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The inhibitory effect of somatostatin peptides on the rat anococcygeus muscle *in vitro*

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1 Electrically evoked contractions of the rat anococcygeus muscle were inhibited in a concentration-dependent manner by somatostatin-14 (SS14), -28 (SS28) and two synthetic hexapeptide analogues: L-363,301 (Pro-Phe-D-Trp-Lys-Thr-Phe) and L-363,586 (N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe), with pIC_{50} values of 7.41, 7.38, 7.07 and 8.34, respectively.

2 The inhibitory effects of SS14 were dependent on stimulation frequency and external calcium ion concentration. Calcium behaved as a non-competitive antagonist of SS14, it reduced the maximal inhibitory effect of the peptide and at a concentration of 5.08 mM it significantly affected the pIC_{50} value.

3 SS14 (3×10^{-7} M) did not affect the tonic actions of bath-applied noradrenaline in the absence of field stimulation.

4 The effects of SS14 persisted in naloxone (10^{-5} M) and were, therefore, not due to an action at opiate receptors. Furthermore, experiments involving the lyophilisation of bath contents, showed no evidence to support an indirect mechanism involving the release of an endogenous inhibitory substance.

5 High concentrations (10^{-5} M) of SS14 or L-363,301 inhibited the relaxation response evoked by electrical stimulation of guanethidine (3×10^{-4} M)-treated preparations.

6 These results are consistent with similar actions of SS14 on other smooth muscle preparations and are presumed to reflect a presynaptic inhibition of transmitter release by a direct action on somatostatin receptors. The antagonistic effect of calcium on this response is discussed with reference to a possible role in receptor desensitization.

Introduction

Somatostatin is a cyclic tetradecapeptide originally isolated from bovine hypothalamic tissues (Brazeau *et al.*, 1973) and so-named because of its potent inhibitory effects on the secretion of growth hormone (somatotropin). It is now recognized that the originally isolated somatostatin-14 (SS14) is only one of a family of related peptides which are widely distributed throughout the mammalian body (see reviews by Reichlin, 1983; Delfs & Dichter, 1985; Epelbaum, 1986). In addition to potent neuroendocrine actions (Arimura & Fishback, 1981; Dileepan & Wagle, 1985) somatostatin peptides have diverse biological effects commensurate with their widespread distribution. One such effect is a striking ability to inhibit evoked and spontaneous contractions of smooth muscle preparations *in vitro*, including the rat (Cohen *et al.*, 1978; Magnan *et al.*, 1979; Vizi *et al.*, 1984) and mouse (Meyers *et al.*, 1981) vas deferens, the guinea-pig ileum (Cohen *et al.*, 1978; Kromer & Woinoff, 1981; Yau *et al.*, 1983; McIntosh *et al.*, 1986) and the rabbit ear artery (Cohen *et al.*, 1978). We now describe a similar inhibitory action of somatostatin on electrically-evoked responses in the rat anococcygeus muscle *in vitro*.

The *in vitro* anococcygeus muscle preparation has certain advantages over the other *in vitro* smooth muscle preparations: firstly, it is known that the contractile response to electrical stimulation is exclusively mediated by the neurogenic release of noradrenaline (Gillespie, 1980). Furthermore, when noradrenergic transmission is blocked and muscle tone raised by guanethidine, relaxations are elicited in response to electrical stimulation (Gillespie, 1972). It is possible therefore, to evaluate the effect of drugs

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on two mutually antagonistic responses within the same tissue. In the present series of experiments we have compared the actions of the endogenous somatostatin peptides SS14, somatostatin-28 (SS28) and somatostatin-$28_{(1-12)}$ ($SS28_{(1-12)}$) with two synthetic cyclic hexapeptide analogues, Pro-Phe-D-Trp-Lys-Thr-Phe (L-363,301, Veber *et al.*, 1981) and N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe (L-363,586, Veber *et al.*, 1984) on the electrically-evoked responses in the rat isolated anococcygeus muscle. These novel hexapeptide analogues have previously been shown to inhibit the binding of [^{125}I]-Tyr^{11}-somatostatin to brain cortical membranes (Veber *et al.*, personal communication) and are more potent than somatostatin as inhibitors of growth hormone, insulin and glucagon release (Veber *et al.*, 1984).

A preliminary account of this work has been published elsewhere (Priestley & Woodruff, 1986).

Methods

Male Sprague-Dawley rats (300–350 g) were killed by a blow to the head followed by exsanguination. Anococcygeus muscles were surgically removed by the procedure of Gillespie (1972). Isolated muscle pairs were separated, mounted in conventional 3 ml organ baths and arranged for isometric recordings under an initial resting tension of 0.5 g. Preparations were continuously perfused at a rate of 1 ml min^{-1} (except during drug application periods) with a gassed (95% O_2, 5% CO_2) modified Krebs-Henseleit solution at 37°C (composition in mM: NaCl 118, KCl 4.74, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.20, glucose 11). Transmural field stimulation was applied through platinum electrodes at a rate of 10 Hz, 80 V for 1 s every 10 s for the contractile response and 5 Hz, 80 V for 1 s every 50 s for the relaxation response. In the latter case preparations were treated with either guanethidine (30 μM) alone or in combination with carbachol (10 μM) in order to increase resting muscle tone and abolish the contractile response. Muscle tension was continuously monitored on a Kipp and Zonen chart recorder.

Peptides were prepared as stock solutions in distilled water and stored at −20°C. Drug solutions were applied to the tissue between stimulation periods and in a volume not exceeding 30 μl.

Lyophilisation procedure

In some experiments the bath fluid was removed and rapidly frozen in liquid nitrogen. Frozen samples were lyophilised at 4°C and the resulting powder stored under dessicant at −20°C until required. The lyophilised material was dissolved in 0.45 ml distilled water immediately before use and stored on ice.

Drugs

All of the chemicals used in the preparation of Krebs-Henseleit solutions were of analytical grade (BDH). Somatostatin-14, -28 and -$28_{(1-12)}$ were obtained from Bachem U.K.; in each case purity was assessed by FAB mass-spectrometry and peptide content was measured by amino acid analysis. The synthetic hexapeptide somatostatin analogues, Pro-Phe-D-Trp-Lys-Thr-Phe (L-363,301) and N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe (L-363,586) were obtained from Merck Sharp and Dohme, West Point Laboratories. Guanethidine monosulphate was diluted from a 10 mg ml^{-1} solution in saline (Ismelin, Ciba). The remaining drugs used were: morphine hydrochloride (May & Baker), naloxone hydrochloride (Endo Laboratories) and (−)-noradrenaline hydrochloride (Sigma).

Data analysis and statistics

Concentration-response curves for somatostatin analogues were generated on a VAX computer using an iterative procedure called 'Allfit' (DeLean *et al.*, 1978) which enabled the simultaneous statistical analysis of several curves. Variance ratios of the slope and maximal inhibition parameters were assessed for statistical significance using an F-test. Potency estimations are quoted as mean pIC_{50} values (defined as: the negative logarithm of that concentration of agonist required to produce a 50%-maximum inhibition of the contraction response) ± standard error of the mean (s.e. mean). Potency ratios were calculated for each analogue compared to somatostatin-14 and ratios are quoted with their respective standard errors from which statistical significance was evaluated using Student's *t* test.

Results

Inhibition of electrically-evoked contractions

SS14 (10^{-6} M) produced a marked but transient inhibition of the contractile response to electrical stimulation, the degree of inhibition waned even if the peptide was left in contact with the tissue (Figure 1). When a similar application was repeated 20 min after washing out the first, the inhibitory effects of the peptide were greatly diminished and, indeed, showed only partial recovery after a further 60 min period (Figure 1). In subsequent experiments this pronounced tachyphylaxis was overcome by washing out somatostatin analogues immediately upon obtaining the maximal inhibition at any given concentration and by leaving a period of 1 h between applications. Using this protocol it was possible to

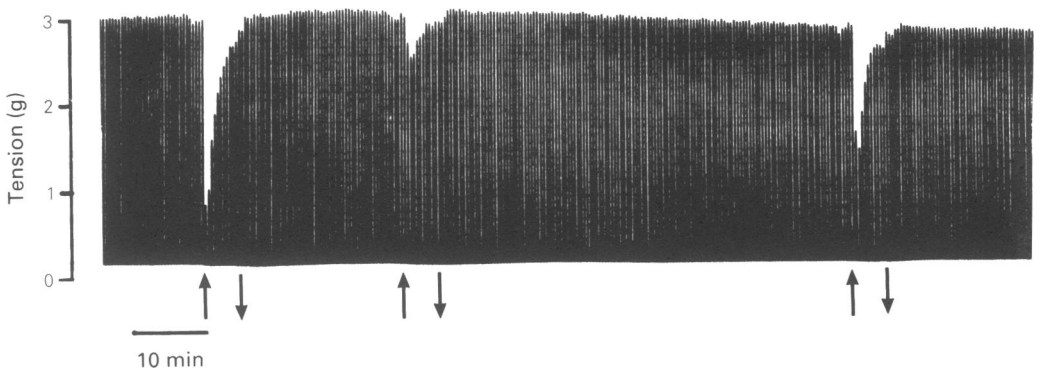

Figure 1 Continuous chart record showing the inhibition of electrically-evoked contractions of the anococcygeus muscle by somatostatin-14 (SS14). The preparation was stimulated at a frequency of 10 Hz for 1 s every 20 s, the resulting contractions are represented as upstrokes on the pen recorder. SS14 (10^{-6} M) was added to the bath for a duration illustrated by the arrows. The preparation was then washed at a rate of 5 ml min^{-1} for 1 min followed by 1 ml min^{-1} until the next application. Note the pronounced tachyphylaxis to a second 10^{-6} M application of SS14. A further application of 10^{-6} M, made 1 h 20 min after the first, still showed signs of tachyphylaxis.

obtain reproducible responses over several hours. Preparations which were desensitized to SS14 showed cross-desensitization to SS28 and the hexapeptide analogues.

Concentration-response curves were constructed for SS14, SS28, L-363,301 and L-363,586, in each case concentrations of peptides were applied in a pseudorandomised order. Preparations were exposed to no more than six applications of any one peptide. Best-fit lines were computed from concentration-response data for each analogue (Figure 2) using the method of DeLean *et al.* (1978); this procedure also enabled the calculation of the pIC_{50} values for each analogue, as listed in Table 1, together with potency ratios compared to SS14.

Effect of bacitracin on the response to somatostatin

The effect of the enzyme inhibitor, bacitracin (20 $\mu g\,ml^{-1}$), was examined on three preparations. Bacitracin was without effect on the contractile response. However, when perfused for at least 20 min, subsequent responses to an IC_{50} concentration (4×10^{-8} M) of SS14 were potentiated by over 19% (mean control response ± s.e. mean = 36 ± 7.8, response after bacitracin = 43 ± 10.3, $n = 3$) although this failed to reach statistical significance at the 5% level.

Lack of effect of somatostatin on bath-applied noradrenaline

Applications of (−)-noradrenaline (3×10^{-8}–10^{-6} M) to unstimulated preparations produced concentration-related and sustained increases in tension. The responses to exogenous noradrenaline were not modified by a 30 s prior exposure to 3×10^{-7} M SS14 (Figure 3). One hour intervals separated combined SS14 and noradrenaline applications in order to avoid desensitization to the peptide. Similar experiments with L-363,301

Table 1 Effect of somatostatin analogues on the contractile response to electrical stimulation

Analogue	n	*Maximum % inhibition* (mean)	pIC_{50}† (mean ± s.e. mean)	*Potency ratio** mean (−s.e. mean, +s.e. mean)
SS14	7	65	7.41 ± 0.04	1
SS28	6	68	7.38 ± 0.05	1.07 (0.92, 1.25)
$SS28_{(1-12)}$	4	7		
L-363,301	5	74	7.07 ± 0.09	0.45 (0.36, 0.56)
L-363,586	4	75	8.34 ± 0.04	8.45 (7.27, 9.83)††

† Values from fitted lines.
* Calculated with respect to somatostatin 14.
†† $t = 14.1$, $P < 0.0001$.
n = No. of animals.

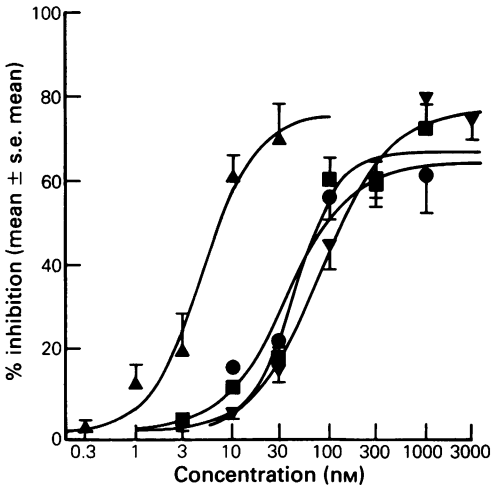

Figure 2 Concentration-response curves showing the inhibitory effect of somatostatin analogues on the contractile response. Best-fit curves were computed for SS14 (■), SS28 (●), L-363,301 (▼) and L-363,586 (▲), according to the method of DeLean *et al.* (1978). Slopes of the fitted lines did not differ significantly from each other or from unity.

(3×10^{-7} M) also failed to show any effect on responses to (−)-noradrenaline (not shown).

Effect of lyophilized bath fluids

Applications of reconstituted lyophilized bath fluids (100 μl) obtained from untreated anococcygeus preparations, inhibited the contractile response to electrical stimulation, in contrast to the reported situation in the guinea-pig ileum (Vizi *et al.*, 1984). Similar applications of lyophilized bath fluids from anococcygeus preparations previously exposed to SS14 (10^{-5} M) produced greater reductions in the contractile response (Figure 4). The extra inhibitory effect of the bath fluid from SS14-exposed preparations was eliminated by prior desensitization of the tissue with a high concentration (10^{-6} M) of SS14 (Figure 4).

Table 2 Lack of effect of naloxone on the inhibitory response to somatostatin

	% *inhibition of contractile response* (mean ± s.e. mean)		
	Control	*Naloxone* (10^{-5} M)	n
SS14 (4×10^{-8} M)	36 ± 7.8	37 ± 9.0	3
Morphine (10^{-5} M)	0.8 ± 0.8		5

n = No. of animals.

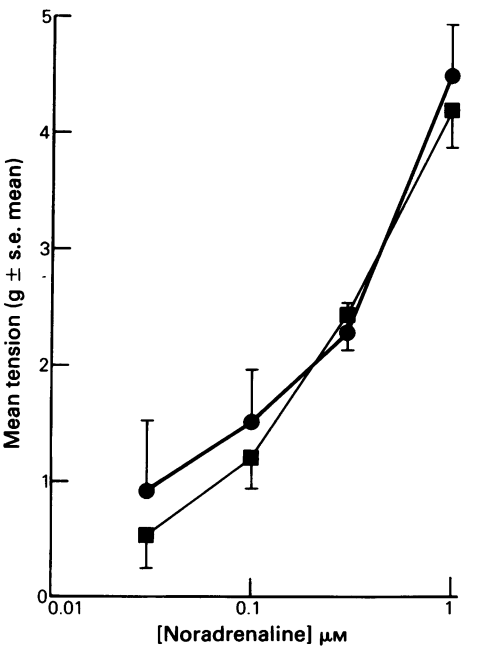

Figure 3 Concentration-response curve for the tonic effects of bath applied (−)-noradrenaline in the absence of field stimulation. Peak contractile force (g tension) is plotted against (−)-noradrenaline concentration for control (●) and somatostatin-14 (SS14)-treated (■) preparations. In the latter case SS14 (3×10^{-7} M) was added to the bath 30 s before (−)-noradrenaline.

Effect of antagonists

In order to address the possibility that the inhibitory effects of somatostatin on the contractile response were mediated by opiate receptors, preparations were exposed to naloxone (10^{-5} M, 5–10 min). The high concentration of naloxone failed to modify the inhibitory effects of an IC_{50} concentration (4×10^{-8} M) of SS14 (Table 2). Furthermore, the μ-receptor opiate, morphine, failed to demonstrate any appreciable somatostatin-like activity against the contractile response, at concentrations up to 10^{-5} M (Table 2).

Effect of varying stimulation frequency

The contractile response and inhibitory effects of SS14 were influenced by stimulation frequency. Reducing the frequency from 10 Hz to 5 Hz attenuated the mean (±s.e. mean) contractile response (from 3.06 ± 0.35 g to 2.09 ± 0.3 g, $n = 6$) and potentiated the inhibitory response to 10^{-7} M SS14

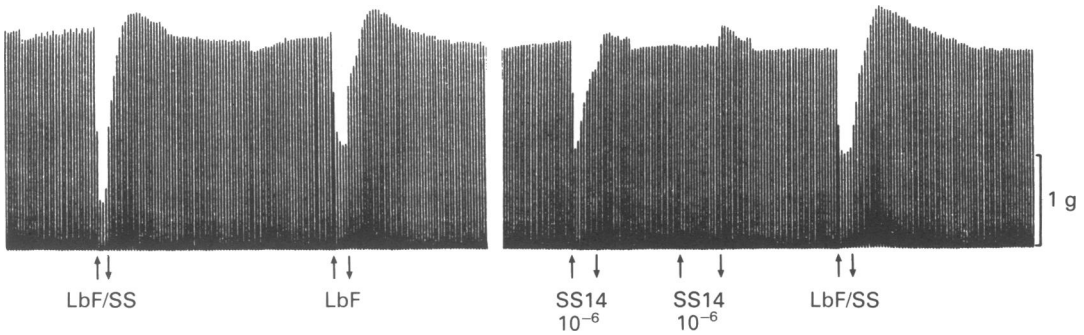

Figure 4 Trace illustrating that the inhibitory response to somatostatin is not secondary to the release of an endogenous inhibitory substance. The trace shows a single chart record which has been broken for clarity. Lyophilized bath fluid (Lbf) was obtained from a separate anococcygeus preparation and freeze-dried as described in Methods, the resulting powder was reconstituted in 0.45 ml distilled water immediately before use. The addition of 100 μl of Lbf to the bath (duration indicated by the arrows) evoked an inhibitory response. Application of Lbf from an anococcygeus preparation which had been exposed to 10^{-5} M somatostatin 14 (SS14) (Lbf/SS), produced a larger inhibitory response. The preparation was then desensitized to SS14 by two 10^{-6} M applications, following which Lbf/SS (100 μl) evoked a response of comparable magnitude to the previous Lbf application. The greater inhibitory response to Lbf from preparations which had been exposed to SS14 was, therefore, abolished by desensitization to SS14. Furthermore, inhibitory responses to Lbf were mimicked by 100 μl of a solution of NaCl, KCl and $CaCl_2$, calculated to contain the same ionic concentrations as reconstituted Lbf (not shown).

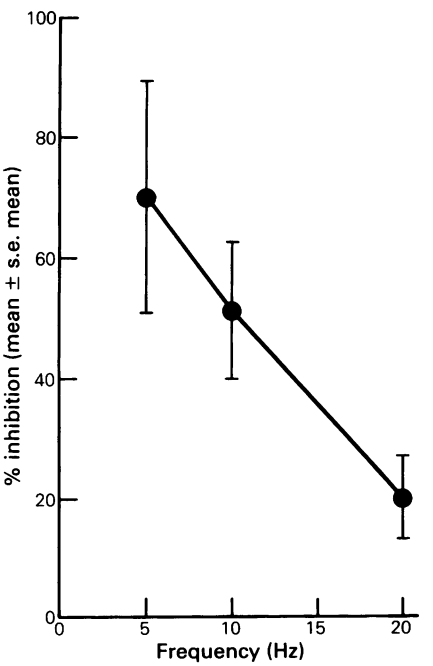

Figure 5 Effect of varying stimulation frequency on the inhibitory response to 10^{-7} M somatostatin-14 (SS14). The figure shows % inhibition by 10^{-7} M SS14 of the contractile response as a function of stimulation frequency. Each point represents the mean value from six separate preparations, with s.e. mean shown by vertical lines. In order to avoid desensitization, intervals of 1 h separated SS14 applications.

(Figure 5). Increasing the stimulation frequency to 20 Hz increased the mean contractile force (4.02 ± 0.46 g, $n = 6$) and attenuated the degree of inhibition produced by 10^{-7} M SS14 (Figure 5).

Effect of varying external calcium concentration

Concentration-response curves to SS14 were constructed at three different external calcium concentrations. 0.63 mM, 2.54 mM and 5.08 mM. In high calcium (5.08 mM) perfusates the concentration-response curve to SS14 was displaced slightly to the right (Figure 6c) and the maximum inhibitory effect was significantly reduced (Table 3). In low calcium (0.63 mM) solutions the opposite effect was observed, thus the concentration-response curve to SS14 was slightly shifted to the left with a significant increase in maximum. Only the high calcium perfusate significantly affected the pIC_{50} value for the inhibition of the evoked contraction by SS14 (Table 3).

Calcium also appeared to influence the degree of desensitization to SS14. In high calcium solutions the inhibitory response was particularly transient (Figure 6b). It should be noted, however, that these experiments were performed under conditions which were not ideal since, as with the frequency experiments, the magnitude of the contractile response to field stimulation was profoundly affected by manipulations of external calcium concentrations (Table 3).

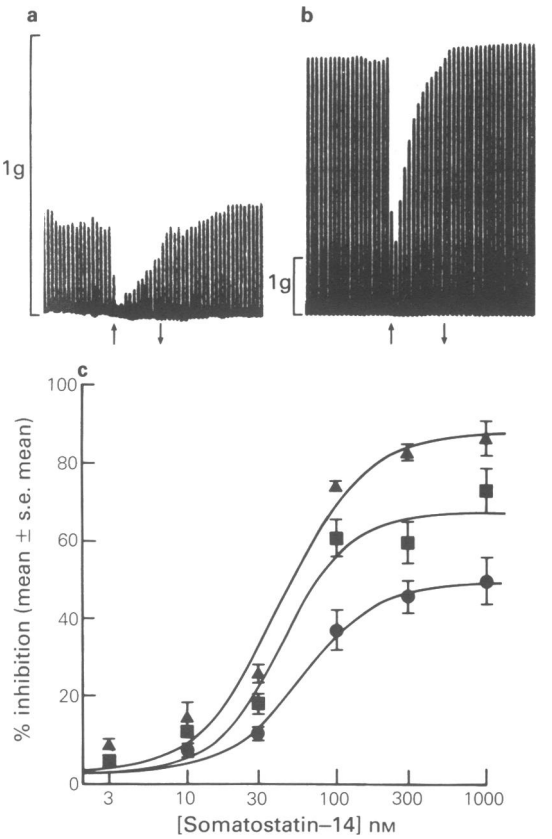

Figure 6 Effect of calcium on responses to somatostatin-14 (SS14). (a) Chart record showing the effect of 10^{-6} M SS14 (arrows) on the contractile response to electrical stimulation in 0.63 mM Ca^{2+}. (b) Effect of 10^{-6} M SS14 (arrows) in 5.08 mM Ca^{2+}. Note that increasing the Ca^{2+} concentration reduces both the amplitude and duration of the inhibitory response to the peptide. (c) Concentration-response curves for inhibition of the contractile response by SS14 in 0.63 mM (▲), 2.54 mM (■) and 5.08 mM (●) Ca^{2+} perfusates. The curves are fitted lines generated by the 'Allfit procedure, the parameters are given in Table 3.

Experiments on the relaxation response

When preparations were treated with carbachol (10^{-5} M) and/or guanethidine (3×10^{-5} M), baseline tension was increased and the contractile response to electrical stimulation was replaced by a relaxation response. Both SS14 (10^{-5} M) and L-363,301 (10^{-5} M) inhibited the relaxation response (Figure 7). However, SS14 was clearly less efficacious against the relaxation response, a concentration of 10^{-6} M produced an 18% inhibition (mean, $n = 4$) whereas this same concentration inhibited the contractile response by 65% (mean, $n = 7$). Extensive concentration-response evaluations were not undertaken because of pronounced tachyphylaxis and because the relaxation response was poorly maintained. Indeed, it was often possible to study the effects of only a single peptide application on any given tissue.

Discussion

SS14 and SS28 were shown to be potent inhibitors of the electrically-evoked contractile response of the isolated anococcygeus muscle of the rat, with similar pIC_{50} values in the nanomolar range (Table 1). The hexapeptide analogues L-363,301 and L-363,586, which have previously been found to possess somatostatin-like neuroendocrine properties (Veber *et al.*, 1984), also inhibited the contractile response. L-363,301 was slightly, but not significantly, less potent than SS14 or SS28, whereas L-363,586 was more than 8 fold more potent than the naturally occurring peptides (Table 1). Interestingly, the rank order of potency (L-363,586 > SS14, SS28 > L-363,301) does not correlate with the reported neuroendocrine profile of these compounds as inhibitors of growth hormone-, insulin- or glucagon-release (Veber *et al.*, 1984). Previously, the inhibitory potency of a number of somatostatin analogues on gastric acid secretion in the cat has been shown to correlate with their inhibitory effects on the field-stimulated mouse vas deferens (Meyers *et al.*, 1981).

Table 3 Effect of varying external Ca^{2+} on contractile force and its inhibition by somatostatin-14

$[Ca^{2+}]$ (mM)	n	*Slope*	pIC_{50}	*Maximum inhibition*	*Contraction amplitude* (g)
0.63	4	1.47 ± 0.30	7.47 ± 0.09	90.5 ± 4.0*	0.41 ± 0.08
2.54	7	2.97 ± 0.77	7.41 ± 0.04	64.8 ± 4.6	1.85 ± 0.12
5.08	5	1.80 ± 0.27	7.22 ± 0.02††	49.8 ± 5.8†	2.35 ± 0.21

Each value is quoted as the mean ± s.e. mean, where n = number of animals used.
* $t = 3.72$, d.f. = 9, $P < 0.005$ with respect to normal (2.54 mM) Ca^{2+}.
† $t = 2.05$, d.f. = 10, $P < 0.05$ with respect to normal (2.54 mM) Ca^{2+}.
†† $t = 3.54$, d.f. = 10, $P < 0.01$ with respect to normal (2.54 mM) Ca^{2+}.
Slopes of the curves did not differ significantly from each other or from unity ($P > 0.05$).

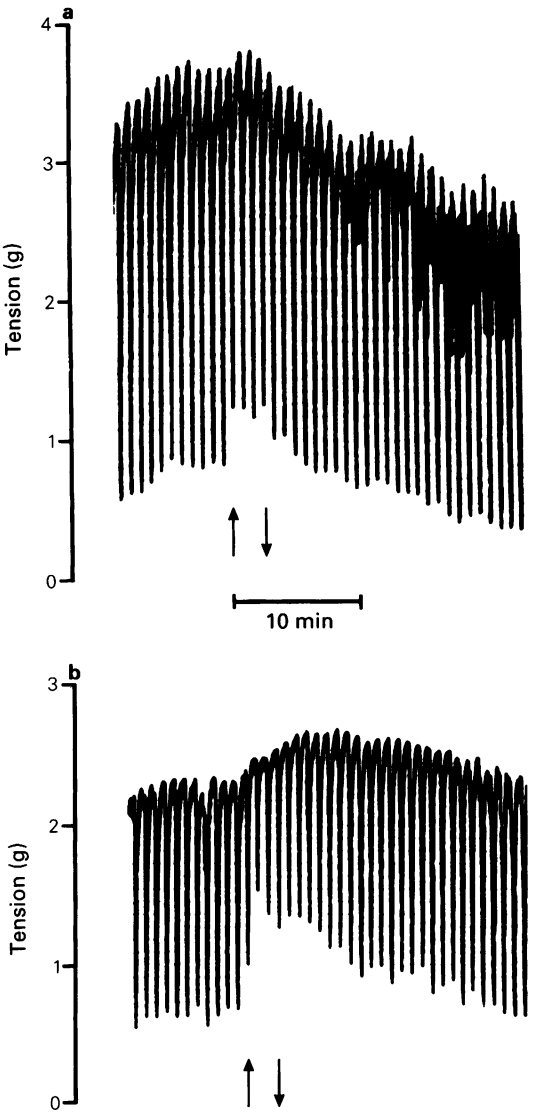

Figure 7 Inhibition of the electrically-evoked relaxation response by (a) somatostatin-14 (SS14, 10^{-5} M) and (b) L-363,301 (10^{-5} M). The preparation was treated with guanethidine (3×10^{-5} M) and stimulation parameters modified (5 Hz for 1 s every 50 s) in order to obtain consistent relaxation responses. The peptides were applied for the duration indicated by the arrows, relaxation responses are represented by downstrokes of the pen. The figure shows responses of two separate preparations.

This observation has prompted the suggestion that the receptors involved in the mediation of these two responses may be the same (Hirst *et al.*, 1980). Data are available for the potency of L-363,301, but not L-363,586, for the inhibition of gastric acid secretion. Thus, L-363,301 is approximately 50% as potent as SS14 in the cat (Hirst *et al.*, 1984) and dog (A.V. Schally, personal communication) *in vivo* gastric acid assays. Such a potency ratio compares favourably with that obtained in the present experiments. It is tempting to speculate, therefore, that inhibitory potency of SS14 analogues on the rat anococcygeus may also correlate with their potencies for the inhibition of gastric acid secretion.

$SS28_{(1-12)}$ was essentially devoid of activity at concentrations up to 3×10^{-6} M (Table 1), consistent with similar findings of inactivity on the guinea-pig ileum (McIntosh *et al.*, 1986) as well as electrophysiologically in the hippocampus (Watson & Pittman, 1986) and from radioligand binding experiments (Moyse *et al.*, 1984). The physiological role of this N-terminal fragment of SS28 therefore remains unclear, despite an intense immunohistochemical visualisation in rat hypothalamus and pancreas (Benoit *et al.*, 1982).

In common with numerous other reports in the literature (for example, Vizi *et al.*, 1984; McIntosh *et al.*, 1986) the inhibitory response to somatostatin was subject to pronounced tachyphylaxis. Preparations which were intentionally desensitized to SS14 showed cross-desensitization to SS28 and the hexapeptide analogues. Tachyphylaxis appeared to be a calcium-dependent phenomenon since sensitivity to the inhibitory effect of SS14 (10^{-6} M) was restored more quickly in desensitized preparations perfused with low (0.63 mM) calcium solutions than those maintained in high (5.08 mM) calcium perfusates (data not shown). The transient nature of the inhibitory response in high calcium and the diminished maximum inhibition (Figure 6b) may reflect ongoing receptor desensitization.

Metabolic stability

Endogenous SS14 and SS28 peptides are known to be substrates for the catabolic activity of endopeptidases, the Trp^8-Lys^9 bond being particularly susceptible (Marks *et al.*, 1976). Accordingly, the inhibitory effects of 4×10^{-8} M SS14 (approximate IC_{50}) were consistently but non-significantly potentiated by the peptidase inhibitor, bacitracin. It must be considered unlikely, therefore, that the inhibitory response to SS14 was due to the formation of an active metabolite.

Mechanism of action

The mechanism by which somatostatin exerts its inhibitory actions on smooth muscle contractions has been suggested by several authors to involve, at least empirically, the presynaptic inhibition of trans-

mitter release (Guillemin, 1976; Cohen *et al.*, 1978; Furness & Costa, 1979, Magnan *et al.*, 1979; Yau *et al.*, 1983). The present experiments have provided circumstantial evidence in support of a similar presynaptic action on the anococcygeus; firstly, SS14 (3×10^{-7} M) failed to modify the effects of bath-applied noradrenaline (Figure 3); secondly, the inhibitory response to 10^{-7} M SS14 was markedly affected by the stimulation frequency; and thirdly, the inhibitory potency of SS14 varied inversely with the concentration of calcium in the external perfusate. Taken together, these three characteristics suggest a presynaptic action (Marshall *et al.*, 1979) and have been previously established, to varying extents, for other purported presynaptic effects such as the inhibitory actions of clonidine (Doxey & Everitt, 1977; Magnan *et al.*, 1979), baclofen (Ong *et al.*, 1986), adenosine (Dowdle & Maske, 1980) and morphine (Illes *et al.*, 1980) on smooth muscle preparations. Furthermore, it is unlikely that somatostatins' action on the anococcygeus represents the postsynaptic modulation of a transmitter other than noradrenaline since there is overwhelming evidence to suggest, at least *in vitro*, that the contractile response is due solely to the neurogenic release of this catecholamine (Gillespie, 1980).

Several reports in the literature have suggested that somatostatin (Terenius, 1976) and particularly some short-chain analogues (Pelton *et al.*, 1985) may exert their effects by an action at opiate μ-receptors. This was clearly not the case on the anococcygeus for two reasons; firstly, the prototypic μ-receptor agonist, morphine (10^{-5} M), failed to mimic the inhibitory actions of somatostatin and secondly, the opiate antagonist, naloxone, at a concentration (10^{-5} M) assumed to exert a non-selective antagonist action at all opiate receptors (Kosterlitz *et al.*, 1985), failed to attenuate the inhibitory effects of somatostatin. This observation is consistent with similar findings in the guinea-pig ileum (Kromer & Woinoff, 1981). A separate study using the guinea-pig ileum suggested an indirect mechanism of action of somatostatin, involving the release of an unidentified endogenous inhibitory substance (Vizi *et al.*, 1984). This is patently not the case in the anococcygeus since similar experiments involving the lyophilization of bath contents failed to demonstrate any inhibitory effects which could not be explained entirely in terms of the high ionic strength imparted by the reconstituted bath fluid or residual, presumably undegraded, SS14 (see legend Figure 4).

The role of calcium ions

The precise mechanism involved in the inhibitory effect of somatostatin may be intimately related to calcium mobilization. Indeed, there is substantial evidence from both biochemical (Curry & Bennet, 1974; Oliver, 1976; Schofield & Bicknell, 1978) and electrophysiological (Quirion *et al.*, 1979; Diez *et al.*, 1985; Luini *et al.*, 1986; Tsunoo *et al.*, 1986) studies to suggest that somatostatin interferes with the entry of calcium into cells and/or its intracellular availability. In the present experiments, calcium was found to act as a non-competitive inhibitor of somatostatins' action (Figure 6c). A similar effect resulted if magnesium ions were omitted from the perfusate (data not shown). These observations are consistent with recent reports that somatostatin binding to pancreatic acinar cell membranes is both calcium-dependent (Susini *et al.*, 1985) and is attenuated by a calcium-activated phospholipid-dependent protein kinase (i.e. protein kinase C, Matozaki *et al.*, 1986; 1987). Furthermore, somatostatin binding to acinar cell membranes is regulated by cholinomimetic drugs (Esteve *et al.*, 1984) which are known to increase intracellular calcium (Williams, 1980) and to stimulate protein kinase C (Worley *et al.*, 1987). Whilst further experiments would be required to confirm the existence of a similar regulatory mechanism in the anococcygeus, such a mechanism would explain the antagonistic actions of the calcium in the present experiments. However, an important caveat in the interpretation of experiments involving calcium ion manipulations is necessary because of the marked effects such manoeuvres have on contractile force (Table 3).

Experiments on the relaxation response

A comprehensive evaluation of the inhibitory actions of somatostatin on the relaxation response was not undertaken because of the labile nature of the response, compounded further by a pronounced tachyphylaxis to the effects of the peptide. High concentrations ($\geqslant 10\,\mu$M) of SS14 have previously been reported to antagonize the tonic effects of carbachol on the unstimulated mouse anococcygeus (Gibson *et al.*, 1984). This effect was not confirmed by the present study, in which concentrations of either SS14 or L-363,301 up to $10\,\mu$M failed to effect baseline tone but did attenuate the relaxing effect of field stimulation on guanethidine-treated preparations. Whilst it has been demonstrated that the relaxation response is neurogenic in origin (Gibson & James, 1977), the nature of the transmitter substance(s) involved remains unclear (Gibson & James, 1977; Hunter *et al.*, 1984, Bowman, 1984). Accordingly, it is only possible to speculate that the inhibitory effect of somatostatin peptides involves a presynaptic modulation of transmitter release.

In conclusion, therefore, it has been shown that somatostatin and related peptides exert a potent inhibitory action on both the contraction and relaxation responses in the anococcygeus. The physiological relevance of these observations would be clarified by the immunohistochemical demonstration of somatostatin-like peptides in neurones of the rat anococcygeus muscle.

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