Br. J. Pharmac. (1984), **82**, 259–267

Substance P can contract the longitudinal muscle of the guinea-pig small intestine by releasing intracellular calcium

P. Holzer & Irmgard Th. Lippe

University Department of Experimental and Clinical Pharmacology, Universitätsplatz 4, A-8010 Graz, Austria

1 The contraction of the longitudinal muscle of the guinea-pig isolated ileum in response to substance P (SP) in high [K^+] medium and in Ca^{2+}-free solution which contained EGTA has been investigated in order to examine whether excitation-contraction coupling involves the release of Ca^{2+} from an intracellular store.

2 In tissues contracted by K^+, high concentrations of SP ($> 0.1\ \mu M$) were still capable of causing a slight, transient contraction.

3 Bathing the ileum in a Ca^{2+}-free medium for 2.5 min greatly diminished the potency and efficacy of SP in contracting the longitudinal muscle but concentrations of 2.2–22 μM SP were still able to produce a response 30–40% of maximal.

4 The responsiveness to SP was completely lost within 10 min of bathing in Ca^{2+}-free medium but was partially restored by a brief exposure (0.5–2 min) to high concentrations of Ca^{2+} (9–72 mM). The restorative effect of Ca^{2+} depended on the concentration of Ca^{2+} and on the time for which the tissue was exposed to this Ca^{2+} concentration.

5 The fact that high concentrations of SP were able to elicit a contraction in media containing high [K^+] or no Ca^{2+}, suggested that they may do so by releasing Ca^{2+} from an intracellular store which is not as sensitive to removal of extracellular Ca^{2+} or as easily accessible to EGTA as the extracellular space of the muscle. The location of this store is not known; it may be associated with the internal side of the cell membrane.

6 There is apparently an overlap between the Ca^{2+} pool associated with the action of SP and the Ca^{2+} pools utilized by acetylcholine, histamine or tetraethylammonium, which accounts for the cross-desensitization observed between these agonists. It was not possible to determine whether autodesensitization to SP also results from depletion of an intracellular Ca^{2+} store.

Introduction

There is good evidence that substance P (SP) is a neurotransmitter in the mammalian gastrointestinal tract where it seems to be involved in both neuro-neuronal and neuro-muscular transmission. SP contracts the longitudinal muscle of the guinea-pig small intestine by a direct action on the smooth muscle cells, depolarizes the muscle membrane and increases the frequency of spontaneous spike discharges; it has been suggested that these effects are due to inactivation of K^+ conductance (Fujisawa & Ito, 1982). Studies of the effect of SP on the ^{86}Rb efflux from intestinal smooth muscle have also indicated such a mode of action, but it appears that this is not the only mechanism by which SP causes contraction (Holzer & Petsche, 1983). The response of intestinal smooth muscle to SP reaches a peak and then fades. The fading of the contraction reflects, in all probability, desensitization of the muscle to SP (Huidobro-Toro *et al.*, 1982; Holzer & Petsche, 1983) which may arise from a block in excitation-contraction coupling (Holzer & Petsche, 1983; Watson & Downes, 1983). It has been argued that the contraction in response to SP involves the mobilization of Ca^{2+} from an intracellular store and that depletion of this store might be responsible for desensitization but there was no direct experimental support for this proposal. In addition, SP was found to alter the transmembrane movements of $^{45}Ca^{2+}$, but the changes were so complex that they could not be fully explained (Holzer & Petsche, 1983).

Activation of the contractile mechanism in smooth muscle is triggered by an increase in the concentra-

tion of free Ca^{2+} in the cytoplasm and it now seems well established that drugs may bring about such an increase not only by a depolarizing action on the cell membrane but also by release of Ca^{2+} from an intracellular store (see Brading & Sneddon, 1980). Much evidence for the latter has come from studies of the responses of vascular smooth muscle to noradrenaline (e.g. Karaki *et al.*, 1979; van Breemen *et al.*, 1982) or of intestinal smooth muscle to acetylcholine or carbachol (e.g. Ohashi *et al.*, 1974; Casteels & Raeymaekers, 1979; Brading & Sneddon, 1980) which persist in Ca^{2+}-free media for some time.

The present study examines whether the contraction of intestinal smooth muscle elicited by SP may be due in part to release of Ca^{2+} from an intracellular store. Further experiments were undertaken to determine whether the Ca^{2+} store is selectively affected by SP and to show whether depletion of the store could be a cause for the desensitization to SP.

Methods

Recording of contractions

Segments of ileum, at least 5 cm distant from the ileocaecal valve, were obtained from albino guinea-pigs (300–400 g, either sex). The segments, 1.5–2 cm in length, were cut open along the mesenteric attachment and mounted in a 6 ml glass organ bath containing oxygenated Tyrode solution at 37°C. The bath fluid could be changed rapidly by overflow. The preparations were kept under a resting load of 0.5 g, and longitudinal contractions were recorded isotonically (for further details see Holzer & Petsche, 1983).

After 30 min equilibration, standard control responses to 30 s applications of histamine (9 μM) were recorded and subsequent responses expressed as a percentage of these. The ileum was then repeatedly for up to 30 min exposed to SP (22 nM, contact time 30 s) until a constant response was obtained. Concentration-response curves for SP in normal Tyrode solution were generated by exposing the ileum to increasing concentrations of SP at 5 min intervals, each addition of SP being followed by a wash after the response had reached a peak. Further details of the experimental protocol are given under results and in the figures.

Measurement of $^{45}Ca^{2+}$ efflux

Strips (approximately 5 cm long) of longitudinal muscle with adhering myenteric plexus were prepared from the jejunum and ileum of the guinea-pig according to the method of Bolton (1972). The tissues were equilibrated for 30 min in oxygenated Tyrode solution and then incubated for 2–3 h in oxygenated Tyrode solution containing 74 KBq ml^{-1} $^{45}Ca^{2+}$ at 37°C (specific activity of $^{45}Ca^{2+}$: 24 MBq μmol^{-1}; Amersham). After this, the strips were placed for 30 s in Ca^{2+}-free medium, and the efflux of $^{45}Ca^{2+}$ from the tissue was followed by transferring the strips every minute through a series of vials containing Ca^{2+}-free medium. The $^{45}Ca^{2+}$ remaining in the tissue was extracted by incubating the strips for 15 h in 2 ml of 5 mM EDTA (ethylenediaminetetraacetic acid), a procedure which transfers the $^{45}Ca^{2+}$ contained in the tissue into the vial solution (Aaronson & van Breemen, 1981). Subsequently, 7 ml scintillator (Unisolve I, Koch-Light Laboratories) was added to each vial and the radioactivity determined by scintillation spectrometry.

Solutions

The normal Tyrode solution used in this study was of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6; pH 7.4. It was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. In order to avoid effects of acetylcholine released by SP (Holzer & Lembeck, 1980; Yau & Youther, 1982), atropine (0.35 μM) was added in all experiments except those in which the effect of acetylcholine was studied. Ca^{2+}-free Tyrode solution was prepared by omitting $CaCl_2$ from and adding 1 mM EGTA (ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid) to the solution. In some experiments the K^+ concentration of the medium was increased by a factor of 20 (54 mM K^+). In this instance the Tyrode solution was kept isosmotic by appropriate changes in the NaCl concentration. Only in those experiments where short (0.5–2 min) applications of Ca^{2+} (9–72 mM) were used, was $CaCl_2$ added so as to result in a hyperosmotic solution.

Substances

The following drugs were used: acetylcholine (Becker), atropine sulphate, EDTA and EGTA (Merck), histamine dihydrochloride and substance P (Serva), and tetraethylammonium chloride (Merck-Schuchardt). SP was dissolved (1 mg ml^{-1}) and diluted in 0.01 M acetic acid. When added to the bath in volumes not exceeding 3% of the bath volume, this solvent had no effect on the pH of the medium nor on the contractile activity of the ileum.

Statistics

All values are expressed as the mean ± s.e.mean. The two sample or paired t test was used for statistical comparisons, where appropriate; P values < 0.05 were regarded as significant.

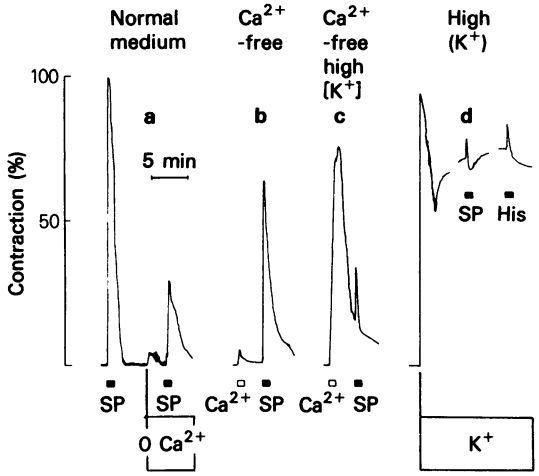

Figure 1 Contractions of the guinea-pig ileum longitudinal muscle in response to substance P (SP). (a) Effect of SP (2.2 μM) in normal medium (1.8 mM Ca^{2+}, 2.7 mM K^+) and after 2.5 min of bathing in Ca^{2+}-free medium (O Ca^{2+}). (b) Effect of SP (2.2 μM) in Ca^{2+}-free medium (O Ca^{2+}, 2.7 mM K^+), SP being added 2.5 min after exposure of the Ca^{2+}-depleted ileum to Ca^{2+} (36 mM) for 1 min. (c) Effect as in (b) but in the continuous presence of 54 mM K^+. (d) Effect of SP (22 μM) and histamine (His 0.9 mM) on a preparation contracted by 54 mM K^+ in the presence of Ca^{2+} (1.8 mM). SP and histamine were applied at 10 and 20 min, respectively, after exposure to high [K^+]. Ordinate scale: contraction expressed as a percentage of the effect of 9 μM histamine in normal medium.

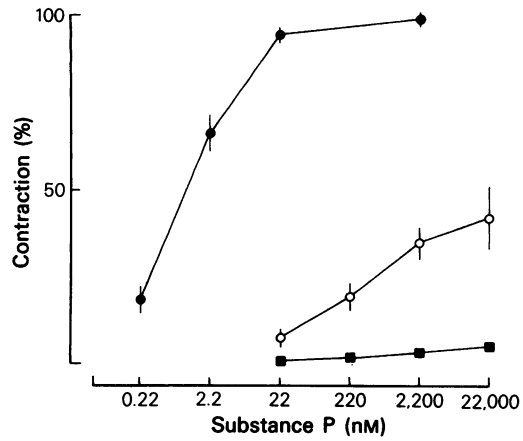

Figure 2 Concentration-response curves for substance P in normal medium (1.8 mM Ca^{2+}, 2.7 mM K^+, ●), in high [K^+] medium (1.8 mM Ca^{2+}, 54 mM K^+, ■), and 2.5 min after removal of extracellular Ca^{2+} (O Ca^{2+}, 2.7 mM K^+, ○). Ordinate scale: as in Figure 1. Means of $n = 6-10$; vertical lines show s.e.mean.

Results

Effect of removal of extracellular Ca^{2+} on responses to substance P

Replacement of the normal Tyrode solution with Ca^{2+}-free medium caused a slight contraction and sometimes increased the spontaneous activity of the ileal longitudinal muscle but these effects waned and disappeared within 2 min (Figure 1a). SP, added 2.5 min after the change to the Ca^{2+}-free medium, was still able to produce a transient contraction (Figure 1a) but the potency and efficacy of SP in eliciting a contraction were greatly diminished (Figure 2). The isotonic response to SP in normal Tyrode solution develops very rapidly and a peak response is usually reached 10–15 s after exposure to the peptide (Holzer *et al.*, 1981). In contrast, the response to a 1 min application of SP after removal of extracellular Ca^{2+} was delayed by up to 10 s and developed more slowly, taking up to 30 s to peak. The contraction then faded rapidly and the muscle relaxed to a near base-line level within 1.5 min following the washout of SP (Figure 1a).

Figure 3 shows that, in the prolonged absence of extracellular Ca^{2+}, the response of the guinea-pig ileum to SP (2.2 μM) became smaller with time and that the ability of the tissue to respond to SP with a contraction was lost within 10 min. Repetitive exposure of the ileum to SP (2.2 μM) in Ca^{2+}-free medium accelerated the loss of the response to SP. Thus, when a 1 min application of SP 5 min after the change to the Ca^{2+}-free medium had been preceded by a 1 min application of SP at 2.5 min, the response was only $2 \pm 1\%$ ($n = 24$) instead of $13 \pm 4\%$ ($n = 10$; $P < 0.01$) of that in tissue not previously exposed to SP.

Experiments, in which the loss of $^{45}Ca^{2+}$ was studied, demonstrated a rapid depletion of $^{45}Ca^{2+}$ from the tissue. The loss of $^{45}Ca^{2+}$ seemed, however, to proceed more slowly than the loss of the ability of the muscle to contract in response to SP (Figure 3).

Loading of an internal Ca^{2+} store

The fact that a response could still be elicited by SP in the absence of extracellular Ca^{2+} suggested that this response might be due to mobilization of Ca^{2+} from an intracellular store. As stated above, the responsiveness of the muscle to SP was lost after 10 min of bathing in Ca^{2+}-free medium; it could be restored completely by bathing the tissue in normal Tyrode solution (1.8 mM Ca^{2+}) for 10–15 min. However, if higher Ca^{2+} concentrations (9–72 mM) were used, 0.5–2 min applications of Ca^{2+} sufficed to restore substantially the responsiveness of the muscle to SP in Ca^{2+}-free medium (Figures 1b and 4). The addi-

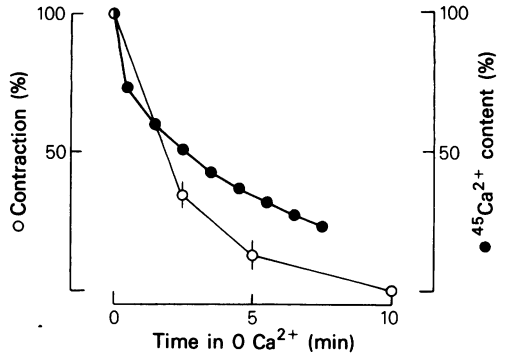

Figure 3 Effect of removal of extracellular Ca^{2+} on the responsiveness of the guinea-pig ileum longitudinal muscle to substance P (SP, 2.2 μM) and on the loss of $^{45}Ca^{2+}$ from muscle preloaded with the isotope into Ca^{2+}-free Tyrode solution. (○) Left ordinate scale: relative contraction height; 100% = initial response to SP (2.2 μM). (●) Right ordinate scale: relative $^{45}Ca^{2+}$ content; 100% = content prior to incubation in Ca^{2+}-free medium (calculated from the loss of label into Ca^{2+}-free solution). Means of $n = 6-10$ for contraction and $n = 22$ for $^{45}Ca^{2+}$ content; vertical lines show s.e.mean.

tion of $CaCl_2$ to the bath resulted in a hyperosmotic bathing medium and produced a transient contraction on its own. This response to $CaCl_2$ seemed unrelated to the Ca^{2+} concentrations and disappeared about 1.5 min after washout of $CaCl_2$ (Figures 1b and 4). SP (2.2 μM), given 2.5 min after washout of $CaCl_2$, caused a transient contraction, the magnitude of which depended on the $[Ca^{2+}]$ (Figures 4 and 5a), the time for which this $[Ca^{2+}]$ had been applied (Figures 4 and 5b), and the time interval between washout of $CaCl_2$ and application of SP (Figure 5c).

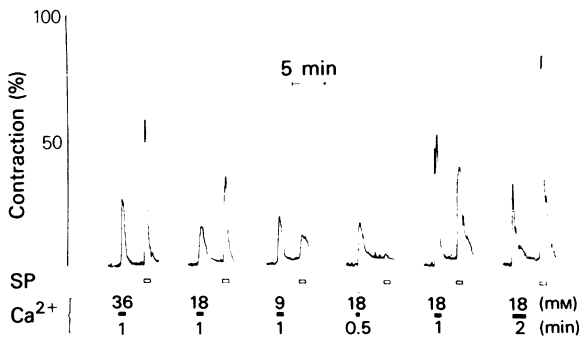

Figure 4 Contractions of the guinea-pig ileum longitudinal muscle to substance P (SP, 2.2 μM) in Ca^{2+}-free solution. SP was added 2.5 min after exposure of the Ca^{2+}-depleted ileum to various concentrations of Ca^{2+} (hyperosmotic addition of 9–36 mM $CaCl_2$) for different time periods (0.5–2 min). Ordinate scale as in Figure 1.

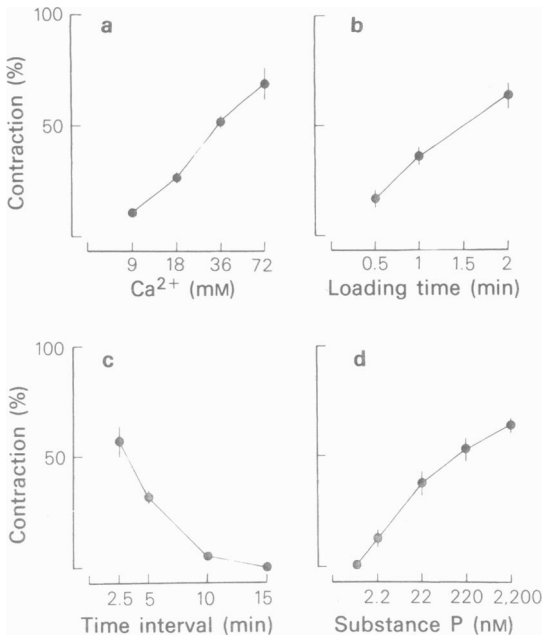

Figure 5 Quantitative results obtained from the experiments illustrated in Figure 4. Relation between the response of the guinea-pig ileum to substance P (SP, 2.2 μM) in Ca^{2+}-free solution and (a) the concentration of Ca^{2+} to which the ileum had been exposed for 1 min, 2.5 min prior to the application of SP; (b) the time for which the ileum had been exposed to Ca^{2+} (18 mM) 2.5 min prior to the application of SP, and (c) the time interval which elapsed between the 1 min application of Ca^{2+} (36 mM) and the subsequent application of SP. (d) Relation between the concentration of SP when applied 2.5 min after a 1 min application of Ca^{2+} (36 mM) and the size of the contraction observed. Ordinate scale as in Figure 1. Means of $n = 6-10$; vertical lines show s.e.mean.

The response to SP was, as might be expected, also dependent on the concentration of SP (Figure 5d). The concentration-response curve for SP, when the Ca^{2+} store had been reloaded by a 1 min application of 36 mM $CaCl_2$, was further to the left, steeper and showed a higher maximum than that observed when the normal Tyrode solution had been replaced by Ca^{2+}-free medium (compare Figures 2 and 5d).

Figure 5c suggests that the Ca^{2+} store mobilized by SP has been depleted within 10–15 min following the washout of $CaCl_2$. Reapplication of $CaCl_2$ refilled the Ca^{2+} store. This cycle of filling and depleting the Ca^{2+} store could be repeated at 15–20 min intervals for 2–3 h. During this time, the responses to SP, applied shortly after washout of $CaCl_2$, were reproducible; after 2–3 h the preparations usually began to deteriorate and were then discarded. The re-

sponses to SP obtained after the first two cycles of refilling the Ca^{2+} store were also somewhat erratic and have, therefore, been excluded from the evaluation of the results.

Separate experiments were carried out to investigate whether there was a difference when the Ca^{2+} store mobilized by SP was filled by hyperosmotic or isosmotic application of $CaCl_2$. This was done by loading the Ca^{2+} store with $CaCl_2$ (18 mM for 2 min) hyperosmotically or isosmotically and by testing with an application of SP (2.2 μM) 2.5 min after washout of $CaCl_2$. The response after isosmotic loading was $64 \pm 5\%$ ($n = 6$) and after hyperosmotic loading $62 \pm 2\%$ ($n = 6$). Thus, short exposures of the ileum to hyperosmotic media appeared not to have a deleterious effect on the responsiveness of the tissue to SP. All other experiments reported here were performed by adding $CaCl_2$ hyperosmotically.

Another series of experiments was carried out to investigate whether the refilling of the Ca^{2+} store and/or the release of Ca^{2+} from the store by SP is affected by depolarization of the muscle with high $[K^+]$. As shown in Figure 1c, the application of $CaCl_2$ (36 mM) in the presence of 54 mM K^+ resulted in a much larger contraction than in the presence of 2.7 mM K^+ (Figure 1b). In the presence of 54 mM K^+, although the $CaCl_2$ was washed out after 1 min, the muscle continued to contract before a rapid relaxation ensued (Figure 1c). In contrast, the response to SP (2.2 μM), given 2.5 min after washout of $CaCl_2$, was depressed in high $[K^+]$ medium (Figure 1b, c): the response was only $19 \pm 3\%$ ($n = 7$) compared with $55 \pm 6\%$ ($n = 7$; $P < 0.01$) in normal $[K^+]$ medium. It was also noted that in the presence of 54 mM K^+, SP caused only a short twitch-like contraction which disappeared completely within 30–40 s of exposure to the peptide, i.e. whilst SP was still present in the bath (Figure 1c). Furthermore, when 54 mM K^+ was present only during the loading of the Ca^{2+} store, the response to SP was also decreased and was only $22 \pm 3\%$ ($n = 9$) compared with $57 \pm 3\%$ ($n = 9$; $P < 0.01$) when the store had been loaded in normal $[K^+]$ medium, although the time courses of the responses were the same.

Exposure of the ileum to K^+ (54 mM) in normal Tyrode solution caused a sustained contraction and even under these conditions SP, like histamine, was still able to elicit a slight, short-lasting contraction superimposed on the K^+-evoked contracture (Figure 1d). The responses to SP and histamine were followed by a rapid relaxation below the level observed before drug application. The concentration-response curve for SP in this high $[K^+]$ medium was very flat and to the right of the curve obtained in normal medium (Figure 2).

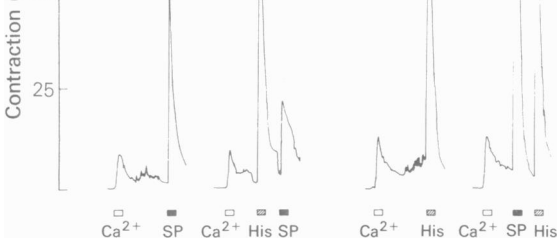

Figure 6 Responses of the guinea-pig ileum to substance P (SP, 2.2 μM) and histamine (His 0.27 mM) in Ca^{2+}-free solution (for at least 15 min). The substances were added in the indicated order, 2.5 or 5 min after a 1 min application of 36 mM Ca^{2+} so as to examine whether the internal Ca^{2+} store used by one substance is also used by the other. Ordinate scale as in Figure 1.

Effect of other spasmogens on the Ca^{2+} store mobilized by substance P

It was found that in addition to SP, histamine, acetylcholine (in the absence of atropine), tetraethylammonium and K^+ were able to elicit a contraction of the ileum in Ca^{2+}-free medium, when they were applied shortly after a 1 min application of $CaCl_2$ (36 mM). The responses to these agonists, like those to SP, disappeared within 15 min of bathing in Ca^{2+}-free medium. These findings raised the question whether the Ca^{2+} store that is mobilized by SP is identical to those which are mobilized by the other agonists. The experiments designed to resolve this question are illustrated in Figure 6 and the quantitative results obtained are shown in Figure 7. In brief, the response to SP, or of the agonist being investigated, was tested 5 min after placing the tissue in Ca^{2+}-free medium. Similar tests were made with tissues that had been previously exposed to SP, or agonist, 2.5 min after being placed in Ca^{2+}-free medium. The concentrations of SP and the agonists were such that they produced contractions of similar magnitude. The response to SP was significantly depressed by the prior application of histamine (Figures 6 and 7) or acetylcholine (Figure 7) whereas the responses to histamine or acetylcholine remained unchanged by prior application of SP.

Prior application of K^+ did not alter the response to SP nor was the response to K^+ changed by a preceding application of SP. In contrast, the response to tetraethylammonium was significantly inhibited when the muscle had been previously exposed to SP whereas the response to SP remained unaffected by a preceding exposure to tetraethylammonium (Figure 7).

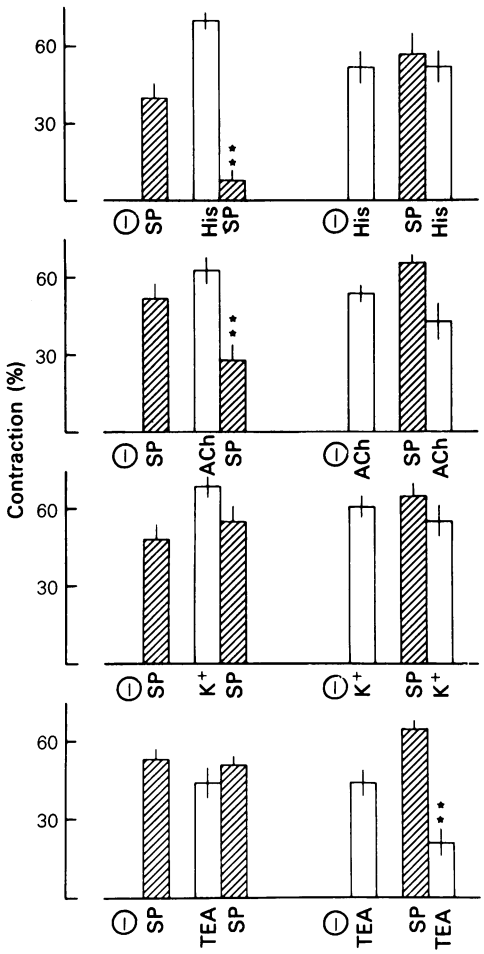

Figure 7 Quantitative results obtained in experiments of the type illustrated in Figure 6. The figure shows the effects of substance P (SP, 2.2 μM) and of histamine (His, 0.27 mM) acetylcholine (ACh, 0.18 mM), K^+ (54 mM) or tetraethylammonium (TEA, 30 mM) added to the Ca^{2+}-free medium 2.5 or 5 min after application of Ca^{2+} (36 mM, 1 min). The left hand column of each pair denotes the contraction elicited by an agonist 2.5 min after the application of Ca^{2+} (Θ: no agonist was added), and the right hand column denotes that elicited by a second agonist 5 min after the application of Ca^{2+}. Each of the four panels thus exhibits the influence that is exerted by one of the agonists on the response to the subsequent application of SP and *vice versa*. Ordinate scale as in Figure 1. Means of $n = 6-10$; vertical lines show s.e.mean. $^{**}P < 0.01$.

Desensitization to substance P in Ca^{2+}-free medium

In a previous study (Holzer & Petsche, 1983) it was suggested that the desensitization of the ileal longitudinal muscle to SP, which is observed during the prolonged presence of high SP concentrations, might be due to a depletion of an intracellular Ca^{2+} store. This possibility was tested in the present experiments. Desensitization to SP was induced by exposing the ileum to SP (2.2 μM) for 5 min and quantified by measuring the inhibition of the response to SP (22 nM) applied 3.5 min after washout of the solution containing the desensitizing concentration of SP. In normal Tyrode solution (1.8 mM Ca^{2+}) the response to SP (22 nM) was $69 \pm 3\%$ ($n = 9$) prior to, and $34 \pm 4\%$ ($n = 9$; $P < 0.01$) after desensitization to SP. This experiment was repeated in Ca^{2+}-free medium. After exposure of the Ca^{2+}-depleted tissue to the desensitizing concentration of SP for 5 min, which by itself evoked no response, a 1 min application of 18 mM $CaCl_2$ was made and SP (22 nM) applied 2.5 min after washout of $CaCl_2$ (i.e. 3.5 min after washout of the desensitizing solution of SP). In Ca^{2+}-free medium, the response to SP (22 nM) was $39 \pm 5\%$ ($n = 5$) without, and $15 \pm 2\%$ ($n = 5$; $P < 0.01$) with a preceding exposure to 2.2 μM SP.

Discussion

The present results show that after removal of extracellular Ca^{2+}, SP is still able to elicit a contraction of the longitudinal muscle of the guinea-pig ileum. The time course of the disappearance of these responses in Ca^{2+}-free solution is very similar to that seen with responses to acetylcholine in the guinea-pig taenia coli (Casteels & Raeymaekers, 1979). How fast is extracellular Ca^{2+} depleted from the guinea-pig ileum longitudinal muscle under these conditions? It is probable that extracellular, free Ca^{2+} was reduced very rapidly, since firstly the longitudinal muscle layer with attached myenteric plexus is very thin (20–50 μm; Paton, 1975), secondly EGTA (able to diffuse into the extracellular but not intracellular space, see Casteels & Raeymaekers, 1979) will form a complex with free extracellular Ca^{2+}, and thirdly the ileum was cut open to facilitate removal of mucosal extracellular Ca^{2+}. Thus, if the diffusion constant for Ca^{2+} in the extracellular space of smooth muscle is assumed to be 173 $\mu m^2 s^{-1}$ (Keatinge, 1972) and the calculations outlined by Hill (1928) are used, the time required for a 90% reduction in the extracellular [Ca^{2+}] in a muscle sheet of 50 μm thickness would be 12.5 s. However, while EGTA will accelerate removal of free Ca^{2+}, diffusion of Ca^{2+} from deep in the ileum wall will delay removal of free Ca^{2+}. Nevertheless, the extracellular [Ca^{2+}] in the longitudinal muscle would be greatly reduced within 1 min. After 10 min in Ca^{2+}-free medium, the tissue content of $^{45}Ca^{2+}$ was reduced by more than 80% and the tissue no longer responded to SP, indicating that Ca^{2+} is necessary for the response to SP. Thus when

SP causes a contraction after the tissue has been in Ca^{2+}-free medium for more than 1 min it is probable that Ca^{2+} is being mobilized from a site other than the extracellular space.

The persistence of responses to smooth muscle stimulants in Ca^{2+}-free solution has been widely accepted as indicating that these substances are capable of mobilizing Ca^{2+} from an intracellular store (Durbin & Jenkinson, 1961; Edman & Schild, 1962; Ohashi *et al.*, 1974; Casteels & Raeymaekers, 1979; Brading & Sneddon, 1980; van Breemen *et al.*, 1982). The present results indicate that SP is also able to release Ca^{2+} from an intracellular store. The response to SP in Ca^{2+}-free medium might serve as a measure of the degree to which this store is filled with Ca^{2+} or of the amount of Ca^{2+} which is released from this store during a contraction, but the exact relation is difficult to determine.

The characteristics of the Ca^{2+} store mobilized by SP can be summarized as follows. Firstly, it is only affected by concentrations of SP higher than those required to produce a near-maximal contraction in normal medium. Repeated exposure of the muscle to such high concentrations of SP in Ca^{2+}-free solution seems to accelerate the depletion of the Ca^{2+} store. Secondly, this store can be replenished very rapidly from extracellular Ca^{2+}. Thirdly, it is rapidly depleted by removal of extracellular Ca^{2+} but this depletion seems to proceed more slowly than the removal of Ca^{2+} from the extracellular space of the muscle. Fourthly, the store can supply sufficient Ca^{2+} to enable a 70% activation of the contractile proteins to occur.

The first indication that excitatory substances may contract smooth muscle other than by a depolarizing action on the cell membrane came from the observation that smooth muscle contracted by high $[K^+]$ would contract further in response to such agonists (Evans & Schild, 1957; Evans *et al.*, 1958). From the present study it appears that the same is true for SP, and it is tentatively concluded that the transient contraction of the K^+-depolarized ileum in response to SP, like that of taenia coli to carbachol (Brading & Sneddon, 1980), arises from the release of Ca^{2+} from an intracellular store. The Ca^{2+} store mobilized by SP can also be refilled in the K^+-depolarized ileum. Likewise, the Ca^{2+} store used by acetylcholine or carbachol in the taenia coli can also be loaded in preparations depolarized by high $[K^+]$ (Casteels & Raeymaekers, 1979; Brading & Sneddon, 1980). However, the store used by acetylcholine in the taenia coli appears to be increased by K^+ depolarization (Casteels & Raeymaekers, 1979), but that used by SP in the ileum seems to be decreased. The contraction in response to $CaCl_2$ was clearly enhanced in the K^+-depolarized tissue which suggests that extra Ca^{2+} must have been taken up by the depolarized muscle, but this Ca^{2+} appeared to be only loosely sequestered in the cell and to be rapidly released on removal of extracellular Ca^{2+} as indicated by a further increase in contraction following the removal of $CaCl_2$ (see Figure 1c).

The evidence available suggests that there is more than one mechanism by which SP can increase intracellular $[Ca^{2+}]$. Low, submaximally effective, concentrations of SP elicit a sustained contraction (Holzer & Lembeck, 1980), membrane depolarization (due to reduced G_{K+}) and Ca^{2+} influx (Bury & Mashford, 1976; Szeli *et al.*, 1977; Fujisawa & Ito, 1982; Holzer & Petsche, 1983). Higher concentrations of SP, producing a contraction that after reaching a maximum fades away (Holzer & Lembeck, 1980), may release Ca^{2+} from an intracellular store. The present findings do not allow us to locate this Ca^{2+} store, nor decide whether activated SP receptors directly release Ca^{2+} from the store or do so indirectly e.g. by means of an effect on a membrane K^+ conductance. Since SP was also able to cause contraction in the K^+-depolarized muscle, it would appear that the SP-induced release of Ca^{2+} from a store is at least to some extent independent of the membrane potential. Depolarization with high $[K^+]$ seemed to decrease the size of the store and this might indicate that the store is associated with the cell membrane. The Ca^{2+} could be bound either in the membrane or to its internal surface, and depolarization might alter the characteristics of the store. Consistent with this view is the finding that in high $[K^+]$ medium (see Figure 1c, d) the tissue responded to SP with a twitch-like contraction and then relaxed rapidly in spite of the presence of SP in the bath. The present findings could thus be accommodated in the hypothesis proposed by Bolton (1979) that 'there is some calcium associated very closely with the receptors for several stimulants, perhaps bound to the receptor macromolecule or adjacent internal surface of the membrane, not in equilibrium with external ionized calcium and inaccessible to chelating agents'.

Since the desensitization to high concentrations of SP is quite specific, the Ca^{2+} store believed to be depleted during desensitization should be selectively utilized by SP and not by other agonists (Holzer & Petsche, 1983). This hypothesis was tested by comparing the effects of doses of SP and other agonists, that in Ca^{2+}-free solution produced contractions of similar magnitude, on each other. It is to be expected that a similar amount of Ca^{2+} will be released if the contractions in response to the different agonists are of the same size. The experiments indicated that the store which is mobilized by SP overlaps with those mobilized by acetylcholine and histamine whereas the stores necessary to produce contractions by acetylcholine and histamine do not overlap with the store associated with SP contraction. These results

are in good agreement with the findings that responses to acetylcholine or histamine remain practically unaltered in preparations desensitized to SP (Franco *et al.*, 1979; Jordan, 1980; Huidobro-Toro *et al.*, 1982; Holzer & Petsche, 1983) whereas responses to SP are substantially diminished in the ileum desensitized to acetylcholine or histamine (Jordan, 1980). There is also unidirectional cross-desensitization between SP and tetraethylammonium, tetraethylammonium responses being depressed in the ileum desensitized to SP whereas responses to SP are enhanced in the ileum desensitized to tetraethylammonium (Holzer & Petsche, 1983). These findings are paralleled by the present results in that depletion of the Ca^{2+} store utilized by tetraethylammonium had no discernible effect on the store available to SP whereas depletion of the store utilized by SP diminished the store available to tetraethylammonium (Figure 7). A possible explanation for the unidirectional overlap of Ca^{2+} pools which are mobilized by different agonists could be that some of them mobilize more Ca^{2+} than is necessary to produce the contraction.

The Ca^{2+} pools mobilized by SP and K^+ depolarization in Ca^{2+}-free medium seem not to overlap. This result is difficult to reconcile with the finding that responses to high $[K^+]$ are depressed in the ileum desensitized to SP (Holzer & Petsche, 1983). It is possible that the mechanism of cross-desensitization between SP and K^+ is unrelated to the depletion of a Ca^+ store.

The postulated unidirectional overlap between the Ca^{2+} pools used by SP, acetylcholine, histamine and tetraethylammonium and the correspondence with the unidirectional cross-desensitization between these agonists implies that cross-desensitization involves post-receptor events (Jordan, 1980; Holzer & Petsche, 1983), i.e. is due to an interference at an intracellular Ca^{2+} pool from which Ca^{2+} is released by the agonists. Is depletion of the Ca^{2+} store also responsible for SP autodesensitization? The finding that desensitization to SP developed in Ca^{2+}-depleted tissue could be evidence against this hypothesis, but may also indicate that a desensitizing concentration of SP interferes with the loading of the Ca^{2+} store during the application of $CaCl_2$. Evidence is accumulating that autodesensitization to SP results from a block in excitation-contraction coupling, since the effects of SP on ^{86}Rb efflux and ^{45}Ca influx (Holzer & Petsche, 1983) and on inositol phospholipid hydrolysis (Watson & Downes, 1983) still continue when the contractile response has waned. The exact mechanism of SP autodesensitization remains unknown.

We are most grateful to Prof. F. Lembeck for continuously encouraging this study and critically reading the manuscript, to Dr M. Holzbauer and Dr D.F. Sharman for stylistic revision of the English text, and to Mr W. Schluet for preparing the drawings. This study was made possible by financial support from the Austrian Scientific Research Funds (grant 4641).

References

AARONSON, P. & VAN BREEMEN, C. (1981). Effects of sodium gradient manipulation upon cellular calcium, ^{45}Ca fluxes and cellular sodium in the guinea-pig taenia coli. *J. Physiol.*, **319,** 443–461.

BOLTON, T.B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *J. Physiol.*, **220,** 647–671.

BOLTON, T.B. (1979). Mechanism of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59,** 606–718.

BRADING, A.F. & SNEDDON, P. (1980). Evidence for multiple sources of calcium for activation of the contractile mechanism of guinea-pig taenia coli on stimulation with carbachol. *Br. J. Pharmac.*, **70,** 229–240.

BURY, R.W. & MASHFORD, M.L. (1976). Interactions between local anaesthetics and spasmogens on the guinea-pig ileum. *J. Pharmac. exp. Ther.*, **197,** 633–640.

CASTEELS, R. & RAEYMAEKERS, L. (1979). The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.*, **294,** 51–68.

DURBIN, R.P. & JENKINSON, P.H. (1961). The effect of carbachol on the permeability of depolarized smooth muscle to inorganic ions. *J. Physiol.*, **157,** 74–89.

EDMAN, K.A.P. & SCHILD, H.O. (1962). The need for calcium in the contractile responses induced by acetylcholine and potassium in the rat uterus. *J. Physiol.*, **161,** 424–441.

EVANS, D.H.L. & SCHILD, H.O. (1957) Mechanism of contraction of smooth muscle by drugs. *Nature, Lond.*, **180,** 341–342.

EVANS, D.H.L., SCHILD, H.O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol.*, **143,** 474–485.

FRANCO, R., COSTA, M. & FURNESS, J.B. (1979). Evidence for the release of endogenous substance P from intestinal nerves. *Naunyn-Schmiedebergs Arch. Pharmac.*, **306,** 195–201.

FUJISAWA, K. & ITO, Y. (1982). The effects of substance P on smooth muscle cells and on neuro-effector transmission in the guinea-pig ileum. *Br. J. Pharmac.*, **76,** 279–290.

HILL, A.V. (1928). Diffusion of oxygen and lactic acid through tissues. *Proc. R. Soc. B.*, **104,** 39–96.

HOLZER, P., EMSON, P.C., IVERSEN, L.L. & SHARMAN, D.F. (1981). Regional differences in the response to substance P of the longitudinal muscle and the concentration of substance P in the digestive tract of the guinea-pig. *Neuroscience*, **6,** 1433–1441.

HOLZER, P. & LEMBECK, F. (1980). Neurally mediated

contraction of ileal longitudinal muscle by substance P. *Neurosci. Lett.*, **17,** 101–105.

HOLZER, P. & PETSCHE, U. (1983). On the mechanism of contraction and desensitization induced by substance P in the intestinal muscle of the guinea-pig. *J. Physiol.*, **342,** 549–568.

HUIDOBRO-TORO, J.B., CHELALA, C.A., BAHOUTH, S., NODAR, R. & MUSACCHIO, J.M. (1982). Fading and tachyphylaxis to the contractile effects of substance P in the guinea-pig ileum. *Eur. J. Pharmac.*, **81,** 21–34.

JORDAN, C.C. (1980). An examination of desensitization as a basis for defining substance P-like agonist activity. In *Neuropeptides and Neural Transmission.* ed. Ajmone Marsan, C. & Traczyk, W.Z. pp. 131–140. New York: Raven Press.

KARAKI, H., KUBOTA, H. & URAKAWA, N. (1979). Mobilization of stored calcium for phasic contraction induced by norepinephrine in rabbit aorta. *Eur. J. Pharmac.*, **56,** 237–245.

KEATINGE, W.R. (1972). Ca concentrations and fluxes in Ca-deprived arteries. *J. Physiol.*, **224,** 35–59.

OHASHI, H., TAKEWAKI, T. & OKADA, T. (1974). Calcium and the contractile effect of carbachol in the depolarized guinea-pig taenia caecum. *Jap. J. Pharmac.*, **24,** 601–611.

PATON, W.D.M. (1975). Transmural and field stimulation of nerve-smooth muscle preparations. In *Methods in Pharmacology*, vol. 3, *Smooth Muscle.* ed. Daniel, E.E. & Paton, D.M. pp. 313–320. New York: Plenum Press.

SZELI, J., MOLINA, E., ZAPPIA, L. & BERTACCINI, G. (1977). Action of some natural polypeptides on the longitudinal muscle of the guinea-pig ileum. *Eur. J. Pharmac.*, **43,** 285–287.

VAN BREEMEN, C., AARONSON, P., LOUTZENHISER, R. & MEISHERI, K. (1982). Calcium fluxes in isolated rabbit aorta and guinea-pig taenia coli. *Fedn Proc.*, **41,** 2891–2897.

WATSON, S.P. & DOWNES, C.P. (1983). Substance P induced hydrolysis of inositol phospholipids in guinea-pig ileum and rat hypothalamus. *Eur. J. Pharmac.*, **93,** 245–253.

YAU, W.M. & YOUTHER, M.L. (1982). Direct evidence for a release of acetylcholine from myenteric plexus of guinea-pig small intestine by substance P. *Eur. J. Pharmac.*, **81,** 665–668.

(Received November 4, 1983.
Revised December 2, 1983.)