

EFFECTS OF PROCAINE AND EXTRACELLULAR CALCIUM CONCENTRATION ON RESPONSE OF RAT STOMACH FUNDUS MUSCLE TO ACETYLCHOLINE AND 5-HYDROXYTRYPTAMINE

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- 1 When rat stomach fundus muscle was incubated for 30 min in Tyrode solution from which calcium chloride had been omitted, there was an almost complete abolition of the contractile response to 5-hydroxytryptamine (5-HT) while that to acetylcholine (ACh) was still present.
- 2 The maximum tension obtainable with ACh remained the same in external calcium concentrations ranging from 0.45 to 3.6 mM, but the pD_2 value increased.
- 3 A concentration of at least 0.9 mM calcium was needed to maintain a maximum contraction with 5-HT, and the pD_2 for this agent also increased significantly with increase in calcium content of the medium.
- 4 The effects of procaine on the responses of the muscle to 5-HT and ACh were similar to the respective changes induced by lowering the calcium concentration, and were reduced by the addition of calcium.
- 5 Concentrations of 2.2×10^{-7} to 3.6×10^{-5} M procaine reduced the effects of both 5-HT and KCl and suppressed the maximum responses.
- 6 The maximum responses to KCl and 5-HT were restored at higher concentrations of procaine ($> 3.6 \times 10^{-4}$ M), while the effect of ACh was reduced.
- 7 It is suggested that 5-HT, like KCl, is almost entirely dependent on extracellular calcium for inducing muscle contraction, while ACh may utilize calcium from bound stores.

Introduction

Contraction of smooth muscle can be brought about by agents that initiate cellular reactions which result in an increase in the free calcium ion concentration in the cytoplasm (Durbin & Jenkinson, 1961; Edman & Schild, 1962; Bohr, 1964; Hurwitz, Joiner & Van Hagen, 1967; Berridge, 1975).

Several detailed studies with acetylcholine (ACh), noradrenaline (NA) and potassium chloride (KCl), have indicated that, while KCl utilizes mainly extracellular calcium, the other agents can release calcium ions from an intracellular source to cause a contraction (Edman & Schild, 1962; Hurwitz *et al.*, 1967; Hudgins & Weiss, 1968; Bose & Innes 1975; Kirkpatrick, 1975).

Similar studies with 5-hydroxytryptamine (5-HT) seem to be lacking, except for an early report by Woolley (1958), who found a qualitative similarity in the response of rat uterus to 5-HT and to calcium ions. The stimulant effect of both agents was abolished by disodium edetate (EDTA). This led the

author to suggest that 5-HT may contract uterine muscle by increasing the permeability of the muscle to calcium ions in the extracellular fluid.

In preliminary studies, we have found that the response of the rat stomach muscle to 5-HT was antagonized by relatively low doses of procaine (1×10^{-7} to 1×10^{-6} M), which had no effect on the response of the muscle to ACh (Weiss & Weinstock, 1976). Other authors have reported either that procaine antagonizes the response of all contractile agents, including ACh, 5-HT and KCl at similar concentrations (Feinstein & Paimre, 1969), or that it antagonizes the action of NA and ACh, but not that of KCl, because it prevents the amines from releasing calcium from firmly bound membrane stores (Hudgins & Weiss, 1968).

In an attempt to resolve these apparent discrepancies, we have carried out a quantitative study of the effect of 5-HT and ACh on rat stomach (fundus) muscle in the presence of different concentrations of

external calcium and procaine. We also attempted to demonstrate whether or not these agents might utilize calcium ions from two different sources.

The fundus of the rat stomach was chosen since it is extremely sensitive to both 5-HT and ACh, and because 5-HT brings about a contraction by a direct action on the muscle itself and not by releasing ACh, as in guinea-pig ileum (Gershon, 1967).

Methods

Male albino rats (Wistar strain), weighing 150 to 200 g, were killed by decapitation and the stomach was removed. A strip was prepared from the fundus as described by Vane (1957) and suspended in a 10 ml isolated organ bath containing Tyrode solution of the following composition (mM): NaCl 136.8, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.6, NaH₂PO₄ 0.4, NaHCO₃ 11.9 and glucose 5.6. For experiments in 'calcium-poor' solution, calcium chloride was omitted from the bath solution.

Bath temperature was maintained at $37 \pm 0.5^\circ\text{C}$ and the Tyrode solution was gassed continuously with 95% O₂ and 5% CO₂.

Before drugs were added, the muscle strips were allowed to equilibrate for 30 to 45 min under a resting tension of 1.5 g. Contractions of the muscle were recorded isometrically on a multichannel polygraph with a Statham strain gauge.

Complete dose-response curves were obtained by adding 5-HT in concentrations ranging from 1×10^{-9} M to 2×10^{-4} M, ACh from 4×10^{-8} M to 4×10^{-2} M, or KCl from 2.7×10^{-3} M to 1.3×10^{-2} M. 5-HT was left in contact with the tissue for 1 min and the preparation was washed 4 to 6 times for 10 s during the following 2 min to ensure that the muscle returned to the initial resting tension before the next dose of drug was injected. The contact time between tissue and ACh or KCl was 30 s; the preparation was washed 3 times for 10 s and the drug injected every 3 min. A different piece of fundus was used for each agonist and each experiment was repeated on at least 5 separate stomach strips.

In other experiments, which were carried out in order to determine the influence of external calcium ion concentration on the responses to 5-HT and ACh, dose-response curves were first obtained after equilibrating the preparation in calcium-poor fluid for 30 min. Then appropriate concentrations of calcium chloride were added to the reservoir of Krebs solution, bathing the tissue. After each change in calcium concentration, the preparation was washed thoroughly and allowed to equilibrate for a further 30 to 45 min. A dose-response curve was obtained after each successive addition of calcium chloride up to a final concentration of 3.6 mM.

In additional experiments, the tissue was incubated for longer periods of time in calcium-poor medium and dose-response curves were obtained to either ACh or 5-HT.

The effect of procaine on the responses of the stomach fundus to 5-HT, ACh or KCl was determined by adding various concentrations of procaine from 2.2×10^{-7} M to 3.6×10^{-3} M to the reservoir containing Tyrode solution with 1.8 mM Ca²⁺ ion concentration. After the tissue had been washed thoroughly for 15 to 20 min, a dose-response curve to one of the three stimulants was obtained and repeated after increasing the concentration of procaine in the Tyrode solution.

In some experiments, the calcium ion concentration in the Tyrode solution was reduced to 0.45 mM or increased to 3.6 mM and the experiments with 5-HT, ACh and procaine repeated as described above. The following drugs were used: acetylcholine bromide (Abic), 5-hydroxytryptamine creatinine sulphate (Sigma), procaine hydrochloride (Hillel).

Acetylcholine and 5-hydroxytryptamine were made up freshly in 0.9% w/v NaCl solution (saline) for each experiment, and stored on ice. Concentrations in the text refer to the molar concentration of the respective salt.

Evaluation of antagonism

The pA₂, pD₂ and pD'₂ values were calculated according to the method of Van Rossum (1963). The pA₂ value indicates the affinity of a competitive antagonist for the receptor and is denoted as the negative logarithm of the molar concentration of antagonist, which causes an elevation of the concentration of the agonist by a factor of 2 in order to obtain the same response. The pD₂ value is given by the negative logarithm of the molar concentration of the agonist, which produces 50% of the maximum effect, and may be related to the affinity in the event that the stimulus-effect relationship is linear. The pD'₂ value indicates the affinity of a non-competitive antagonist for the receptor, and is given by the negative logarithm of the molar concentration of the antagonist, which causes a 50% reduction of the maximum effect of the agonist.

Results

The effects of external calcium concentration on responses of rat fundus to acetylcholine and 5-hydroxytryptamine

In normal Tyrode solution (1.8 mM Ca²⁺), ACh caused graded contractions of the muscle in concentrations ranging from 4.4×10^{-8} to 4.4×10^{-2} M

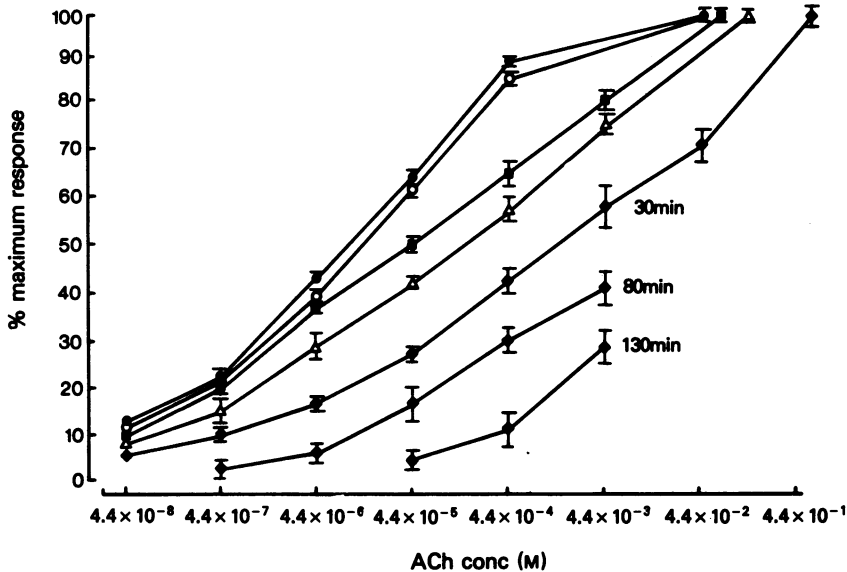


Figure 1 The effects of external calcium ion concentration on the response of the rat stomach fundus to acetylcholine (ACh): Ca^{2+} (●) 3.6 mM; (○) 1.8 mM; (■) 0.9 mM; (△) 0.45 mM; (◆) Tyrode without added Ca^{2+} . Numbers above graphs represent total time in min of exposure of tissue to this solution.

(Figure 1). The maximum increase in tension in the muscle obtained with this agent was 1.4 g. 5-HT contracted the fundus preparation in concentrations ranging from 2×10^{-10} M to 2×10^{-5} M (Figure 2); the maximum increase in tension to 5-HT was 0.7 g, half of that achieved with ACh.

When the muscle was pre-incubated for 30 min in calcium-poor Tyrode solution, the dose-response curve to ACh was shifted by about 2 log units to the right, but it was still possible to obtain the same maximum increase in tension as that achieved in the presence of 1.8 mM Ca^{2+} .

After 80 min or 130 min incubation of the tissue in calcium-poor solution, ACh still caused significant contractions of the muscle (Figure 2).

In contrast to this ability of ACh to maintain its activity despite very low external calcium ion concentration, the response to 5-HT after 30 min preincubation in calcium-poor Tyrode solution was only about 20% of that in normal Tyrode solution, and after a further 50 min, the responses were almost completely abolished (Figure 2).

The addition of calcium to the calcium-poor bathing fluid, increased the response of the muscle to both ACh and 5-HT. ACh caused the same maximum tension in the presence of external calcium concentrations from 0.45 mM to 3.6 mM. 5-HT only caused the tension to increase to its maximum, when a concentration of at least 0.9 mM Ca^{2+} was present.

It was demonstrated by Offermeier & Ariens (1966), that the rat fundus strip has no spare receptors for

5-HT. Since the maximum response to 5-HT remained unaltered in external calcium ion concentrations of 0.9 mM to 3.6 mM, it was possible to compute pD_2 values for this agent in the presence of various amounts of calcium ions (See Table 1). Although we do not know of a similar study carried out with ACh instead of 5-HT, on the rat stomach fundus, examination of the dose-response curve for

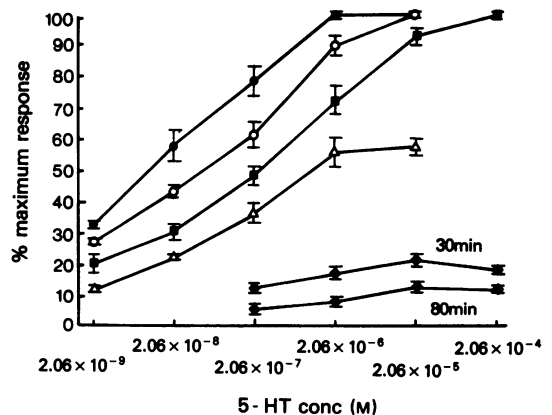


Figure 2 The effects of external calcium ion concentration on the response of the rat stomach fundus to 5-hydroxytryptamine(5-HT): Ca^{2+} (●) 3.6 mM; (○) 1.8 mM; (■) 0.9 mM; (△) 0.45 mM; (◆) Tyrode without added Ca^{2+} . Numbers above graphs represent time in min of incubation of tissue in this solution.

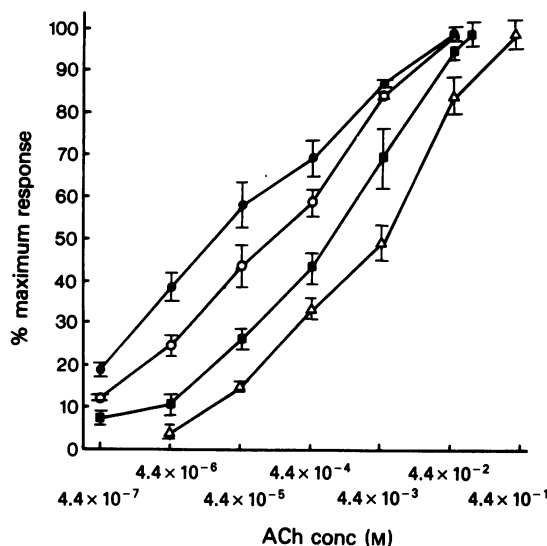


Figure 3 The effects of procaine on the response of the rat stomach fundus to acetylcholine (ACh): (●) control Tyrode solution; procaine (○) 3.6×10^{-4} M; (■) 1.83×10^{-3} M; (△) 3.6×10^{-3} M.

ACh in the presence of 1.8 mM Ca^{2+} ion concentration reveals that it fits the theoretical curve for a direct proportionality between stimulus and effect (Ariens, Simonis & Van Rossum, 1964). Thus if the maximum effect is given by E_m and the maximum stimulus S_m , then the ratio E_a/E_m is proportional to S_a/S_m , where E_a is the effect of any given stimulus S_a . This suggests that there are also no spare receptors for ACh in this preparation. As the maximum response to ACh remained unchanged, we also calculated pD_2 values for this agent in the presence of external calcium ranging from 0.45 to 3.6 mM (see Table 1).

Table 1 pD_2 values for acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) on rat stomach fundus muscle in Tyrode solution containing different concentrations of calcium chloride

Concentration of calcium chloride (mM)	pD_2 values*	
	ACh	5-HT
0.45	3.77	—
0.90	4.30	6.51
1.80	4.85	7.32
3.60	5.03	7.93

* Mean of 5 to 6 experiments.

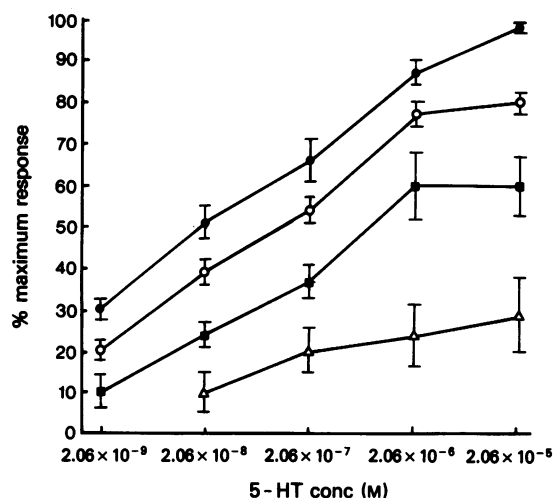


Figure 4 The effects of procaine on the responses of the rat stomach fundus to 5-hydroxytryptamine (5-HT): (●) control Tyrode solution; procaine (○) 2.2×10^{-7} M; (■) 3.6×10^{-7} M; (△) 1.1×10^{-6} M.

The effects of procaine on responses of the rat fundus to acetylcholine, 5-hydroxytryptamine and potassium chloride

Procaine in concentrations of 3.6×10^{-4} to 3.6×10^{-3} M caused a parallel shift to the right in the dose-response curve to ACh with no suppression of the maximum response. Lower concentrations of procaine had no significant effect (Figure 3). The pA_2 value for procaine antagonism of ACh was 3.82. The relationship between $\log(\text{dose-ratio} - 1)$ and \log procaine concentration had a slope of 1.07, further testifying to the competitive nature of acetylcholine antagonism by procaine.

When the calcium ion concentration of the Tyrode solution was reduced to 0.45 mM, procaine antagonized the responses to ACh at lower concentrations (pA_2 4.4), but there was still no suppression of the maximum response. The slope of the relationship $\log(\text{dose-ratio} - 1)$ and \log procaine concentration was, however, significantly greater than 1.0, being 1.56.

Responses of the rat stomach muscle to 5-HT in normal Tyrode solution were antagonized by 2.2×10^{-7} M to 1.8×10^{-6} M procaine. The maximum response to 5-HT was suppressed at even the lowest concentrations of procaine (Figure 4). When the calcium concentration in the bath fluid was increased to 3.6 mM, procaine (1.09×10^{-6} M) produced a shift to the right of the 5-HT dose-response curve of almost 1 log unit without suppressing the maximum effect. The pD'_2 values (\pm s.e.) for non-competitive antagonism of 5-HT by procaine were calculated

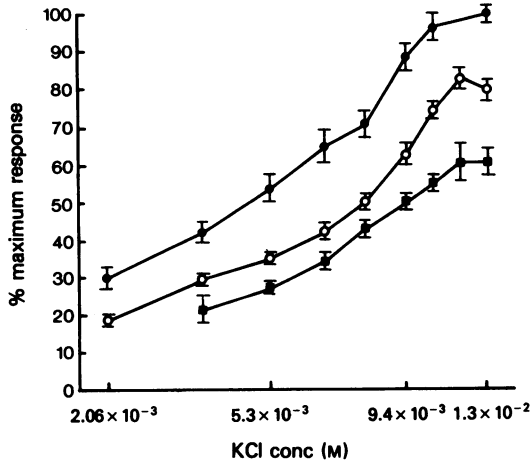


Figure 5 The effects of procaine on the responses of the rat stomach fundus to potassium chloride (KCl): (●) control Tyrode solution; procaine (○) 3.6×10^{-7} M; (■) 1.1×10^{-6} M.

in the presence of different calcium ion concentrations and were found to be: Ca^{2+} 0.45 mM, 6.4 ± 0.09 ; 1.8 mM, 5.93 ± 0.08 .

At these same low concentrations (3.6×10^{-7} M to 1.1×10^{-6} M) procaine also antagonized the responses to potassium chloride (Figure 5). The pD_2 for procaine against KCl was 5.47 ± 0.12 . When the concentration of procaine in the bath fluid was increased to 3.6×10^{-3} M, the muscle contracted spontaneously to a tension of 0.4 to 0.5 g and then slowly relaxed to its baseline tension within 15 to 30 min.

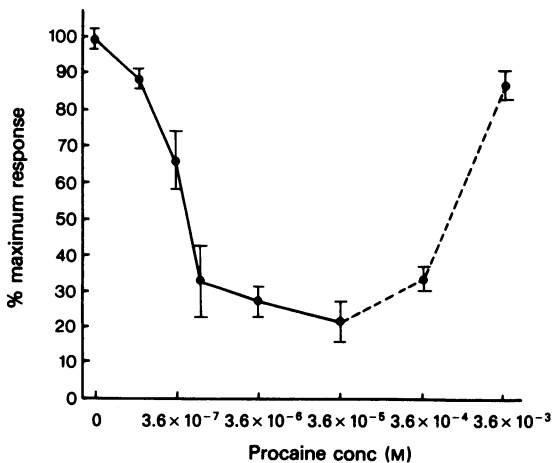


Figure 6 The effect of increasing procaine concentrations on the maximal response of the rat stomach fundus to 5-hydroxytryptamine (5-HT).

The subsequent addition of either 5-HT or KCl resulted in contractions of a similar magnitude to those seen before procaine was introduced to the bathing fluid. The effect of increasing concentrations of procaine on the response to a maximal dose of 5-HT and KCl are shown in Figures 6 and 7.

Discussion

In the present study it was found that 5-HT is much more dependent on the presence of calcium ions in the extracellular fluid to elicit a contraction of rat stomach muscle than is ACh. Lowering the calcium ion concentration of the Tyrode solution to 0.45 mM (25% of normal), did not alter the maximum increase in tension obtainable with ACh, but reduced that produced by 5-HT by almost 50%.

The inability of 5-HT to contract the rat fundus muscle in the absence of significant amounts of calcium ions in the bathing medium, indicates that this agent acts by increasing the permeability of the muscle membrane to external calcium ions, as originally suggested by Woolley (1958). In this respect, the action of 5-HT appears to resemble more closely that of KCl, than those of either ACh or NA (Hudgins & Weiss, 1968; Hinke, 1964).

The present data differ from those of Woolley & Gommi (1963), who used direct measurement of $^{45}\text{Ca}^{2+}$ movements to demonstrate the source of calcium involved in muscle contraction. They found that both 5-HT and ACh increased the uptake of $^{45}\text{Ca}^{2+}$ into rat uterus by the same amount, suggesting that these agents act similarly in uterine muscle. However, adrenaline, which relaxes this preparation, also brought about a similar uptake of $^{45}\text{Ca}^{2+}$. This makes it unlikely that the inward movement of calcium measured in these experiments was the cause of the muscle contraction.

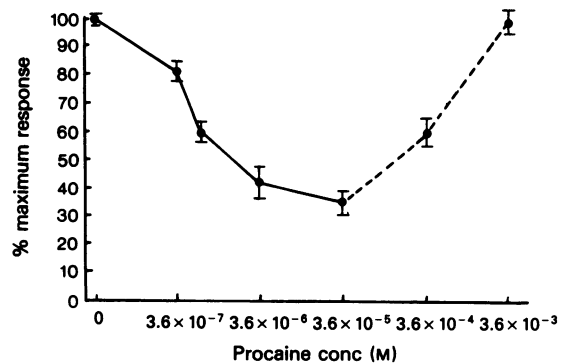


Figure 7 The effects of increasing procaine concentration on the maximal response of the rat stomach fundus to KCl.

In the present study, marked changes in the pD_2 values for both ACh and 5-HT were obtained on altering the external calcium ion concentration within a range which did not influence the maximum response obtainable. This confirms the findings of Swamy & Triggles (1972) on the rat vas deferens, that calcium ions not only serve as essential mediators of the contractile process, but may also be involved in the interaction between agonists and their receptor sites. The antagonism of the response to ACh by relatively high concentrations of procaine appeared to be of a competitive type. There was no change in the maximum response and the slope of the relationship between $\log(\text{dose-ratio} - 1)$ and \log procaine concentration was unity. However, lowering the calcium ion concentration in the extracellular fluid from 1.8 to 0.45 mM raised both the pA_2 and the slope of the above relationship. In this way, antagonism of acetylcholine by procaine differed from that by atropine, since the latter remained unaltered in the presence of different concentrations of calcium (Paton & Rothschild, 1965). The effect of procaine on responses to ACh resembled that produced by lowering the calcium ion concentration alone.

Procaine may therefore antagonize the effect of

ACh, not by competing with the agonist directly for its receptors, but by interfering with the binding of calcium ions to an allosteric site which alters the affinity of the agonist for the receptor (Triggles, 1971).

Procaine also antagonized the effect of both KCl and 5-HT at much lower doses than those which reduced that of ACh. Others had found procaine (10^{-3} M) to be ineffective against KCl-induced contractions (Hudgins & Weiss, 1968). In order to check this apparent discrepancy, we gradually increased the concentration of procaine from 1×10^{-6} to 1×10^{-3} M, and found that its ability to block the effects of both KCl and 5-HT was reduced or abolished.

Antagonism of the effects of KCl and 5-HT by low concentrations of procaine, could result from a change in the fluidity of the muscle cell membrane, thereby making it more difficult for these agents to open Ca^{2+} ion channels in it. At higher concentrations (greater than 1×10^{-3} M) of procaine, general disorganization of the membrane may occur with the release of bound calcium (Seeman, 1972). This could explain the contractile response induced by these amounts of procaine in the present study and in others (Kurihara, 1975).

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