS potentiated the Ca^{2+}-induced contraction, shifting the pCa-tension curve to the left (Fujiwara *et al.*, 1988; Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989). Prostaglandin $F_{2\alpha}$ induces a sustained contraction of rat aorta with a small increase in Fura-2 signals (Karaki *et al.*, 1989).

Phorbol ester induces a contraction without a change in the Fura-2 signal or the aequorin signal, a so called " calcium-independent" contraction, in canine tracheal

smooth muscle (Ozaki *et al.*, 1990), porcine coronary arteries (Mori *et al.*, 1990), human coronary arteries (Morgan *et al.*, 1989), ferret aorta (Jiang and Morgan, 1987, 1989), and rat aorta (Jiang and Morgan, 1989; Karaki *et al.*, 1989; Sato *et al.*, 1989). At a low concentration, phorbol ester slightly increases the $[Ca^{2+}]_i$. However, it stimulates a large contraction even after the increase of $[Ca^{2+}]_i$ has been abolished by verapamil. At a high concentration, the induced contraction is accompanied by a slight decrease in $[Ca^{2+}]_i$. Phorbol ester potentiates the high K^+ -induced contraction, which is abolished in the presence of EGTA. This evidence suggests that phorbol ester greatly increases the calcium sensitivity of the contractile elements, which enables the muscle to develop tension even at resting levels of $[Ca^{2+}]_i$.

b. Calcium desensitization

In contrast, some relaxing agents desensitize certain types of smooth muscle. Forskolin inhibits the tension to a greater extent than it inhibits $[Ca^{2+}]_i$ increased by high K^+ and agonists. Forskolin therefore dissociates tension and $[Ca^{2+}]_i$ in rat aorta and canine tracheal muscle (Abe and Karaki, 1989; Ozaki *et al.*, 1990). Isoprenaline has an action similar to forskolin in canine tracheal smooth muscle (Ozaki *et al.*, 1990). Other relaxing agents producing such a dissociation are nitroglycerin in canine coronary artery smooth muscle contracted with high K^+ (Yanagisawa *et al.*, 1989; Abe *et al.*, 1990), sodium nitroprusside in rat aorta (Karaki *et al.*, 1988b), and the potassium channel openers, pinacidil and nicorandil in canine coronary artery

(Yanagisawa *et al.*, 1990).

Forskolin and isoprenaline are known to increase intracellular cAMP levels (Seamon and Daly, 1981). Nitroglycerin, nitroprusside and nicorandil all stimulate the production of cGMP (Kukovetz *et al.*, 1981; Endoh and Taria, 1983; Holzmann, 1983; Ignarro and Kadowitz, 1985). Nicorandil also has a potassium channel opening effect. However, this effect may not play role in the desensitization of contractile elements to calcium because cromakalim, another potassium channel opener, produces a parallel inhibition of both tension and $[Ca^{2+}]_i$ (Yanagisawa *et al.*, 1990). cAMP has been reported to desensitize the skinned artery (Pfitzer *et al.*, 1985). In the present study, forskolin effects guinea pig ileum lm in a manner similar to that seen with canine tracheal and rat aorta muscle (Abe and Karaki, 1989; Ozaki *et al.*, 1990). This compound may act on these different smooth muscles by similar mechanisms related to the elevation of intracellular cAMP levels. CGRP seems to primarily desensitize the muscle to $[Ca^{2+}]_i$. It is possible that this action of CGRP may also be related to its ability to stimulate cAMP formation, as in rat aorta and gastric muscle (Maton *et al.*, 1988; Fiscus *et al.*, 1991; Ozaki *et al.*, 1992).

The sensitivity of contractile elements could also be decreased by other mechanisms. Himpens *et al.* (1989) observed that depolarization of guinea pig ileum muscle (but not pulmonary artery muscle) in Ca^{2+}-free solution caused a pronounced decline in the Ca^{2+} sensitivity of the contractile apparatus. Decreased Ca^{2+}-sensitivity of myosin light-chain kinase is also seen in extracts from swine common carotid

arteries stimulated by high K^+ solution (Gilbert *et al.*, 1991). Dissociation is seen between $[Ca^{2+}]_i$ and tension development induced by histamine in the present study, i.e. the tonic tension gradually declines while the $[Ca^{2+}]_i$ remained constant. This was not an artifact due to Fura-2 buffering since the unloaded strips have a similar time course of contractile response to histamine. A similar dissociation was also reported in the action of histamine on the porcine coronary artery (Hirano *et al.*, 1991). In porcine coronary artery, although both tension and $[Ca^{2+}]_i$ induced by 10 μM histamine decline, the tension declines faster than $[Ca^{2+}]_i$. Stull *et al.* (1990) have proposed a hypothesis that large increases in $[Ca^{2+}]_i$ in response to depolarization may activate Ca^{2+}-calmodulin dependent protein kinase II, resulting in phosphorylation of MLCK at site A and decreasing the Ca^{2+} sensitivity of MLCK. It seems that this phenomenon is a tissue and stimulant-specific negative feedback regulation of contracting cells.

C. Intracellular cyclic nucleotides and tension

1. Effects of rCGRP on levels of cyclic nucleotides

In the non-stimulated lm, rCGRP increases the cAMP content only by 60% in the absence of a cAMP-phosphodiesterase inhibitor. CGRP increased cAMP to a similar extent in the guinea pig gastric smooth muscle in the absence of cAMP-phosphodiesterase inhibitor (Maton *et al.*, 1988). The cAMP-phosphodiesterase inhibitor only increases the net amount of cAMP produced by CGRP as in the case in

rat heart cells and guinea pig gastric smooth muscle (Fisher *et al.*, 1988; Maton *et al.*, 1988). After rCGRP administration, the cAMP level in histamine pre-contracted lm is not increased significantly compared with that after the addition of histamine but before the addition of rCGRP and that before the application of histamine. On the other hand, histamine tends to decrease the cAMP level of the muscle in the absence of rCGRP. At 12.5 min after histamine challenge, the cAMP level is 76% of that before histamine challenge, although the decrease is not statistically significant. Therefore, there is a possibility that histamine might antagonize the action of rCGRP promoting the production of cAMP in the lm through some mechanism. This is confirmed by the finding that in the histamine pre-contracted muscle, the cAMP level in the rCGRP-treated strip was significantly higher than that in the control tissue at the same time points after the addition of histamine. There appears to be an interaction between cAMP level and $[Ca^{2+}]_i$. cAMP production stimulating effects by adrenoceptor agonists have been found in the non-stimulated guinea pig intestinal muscle (Inatomi *et al.*, 1974) and rat uterus muscle (Verma and McNeill, 1976). However, a high concentration of KCl inhibits the effects on cAMP production by those agonists in the same tissues. cAMP can be hydrolyzed and inactivated by phosphodiesterases. One type of these enzymes, Ca^{2+}/calmodulin-dependent phosphodiesterases, have been found in smooth muscles (Prigent *et al.*, 1988; Silver *et al.*, 1988; Weishaar *et al.*, 1986). Both histamine and a high concentration of KCl increase the $[Ca^{2+}]_i$ which may in turn stimulate the Ca^{2+}/calmodulin-dependent

phosphodiesterases. Both histamine H_1- and muscarinic receptor agonists have been reported to enhance phosphodiesterase activity in human astrocytoma cell line 1321N1 (Nakahata *et al.*, 1986; Masters *et al.*, 1985).

No change in lm cGMP level is observed in the presence of rCGRP. This is consistent with the results obtained by other investigators from vascular (Grace *et al.*, 1987; Shoji *et al.*, 1987; Crossman *et al.*, 1990; Wang *et al.*, 1991) and gastric smooth muscle cells (Maton *et al.*, 1988). Therefore cGMP can be excluded from the action mechanisms of CGRP.

2. cAMP and smooth muscle relaxation

Activation of receptors coupled to G_s activates the catalytic subunit of adenylate cyclase, resulting in an increase in production of cAMP in smooth muscle. Forskolin stimulates the catalytic subunit directly and leads to the same end result with receptor agonists. cAMP activates cAMP-dependent kinase which alters the activity of functional proteins in the cells. The role of cAMP or the exact mechanisms of this second messenger in relaxing smooth muscle is not fully understood. However, cAMP production-stimulating effect of many substances has a good correlation with their muscle-relaxing effect in many smooth muscle preparations. β-adrenoceptor agonists have been reported to increase cAMP and relax the following tissues in a parallel manner: rabbit colon (Andersson, 1972; Andersson and Nilsson, 1972); canine bronchial smooth muscle (Triner *et al.*, 1977); bovine tracheal smooth muscle

(Andersson *et al.*, 1978; Katsuki and Murad, 1977); guinea pig tracheal smooth muscle (Ohkubo *et al.*, 1976), canine coronary artery (Seidel *et al.*, 1975), bovine coronary artery (Silver *et al.*, 1982; Vegesna and Diamond, 1984); and porcine coronary artery (Pfitzer *et al.*, 1985). Their muscle relaxing effect can be mimicked by cAMP or its derivatives. A highly correlated rise of cAMP and relaxation is also produced by forskolin in guinea pig aorta (Silver *et al.*, 1988b), and 5-hydroxytryptamine in vascular muscle (Trevethick *et al.*, 1984).

3. Mechanisms of smooth muscle relaxation

a. Effects of cyclic nucleotides on Ca^{2+} levels

Two classes of smooth muscle-relaxing agents also change the content of smooth muscle cell nucleotides. Nitro compounds (Ignarro and Kadowitz, 1985), endothelium derived relaxing factor (Furchgott, 1984) and atrial natriuretic peptide (Winquist *et al.*, 1984) increase cGMP content. cAMP content is increased by agonists for β-adrenergic receptors (Bülbring and Tomita, 1987) and adenosine receptors (Kukovetz *et al.*, 1978; Silver *et al.*, 1984), the adenylate cyclase activator, forskolin (Seamon and Daly, 1981; Vegesna and Diamond, 1986) and cyclic AMP-phosphodiesterase inhibitors (Murray, 1990). Therefore, the smooth muscle-relaxing effects of these compounds are thought to be mediated by both cAMP and cGMP, through cAMP or cGMP-dependent kinase (Lincoln and Cornwell, 1991). Actually, cAMP or cGMP derivatives themselves can relax certain types of smooth muscles

(Murray, 1990).

It appears that both nucleotides can reduce $[Ca^{2+}]_i$ through different mechanisms. These mechanisms are: 1) direct inhibition of Ca^{2+}-influx as in the case of isoprenaline, forskolin and dibutyryl cAMP in high K^+-stimulated rabbit aorta (Meisheri and van Breemen, 1982; Hwang and van Breemen, 1987); 2) stimulation of Ca^{2+}-efflux, e.g. by nitroglycerin in rabbit and pig vascular smooth muscle (Itoh *et al.,* 1983) and N^6 2'-0-dibutyl 3',5'cAMP in guinea pig taenia intestinal smooth muscle (Tomiyama *et al.,* 1973), and 3) inhibition of Ca^{2+} release or stimulation of Ca^{2+} uptake by SR, for instance, by db-cAMP in rabbit mesentery artery (Saida and van Breemen, 1984), cAMP in skinned rat aortic smooth muscle cells (Hwang and van Breemen, 1987) and db-cAMP and forskolin in rabbit aorta smooth muscle (Twort and van Breemen, 1988). In smooth muscle strips, compounds stimulating the production of cAMP or cGMP depress the $[Ca^{2+}]_i$ increase evoked by stimulants. Forskolin and isoprenaline inhibit the $[Ca^{2+}]_i$ increased by high K^+ and agonists in rat aorta and canine tracheal muscle (Abe and Karaki, 1989; Ozaki *et al.,* 1990). Nitroglycerin and nicorandil lower $[Ca^{2+}]_i$ in canine coronary artery smooth muscle (Yanagisawa *et al.,* 1989; Abe *et al.,* 1990; Yanagisawa *et al.,* 1990), and sodium nitroprusside acts similarly in rat aorta (Karaki *et al.,* 1988b). However, a general property of these compounds is that they depress tension to a greater extent than $[Ca^{2+}]_i$. This indicates the involvement of a mechanism in addition to the $[Ca^{2+}]_i$ lowering effect, such as regulation of the sensitivity of contractile elements to $[Ca^{2+}]_i$.

b. Effects of cAMP on the contractile elements

The action of Ca^{2+} in the contraction of smooth muscle has been proposed to occur via specific phosphorylation of the 20-kD light chain of myosin by Ca^{2+}/calmodulin-dependent MLCK. The activity of MLCK can be regulated. Phosphorylation of the MLCK at a certain site ("A") decreases the affinity of the kinase for Ca^{2+}/calmodulin, therefore, decreasing the activity of MLCK and subsequently the phosphorylation of myosin. One possible mechanism by which cyclic nucleotides might regulate the sensitivity of contractile elements is phosphorylation of MLCK by cAMP or cGMP-dependent kinases. There has not been much consistent data reported in this area. Myosin phosphorylation has been found to be decreased by β-adrenoceptor agonists in tracheal smooth muscle (Silver and Stull, 1982) and by cAMP-dependent kinase in porcine coronary arteries (Pfitzer et al., 1985). Lanerolle *et al.* (1984) found an increased phosphorylation of MLCK after an increase in cAMP in smooth muscle. The MLCK activity in swine common carotid arteries has been reported to be decreased by cAMP-dependent kinase (Gilbert et al., 1991). In studies of phosphorylation sites, cAMP-dependent protein kinase has been found to phosphorylate both the "A" and "B" sites of MLCK from gizzard smooth muscle in the absence of Ca^{2+}/calmodulin and only the "B" site when Ca^{2+}/calmodulin is present (Sellers and Adelstein, 1987). However, Stull *et al.* (1991) found that isoprenaline did not phosphorylate MLCK in bovine tracheal smooth muscle. Therefore, MLCK may

not be the only target of the cAMP pathway in regulating contractile elements.

The cAMP production stimulating effect may be one of the mechanisms underlying smooth muscle relaxation by rCGRP in longitudinal smooth muscle of GPI. This effect may contribute to the desensitization of contractile elements and possibly the slight decrease in $[Ca^{2+}]_i$ induced by rCGRP, therefore, to the relaxation of lm of GPI contracted by different stimulants. The inability of rCGRP to inhibit the contraction induced by activation of muscarinic receptors indicates the existence of differences in mechanisms of contractile actions of the two receptor pathways. Furthermore, there may be an interaction between second messengers produced by muscarinic receptor activation and rCGRP, or between those produced by histamine receptor activation. These interactions may differ from each other.

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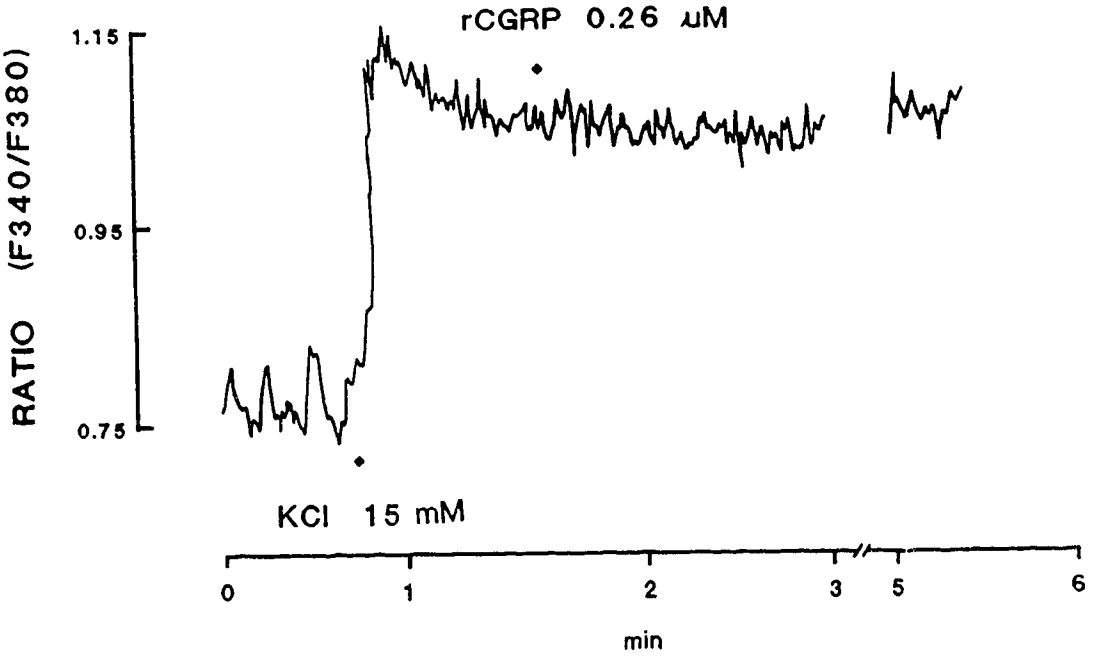


Figure 4-1. Effect of rCGRP on the KCl-induced elevation of $[Ca^{2+}]_i$ in longitudinal smooth muscle.

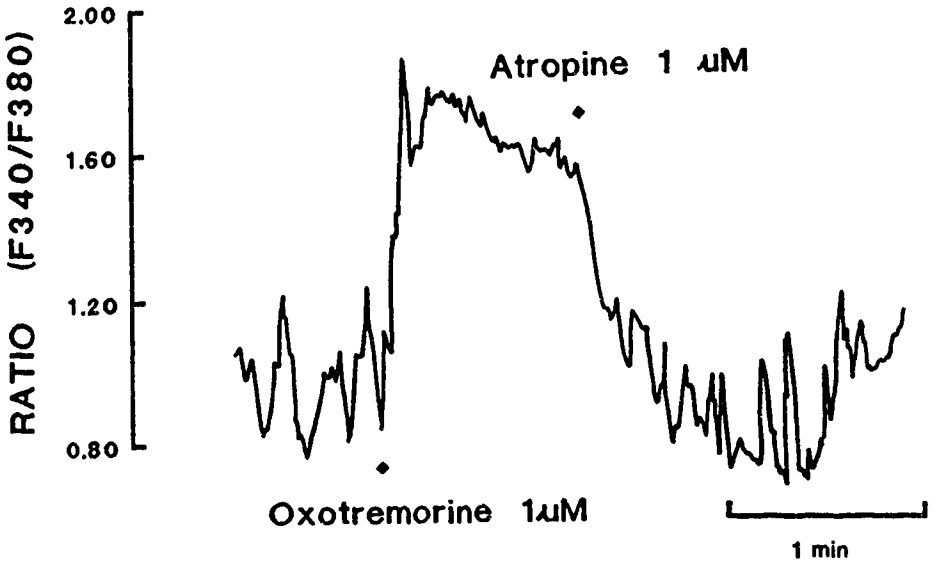
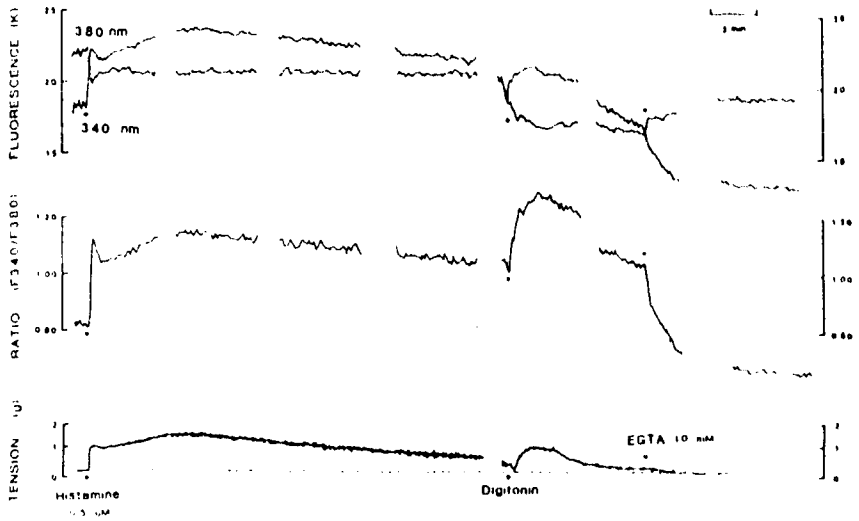


Figure 4-2. Oxotremorine-induced increase in $[Ca^{2+}]_i$ in lm and the effect of atropine on the elevated $[Ca^{2+}]_i$.

a.



b.

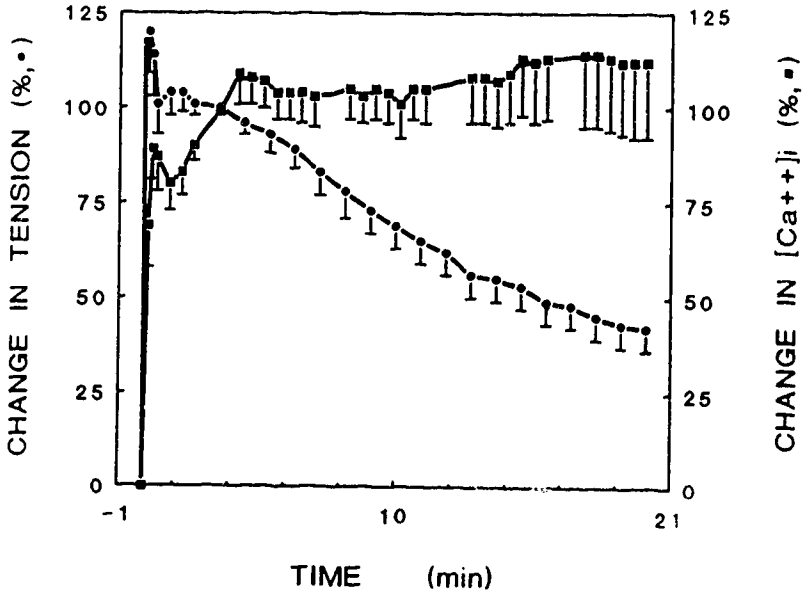
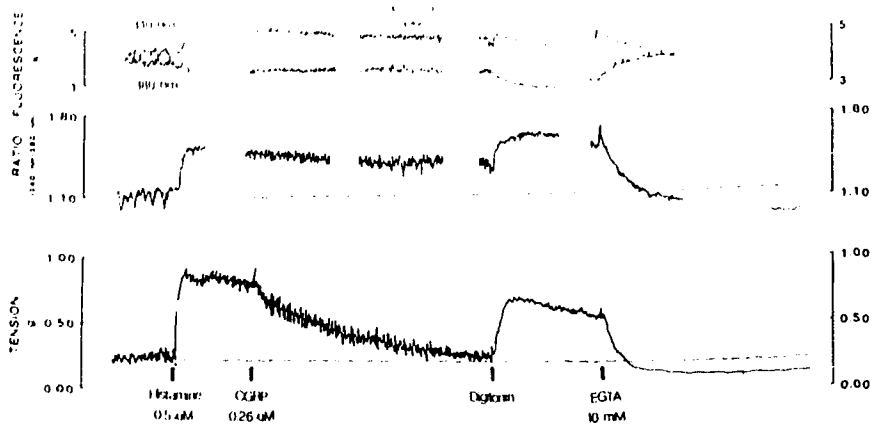


Figure 4-3. Time course of histamine (0.5 μM)-induced change in both tension and $[Ca^{2+}]_i$ of lm. (a) Histamine-induced change in fluorescence excited at both 340 nm and 380 nm, fluorescence ratio and the tension development. (b) Summary of results from eight experiments. Tension and $[Ca^{2+}]_i$ at 2.5 min after the addition of histamine were calculated as 100%. Each point represents the mean ± S.E., n=8.

a.



b.

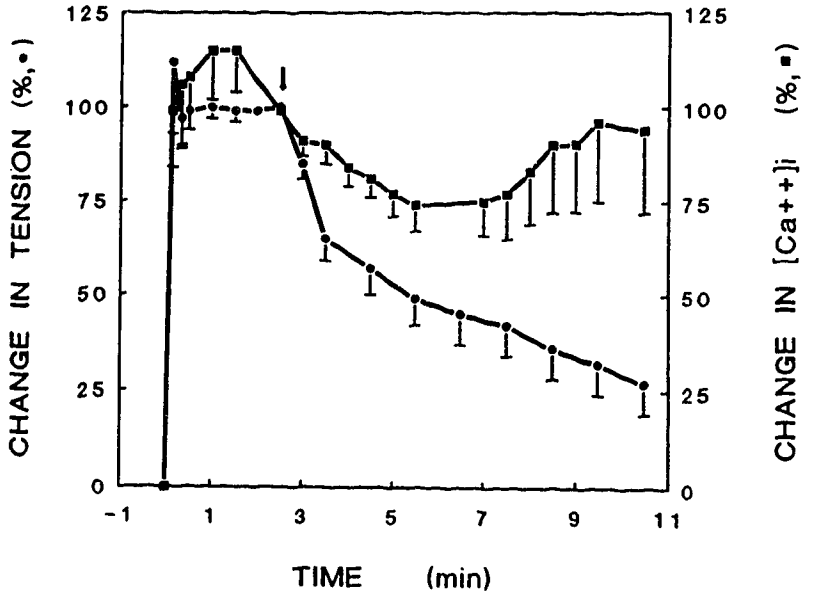
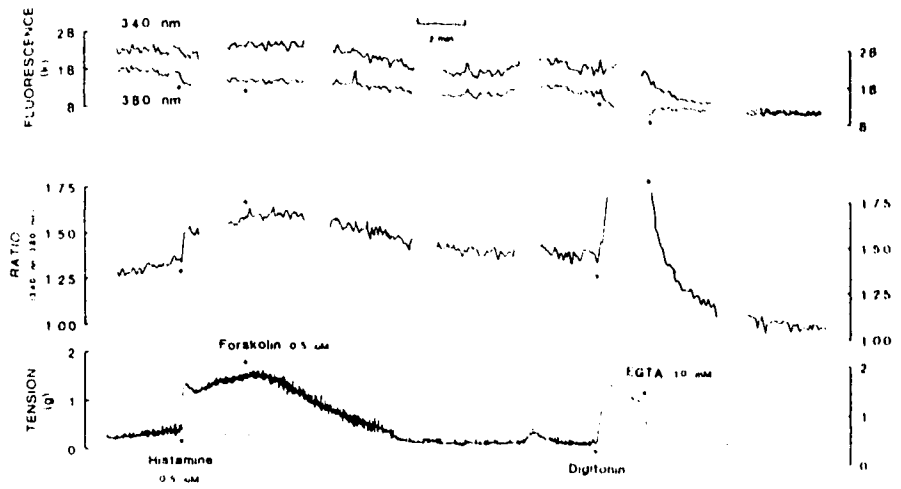


Figure 4-4. Effect of rCGRP (263 nM) on the time course of histamine (0.5 μM) -induced change in both tension and $[Ca^{2+}]_i$ of plexus-free lm. rCGRP was added 2.5 min after the addition of histamine, as indicated by the arrow. (a) Tracing of fluorescence, fluorescence ratio and tension. (b) Summary of the results. Tension and $[Ca^{2+}]_i$ after the addition of rCGRP were calculated as percentage of the responses in a control piece of tissue at the same time point. Data are expressed as mean $\pm$ S.E., n=8. The tonic tension was decreased significantly by rCGRP. The values of $[Ca^{2+}]_i$ at 1.75, 2.08 and 2.58 min, and values of tension at all time points after rCGRP addition were all significantly different from those of the control tissue (Student t-test).

a.



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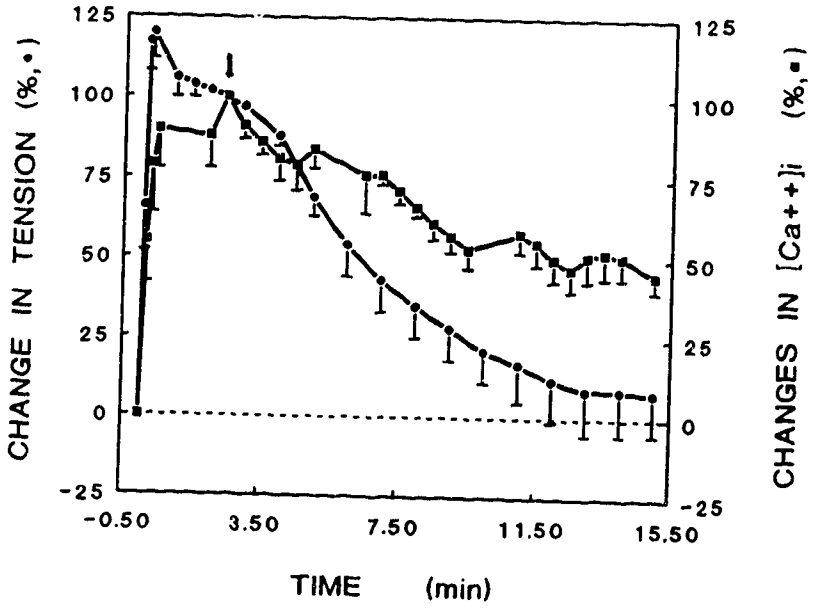
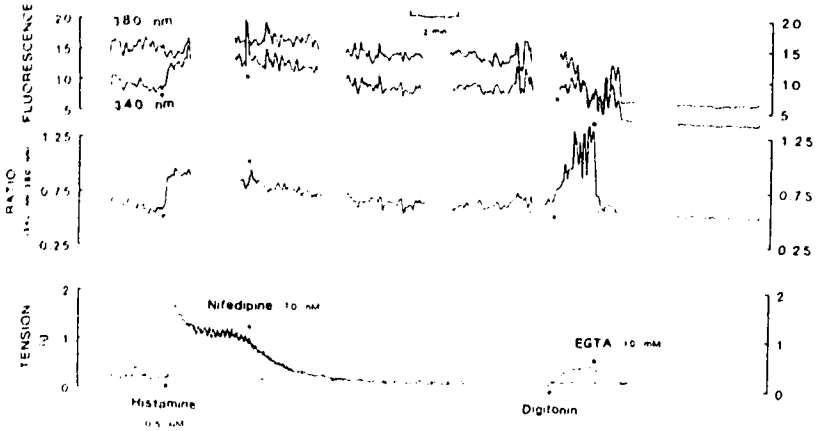


Figure 4-5. Inhibitory effects of forskolin (0.5 μM) on histamine (0.5 μM) -induced change in both tension and $[Ca^{2+}]_i$ of plexus-free lm. (a) Tracing of fluorescence, fluorescence ratio and tension. (b) Summary of the results. Forskolin was added at 2.5 min after the addition of histamine (indicated by the arrow). Tension and $[Ca^{2+}]_i$ were calculated as percentage of those in control pieces of tissue at the same time point. Data are expressed as mean $\pm$ S.E., n=5 (Student t-test).

a.



b.

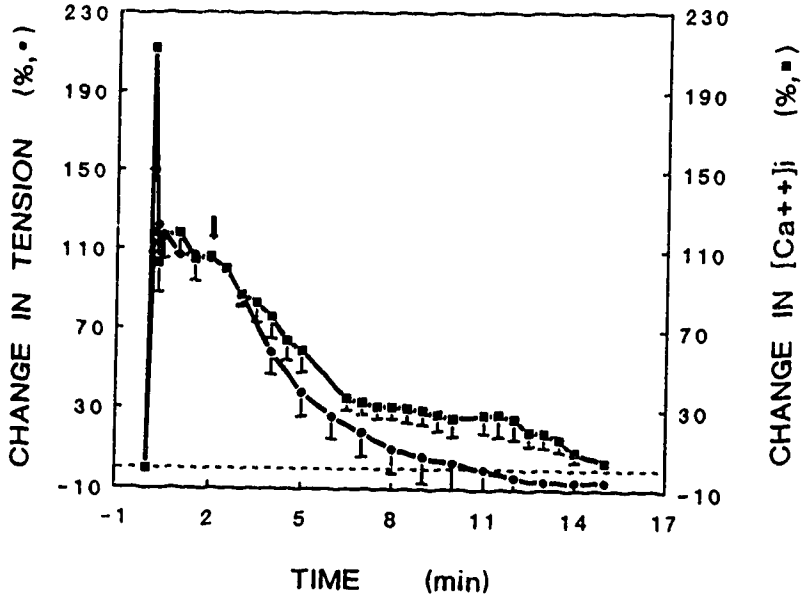


Figure 4-6. Inhibitory effects of nifedipine (10 nM) on histamine (0.5 μM)-induced change in both tension and $[Ca^{2+}]_i$ of plexus-free lm. (a) Tracing of fluorescence, fluorescence ratio and tension. (b) Summary of the results. Nifedipine was added 2.5 min after the addition of histamine (indicated by the arrow). Tension and $[Ca^{2+}]_i$ were calculated as a percentage of those in control piece of tissue at the same time point. Data are expressed as mean ± S.E., n=3. All the values of tension and $[Ca^{2+}]_i$ 1.5 min after rCGRP administration are significantly different from the values of the control tissue (Student t-test).

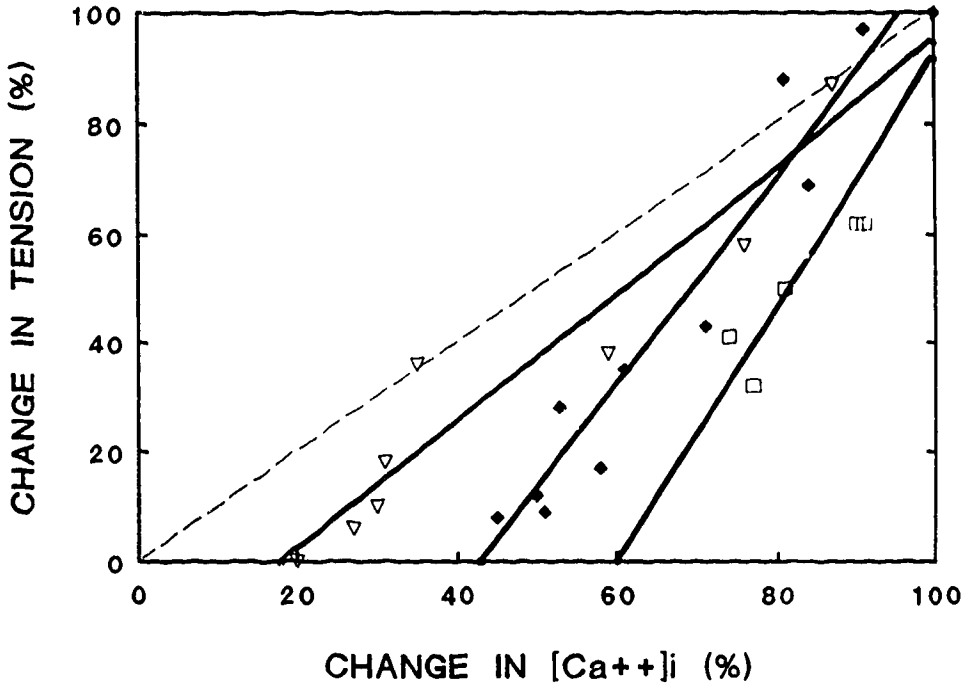


Figure 4-7. Relationship between change in tension and $[Ca^{2+}]_i$ induced by histamine in the presence of rCGRP (▫), forskolin (■) and nifedipine (▿). Plots are calculated from data presented in Fig. 3, 4 and 5. The slopes of lines for forskolin and rCGRP are significantly different from that for nifedipine (line parallelism).

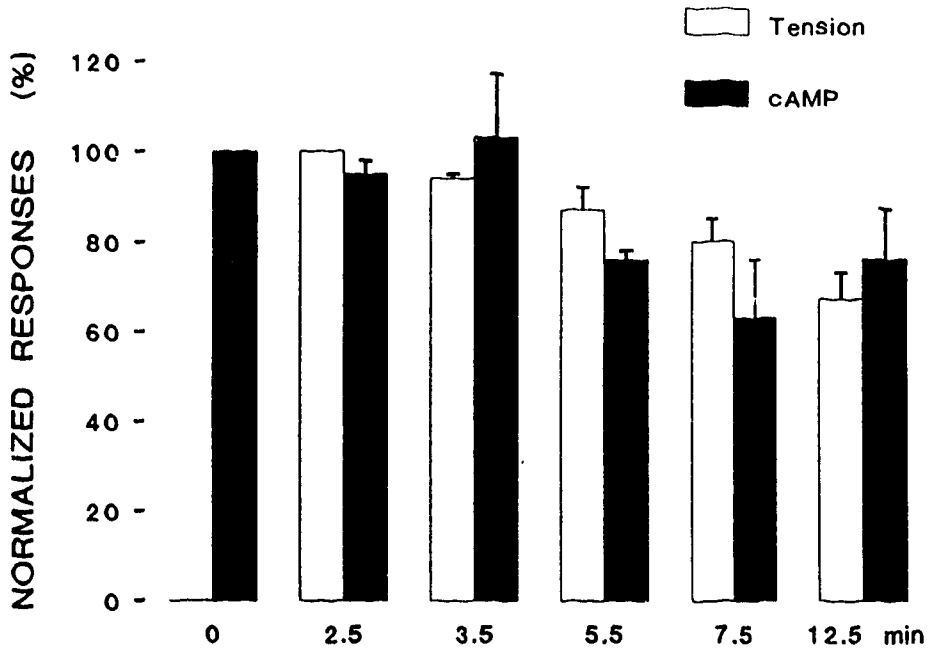


Figure 4-8. Changes in tension and intracellular cAMP level in histamine (0.5 μM)-contracted lm. Each point represents the mean $\pm$ S.E., n=4.

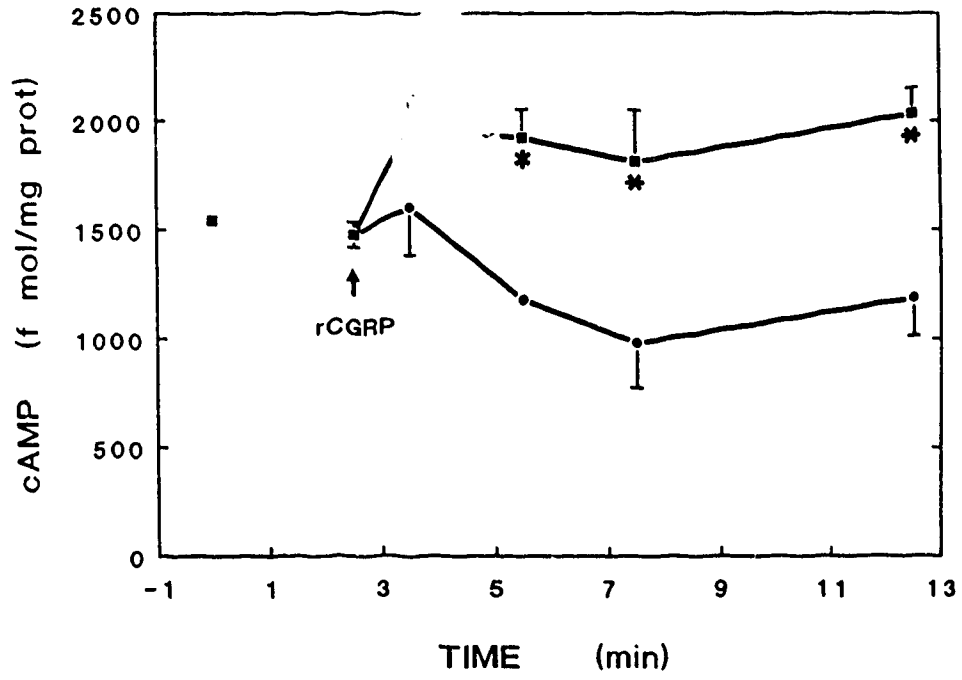


Figure 4-9. Intracellular cAMP levels of histamine (0.5 μM) -precontracted plexus-free lm in the absence (•) or presence (■) of rCGRP (263 nM). The value at time 0 was the cAMP level before histamine stimulation. Data are expressed as mean ± S.E., n=4 *Significantly different from the level in rCGRP-untreated tissue (Student t-test).

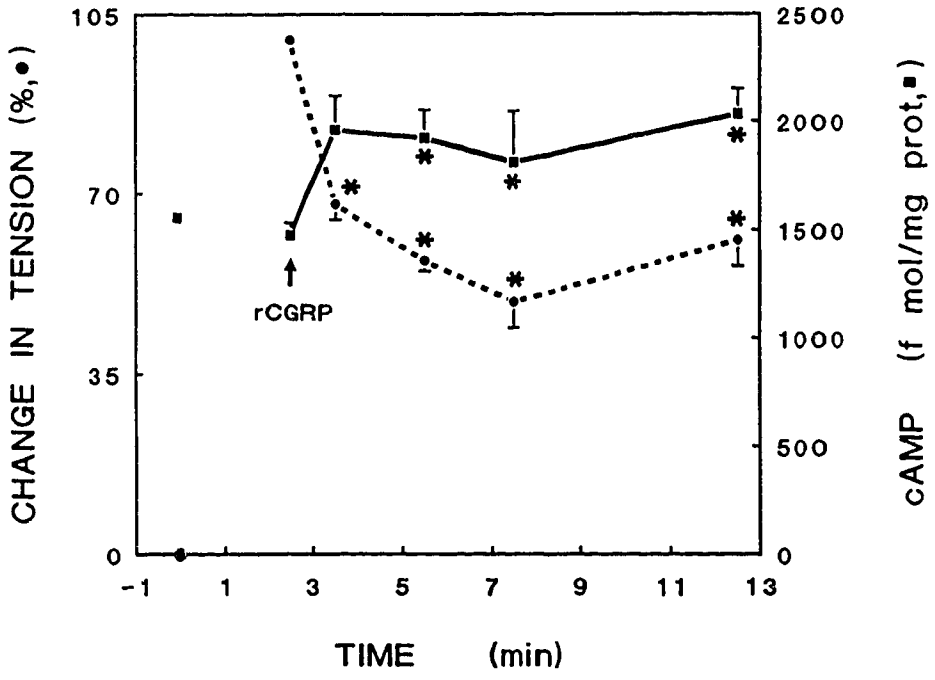


Figure 4-10. Time course for the tension (•) and intracellular cAMP (▪) levels in histamine (0.5 μM) -contracted lm in the presence of 263 nM of rCGRP. Data are mean ± S.E. from four simultaneous measurement experiments. *Significantly different from the level in rCGRP-untreated tissue (Student *t*-test).

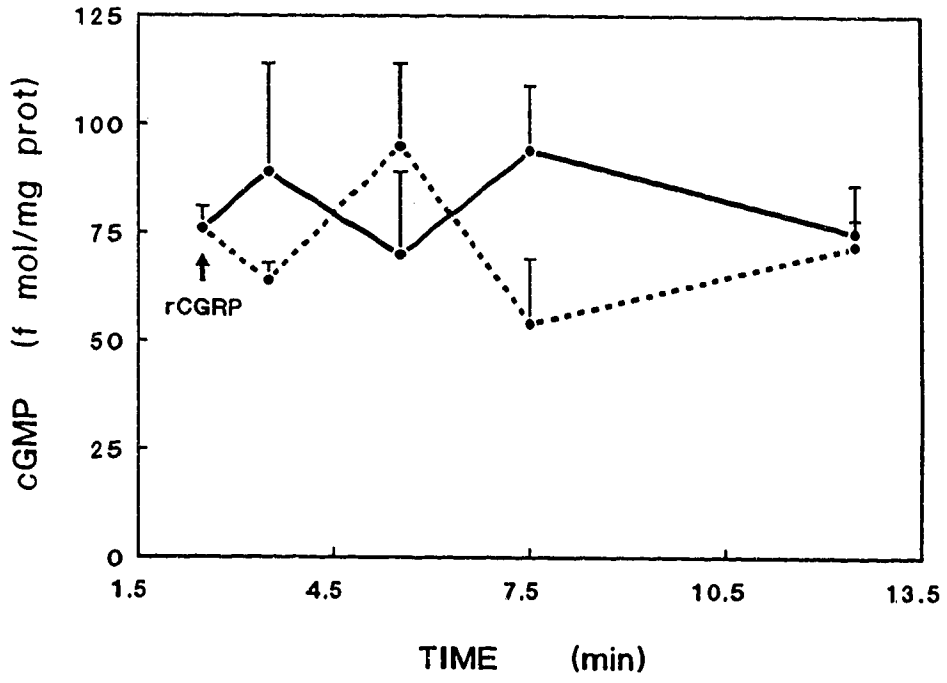


Figure 4-11. Intracellular cGMP levels of histamine (0.5 μM)-precontracted plexus-free lm in the absence (---) or presence (—) of rCGRP (263 nM). rCGRP was added at 2.5 min after the challenge of histamine. Data are expressed as mean $\pm$ S.E., n=4.

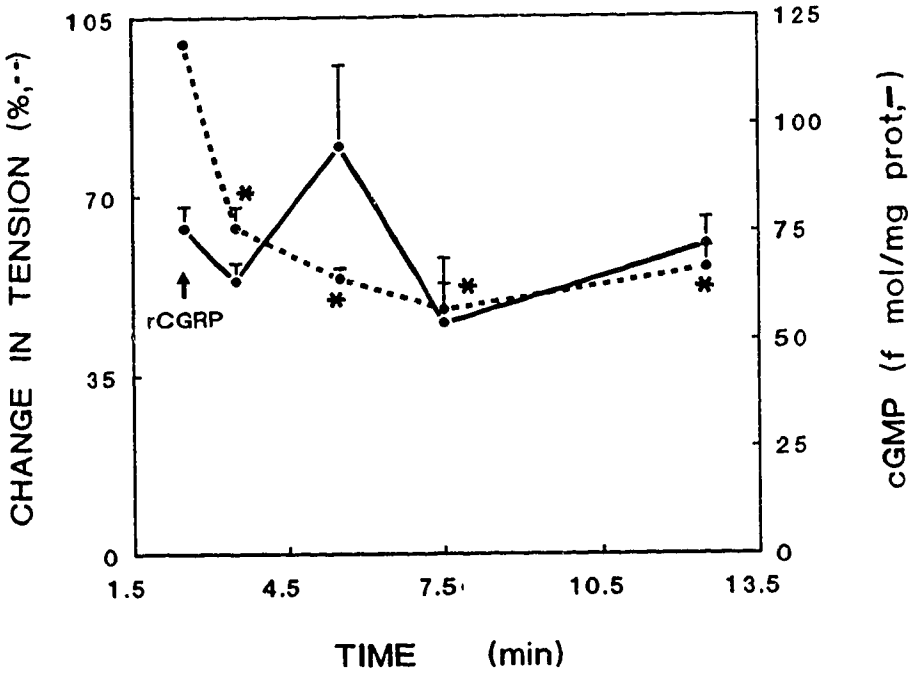


Figure 4-12. Time course for the tension (---) and intracellular cGMP (—) levels of histamine (0.5 μM) -contracted Im in the presence of 263 nM of rCGRP. Data are mean ± S.E. from four simultaneous recording experiments. *Significantly different from the level in rCGRP-untreated tissue (Student *t*-test).

CHAPTER 5. GENERAL DISCUSSION

I. SUMMARY OF PRESENT WORK

The results of the present work may be summarized as follows:

1. The study employed a preparation of the small intestine (lm-mp) which is different from that used by most other investigators. The results indicate that CGRP has influences on lm in the present preparation different from those on lm in the whole segment preparation. The lm-mp is more sensitive to CGRP and responds to a wider range of concentrations of CGRP relative to the whole segment preparation. CGRP-induced changes in muscle activity in lm-mp may not be mediated by exactly same substances as in the case of whole segment preparation. This suggest that, in addition to a difference in the fate of diffusion of this peptide when added to each of the two preparations, there may be additional substances released by rCGRP from submucosal or endocrine cells in the segment preparation.
2. rCGRP affects the activity of lm directly though actions on smooth muscle and indirectly via neuronal pathways.
3. rCGRP may excite enteric neurons to release ACh and another excitatory substance, contracting lm. This peptide may also release an inhibitory substance(s), which is partially responsible for the rCGRP-induced relaxation. It appears that CGRP does not facilitate the release of substances by acting

on nerve terminals. All of these neurons are sensitive to TTX, 2-chloroadenosine and clonidine. Cholinergic interneurons may not play a key role in neuronal pathways used by rCGRP, although the involvement of other types of interneurons cannot be ruled out.

4. Use of the plexus-free lm, instead of lm-mp or whole segment preparations, has, for the first time, provided unambiguous evidence for the direct actions of rCGRP on longitudinal smooth muscle of small intestine. Direct effects on smooth muscle constitute part of the inhibitory actions of rCGRP on motility of the lm of GPI. CGRP has inhibitory effects on non-precontracted lm and also depresses the tension development induced by KCl induced depolarization, activation of histamine receptors and G-proteins. Contraction induced by muscarinic receptor activation is not affected by rCGRP.

5. Competitive blockade of the relaxing effect of rCGRP on histamine-induced contraction of lm by a CGRP receptor blocker, or CGRP antagonist. $hCGRP_{8-37}$, suggests the presence of CGRP receptors on the muscle and their role in mediating the actions of rCGRP on lm.

6. rCGRP increases cAMP levels in lm probably by acting on CGRP receptors. This peptide briefly decreases $[Ca^{2+}]_i$ elevated by stimulant, which may play a role in the early relaxation induced by rCGRP. This $[Ca^{2+}]_i$-lowering effect may be related to the action of CGRP on cAMP levels. That rCGRP induces relaxation of the muscle via an effect on the intracellular cGMP levels has been excluded as a possible mechanism. A very important clue to the predom-

inant mechanism underlying the actions of rCGRP derived from the present study is that this peptide might influence the sensitivity of the contractile elements to cytoplasmic free calcium. This modulation may be mediated via the increase in cAMP levels in lm induced by rCGRP.

II. PHYSIOLOGICAL RELEVANCE OF THE PRESENT STUDY

Studies on the distribution of CGRP in the enteric nervous system have provided an anatomical basis for the physiological role of this peptide in the regulation of the functions of the small intestine, including motility. In addition to those sensory neurons in the dorsal ganglia which synthesize CGRP and have their processes in the small intestine (Mulderry *et al.*, 1988; Sternini and Anderson, 1992; Noguchi *et al.*, 1990), enteric neurons themselves also express CGRP (Mulderry *et al.*, 1988; Sternini and Anderson, 1992). Therefore it is reasonable to deduce that CGRP is likely to play some physiological role in the organ.

We propose several pathways involving CGRP in the regulation of motility of small intestine according to the results obtained in the present study.

In the present study, using the plexus-free lm preparation, we found that CGRP has a direct inhibitory effect on the smooth muscle. CGRP not only regulates the resting muscle, but also the muscle precontracted with some stimulation. Actions of CGRP on smooth muscle can be antagonized by the CGRP receptor blocker, $hCGRP_{8-37}$. The present results indicate that CGRP might be present in the inhibitory neurons innervating lm. These neurons can be sensory neurons or enteric

motor neurons. This suggestion is supported by the following observations. CGRP-IR nerve fibers emanating from myenteric ganglia have been found to innervate both circular and longitudinal smooth muscle (Furness *et al.*, 1985; Ekblad *et al.*, 1987; Lee *et al.*, 1987). On the other hand, a large portion of CGRP in the small intestine is present in nerve fibers of sensory neurons which are located at the dorsal ganglia (Maggi *et al.*, 1986; Mulderry *et al.*, 1988; Sternini and Anderson, 1992). CGRP in these fibers can be released by capsaicin from sensory neurons and then affect the muscle activity. It is possible that CGRP is released from enteric motor neurons, or from sensory neurons through an axon reflex mechanism directly onto the smooth muscle. CGRP then acts as a neuromodulator to selectively modify the responsiveness of lm to some stimulants.

CGRP could also mediate the actions of certain interneurons which synapse with those cholinergic motor neurons, another population of excitatory motor neurons, and inhibitory motor neurons, constituting the other three CGRP pathways in addition to direct actions of CGRP on smooth muscle. Results from the present study show that this peptide can also regulate the muscle activity through neuronal pathways, i.e., by releasing some substances from enteric neurons which contract or relax the lm. In addition, Ekbald and co-workers (1987) found that CGRP-IR nerve fibers terminate on myenteric ganglia. CGRP binding sites are present on those ganglia (Gates *et al.*, 1989). Exogenous CGRP has been reported to excite myenteric neurons (Palmer *et al.*, 1986).

CGRP releases Ach from both cultured myenteric plexus and lm-mp

preparation (Mulholand and Jaffer, 1990; Schworer *et al.*, 1991). These CGRP-sensitive cholinergic motor neurons seem to innervate both lm and circular muscle of small intestine (Holzer *et al.*, 1989). In addition, the motor neurons containing other unknown excitatory substance can be activated by CGRP. CGRP also excites a third population of motor neurons, releasing an inhibitory substance and relaxing smooth muscle.

The unknown stimulatory substance released could be the histamine because in the whole segment preparation, hCGRP-induced contraction of lm is sensitive to inhibition by an antihistamine compound. However, the rCGRP induced contraction in the same type of preparation can not be abolished by the combination of anti-cholinergic and antihistaminergic compounds,suggesting that there could be third excitatory substance in addition to Ach and histamine released by CGRP (Tippins *et al.*, 1984). In addition to histamine, substance P has been reported to be an excitatory modulator in the small intestine (for review see Barthó and Holzer, 1985). ATP has been suggested as inhibitory substance released by capsaicin in the rat duodenum (Maggi *et al.*, 1986). In guinea pig ileum, however, ATP activates P_2 purinergic receptors on lm and contracts the smooth muscle (Wikland and Gustafsson, 1988). Therefore, this substance can be excluded as the inhibitory substance released by CGRP. Other inhibitory substances in enteric neurons are norepinephrine and vasoactive intestinal peptide (Dockray, 1987). Barthó and co-workers (1987) have found that in the whole segment preparation of GPI, the effects of CGRP are not affected by propranolol, a β-adrenergic receptor blocker. NE

therefore, may not be the candidate released by CGRP.

These four proposed pathways are activated simultaneously in the present *in vitro* study because the whole preparation is exposed to exogenous CGRP. This may not be the situation *in vivo*. These pathways may innervate different regions of smooth muscle in vivo. Locally released CGRP from CGRP-containing neurons may discretely activate certain type of pathways, either stimulatory or inhibitory ones. These pathways therefore play a role in the regulation of the motility of small intestine in coordination with other pathways in the enteric nervous system.

The exact role of those CGRP pathways are not clear. It, however, seems reasonable for CGRP to act as a neuromodulator in multiple pathways in the enteric nervous system considering the fact that the small intestine has complex and coordinated movement in the digestive state. Peristalsis, i.e., a movement to propel residual contents into colon, involves alternative contraction and relaxation of lm, and reciprocal contraction and relaxation of lm and circular muscle in the same region. This movement appears to require reciprocal neural excitation and inhibition in a specific time sequence. CGRP may participate both the neural excitation and inhibition together with other neurotransmitters or modulators.

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