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The Sources of Ca^{2+} for Muscarinic Receptor-induced Contraction in the Rat Ileum

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Abstract

The contractile responses obtained by activation of different muscarinic receptor subtypes in the longitudinal muscle of the rat ileum and especially the responses of this muscle to acetylcholine in a Ca^{2+}-free medium have been investigated.

In Ca^{2+}-containing solution, acetylcholine elicited similar concentration-dependent contractile responses in the duodenum, jejunum and ileum strips of the rat intestine. The response to a maximal concentration of the agonist (1 μM) consisted of a rapid phasic response followed by a slower tonic one. Nifedipine completely relaxes or inhibits the sustained response and only partially diminishes the phasic one, which suggests that the phasic contraction depends on the release of internal Ca^{2+} as well as Ca^{2+} entry from the extracellular space through voltage-dependent Ca^{2+} channels, but that the tonic contraction only depends on the influx of the external ion. In Ca^{2+}-free medium, acetylcholine (1 μM) induced phasic contractions that depend on the release of this ion from internal stores.

Participation of different subtypes of receptors (M_1, M_2 and M_3) in these responses depends on the inhibitory action shown by methoctramine, 4-DAMP and atropine but not by pirenzepine in two different experimental models.

The isolated guinea-pig ileum is used to assay muscarinic activity, and is known to contain heterogeneous populations of these receptors (Eglen et al 1994). Radioligand-binding studies have demonstrated that most guinea-pig ileal muscarinic receptors are of the M_2 subtype ($\sim$70%), while a minority are of the M_3 subtype ($\sim$30%), with no measurable quantities of M_1 and M_4 receptors (Giraldo et al 1988; Ford et al 1991; Eglen et al 1994). However, activation by muscarinic agonists of the minority M_3 receptor population is associated with increases in isometric tension, due, in part, to coupling of the M_3 receptor to a phosphoinositide-specific phospholipase.

The function of the predominant muscarinic M_2 receptor population, which couples to guanine nucleotide binding proteins, may be to inhibit β-adrenoceptor-stimulated adenyl cyclase activity and consequently oppose relaxation (Reddy et al 1995). Although no direct contractile response to M_2 receptor activation can be demonstrated in guinea-pig ileum (Eglen & Harris 1993), an indirect influence on contraction via inhibition of β-adrenoceptor-mediated relaxation remains as a modulatory mechanism of the contractile tone of this muscle (Reddy et al 1995).

The aim of our study was to examine the properties of muscarinic receptors in the longitudinal muscle of the rat ileum, especially the participation of each subtype in the contractile response of this muscle to acetylcholine.

Materials and Methods

Female Wistar rats, 200–250 g, were killed by a blow on the head and exsanguinated. The intestine was removed and small pieces of ileum (2–3 cm) were dissected and mounted longitudinally in a 10 mL organ bath chamber filled with Krebs solution at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. In some experiments pieces of duodenum or jejunum were also assayed. Tension was recorded isometrically on a Grass recorder (Serie 7) via force-displacement transducers (FTO_3). An initial load of 1 g was applied to each preparation and the ileum was allowed to equilibrate for 30 min before agonist addition.

The EC50 value of acetylcholine was estimated using a cumulative method with nine concentrations of agonist spaced geometrically every 0·33 log units and calculated from a linear regression plot of all points between 20 and 80% maximal response. After the first concentration-response curve was obtained, washing and incubation for 20 min in Krebs solution yielded reproducible responses to agonist.

The response to different depolarizing solutions was also analysed. The muscle was loaded in a solution in which equimolecular substitution of K^+ for Na^+ made it possible to obtain a final K^+ concentration of 20, 40, 60, 80 or 120 mM. In each case, loading of the muscle in these solutions gave a biphasic response that was measured independently (phasic at 2 min and tonic at 15 min after addition of depolarizing solution). Contractions were expressed as mN.

To study the effects of nifedipine on the contractile response of rat ileum to acetylcholine or depolarizing solutions two experimental procedures were performed. In some experiments concentration-response curves of relaxation to nifedipine were obtained by addition of cumulative concentrations of the compound to ileum strips in which sustained contractions had been induced by depolarizing solution (20 mM KCl) or 1 μM acetylcholine. Concentration-response curves of inhibition to nifedipine were obtained by addition of one concentration of

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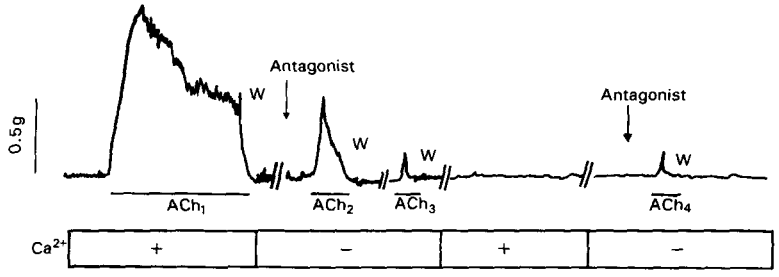

FIG. 1. Experimental procedure used to study the effect of the muscarinic receptor antagonists in the absence of extracellular calcium on acetylcholine-induced contraction. ACh_1: addition of the agonist (1 μM) in Krebs solution (Ca^{2+} 1·8 mM). ACh_2: addition of the agonist after 15 min incubation in calcium-free medium. ACh_3: third addition of the agonist after washing (W) in calcium-free medium. ACh_4: addition of the agonist (in presence or absence of the antagonist) after 20-min resting period in Krebs solution (Ca^{2+} 1·8 mM) and 15 min in calcium-free medium. The antagonists were added 10 min before ACh_2 or 10 min before ACh_4.

nifedipine 15 min before and during 1 μM acetylcholine- or 20 mM KCl-induced contraction. Fig. 1 shows the different experimental procedures designed to study the response to acetylcholine in Ca^{2+}-free medium.

Drugs and solutions

Acetylcholine, methoctramine, atropine, 4-diphenylacetoxy-*N*-(2-chloroethyl)-piperidine (4-DAMP) and EDTA were purchased from Sigma (St Louis MO, USA). All the drugs except nifedipine were prepared daily as aqueous solutions; nifedipine was dissolved in absolute ethanol to prepare a 10 mM solution, which was diluted with distilled water. Solutions containing nifedipine were protected from light and the experiments were carried out in the dark to avoid photodegradation. All other chemicals used were of analytical grade.

The composition of Krebs solution was as follows (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1·8, $MgSO_4.H2O$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25, Glucose 11·1. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0·1 mM) was added.

Analysis of results

Results are expressed as the mean ± s.e. mean of five or more preparations (n) obtained from different animals. Statistical significance of differences between the means was assessed by Student's *t*-test for unpaired data. $P < 0.05$ was considered to represent a significant difference. To determine EC50 values a linear regression analysis was performed (Graph Pad Software; San Diego, CA, USA).

Results

Contractile responses to acetylcholine in Ca^{2+}-containing solution

Cumulative concentrations of acetylcholine (0·003–10 μM) gave a dose response curve of contraction with an EC50 value of 0·213 ± 0·04 μM (n = 5) and similar results were obtained with the duodenum segment (EC50 = 0·295 ± 0·03 μM, n = 5) and the jejunum (EC50 = 0·207 ± 0·04 μM, n = 5). After washing, the concentration-response curve of acetylcholine obtained in each segment was similar to the first one, with an EC50 that was not statistically different. The concentration of agonist that gave a maximal response (11·2 ± 0·59 mN, n = 9) was 1 μM and higher concentrations gave lower responses.

In a separate series of experiments, acetylcholine 1 μM was added and a rapid phasic contraction followed by a plateau with small rhythmic contractions was observed. Addition of cumulative concentrations of different antagonists of muscarinic receptors (atropine: $10^{-11} - 10^{-7}$ M; pirenzepine and methoctramine: $10^{-7} - 10^{-5}$ M; 4-DAMP: $10^{-10} - 10^{-8}$ M) on this sustained contraction to acetylcholine gave concentration-response curves of relaxation that permit us to determine the concentration of antagonist that completely relaxes acetylcholine-induced contraction in our experimental conditions. This concentration was 10^{-8} M for atropine, 10^{-6} M for pirenzepine, 5×10^{-6} M for methoctramine and 10^{-8} M for 4-DAMP.

Depolarizing solutions were also assayed in the different segments of the rat intestine (duodenum, jejunum and ileum) when added to the organ bath; the solution containing K^+ 20 mM when it was added promoted a biphasic response characterized by a phasic peak (10·63 ± 2·97 mN) followed by a tonic contraction (3·97 ± 1·3 mN; n = 5), that was sustained as long as the depolarizing solution was present. This response was similar in the duodenum (n = 5) and jejunum (n = 6).

Higher concentrations of KCl (40, 60, 80 mM) also gave biphasic responses, with a strong phasic response but a lower tonic component that decayed progressively. The highest concentration assayed (KCl 120 mM) only gave a phasic response.

To study the effects of nifedipine on the contractile response of rat ileum to acetylcholine or depolarizing solutions, two experimental procedures were performed as described above. Addition of cumulative concentrations of nifedipine ($10^{-11} - 10^{-7}$ M) on the sustained contractile response induced by KCl 20 mM or acetylcholine 1 μM gave concentration-response curves of relaxation with similar IC50 values: 0·86 ± 0·18 nM (n = 6) and 0·32 ± 0·09 nM (n = 5), respectively. When nifedipine was added 15 min before and during the contractile response induced by KCl 20 mM or acetylcholine 1 μM (concentration-response curves of inhibition), the phasic response was significantly diminished in a dose-dependent manner and the tonic component was completely inhibited (n = 6) at the higher concentrations (0·01, 0·1 μM) of nifedipine.

Contractile responses to acetylcholine in Ca^{2+}-free solution

Fig. 1 shows the experimental procedure used to study the action of acetylcholine on rat ileum loaded in Ca^{2+}-free medium. Acetylcholine (1μM) was added the first time when the ileum was loaded in Ca^{2+}-containing solution and the contractile response obtained was used as a standard for the

contractile responses in Ca^{2+}-free medium. After washing, and loading for 15 min in Ca^{2+}-free solution, addition of acetylcholine 1 μM (ACh_2) promoted a transient response due to the release of intracellular Ca^{2+}. The magnitude of this contractile response was $43{\cdot}0 \pm 7{\cdot}7\%$ (n = 6) relative to the acetylcholine-induced contraction in the presence of Ca^{2+}. In these conditions, participation of extracellular Ca^{2+} can be excluded since addition of KCl 20 mM did not promote any contractile response (n = 8). After washing, subsequent addition of ACh_3 evoked a lower contractile response ($13{\cdot}1 \pm 4{\cdot}9\%$, n = 7) and further addition of the agonist did not evoke any response. This indicates a complete depletion of acetylcholine-sensitive Ca^{2+}-stores. Afterwards, we performed at least three exchanges of the medium at time intervals of 10–15 min, and the tissue was incubated for 20 min in the presence of Ca^{2+} to refill the intracellular Ca^{2+} stores. A new response to acetylcholine in Ca^{2+}-free medium (ACh_4) was obtained after an incubation period of 15 min in the absence of Ca^{2+}, but the magnitude of the contraction was lower than that of ACh_2 and similar to that of ACh_3 ($14{\cdot}9 \pm 4{\cdot}6\%$, n = 5), thus indicating that the refilling of the stores was not complete.

As shown in Fig. 1, in order to study the effects of the different muscarinic antagonists on the acetylcholine-induced contraction in Ca^{2+}-free medium, the maximal concentrations of compounds that completely inhibited acetylcholine-induced contraction in Krebs solution were added at 10 min before the addition of ACh_2 or at 10 min before the addition of ACh_4 (Table 1). No significant differences in the response were obtained in the presence of pirenzepine 1 μM (n = 3) or 4-DAMP 10 nM (n = 5). The addition of ACh_4 in the presence of the same concentrations of 4-DAMP (n = 6), methoctramine (n = 8) and atropine (n = 7) produced a contractile response that was lower than the ACh_3. A diminished response to ACh_4 was observed in the presence of pirenzepine (n = 6) but was not statistically different with respect to ACh_3.

Table 1. The effect of different muscarinic receptor antagonists on contractions induced by acetylcholine in rat ileum loaded in Ca^{2+}-free medium.

Antagonist added	Maximum tension (mean ± s.e.m.)	
	10 min before ACh_2	10 min before ACh_4
Control	44 ± 8	13 ± 5
Pirenzepine	34 ± 6	8·3 ± 1·5
Methoctramine	26 ± 5*	4·6 ± 1·8***
4-DAMP	43 ± 10	3·2 ± 1·2***
Atropine	13 ± 6***	3·2 ± 2·3***

*$P < 0{\cdot}05$, ***$P < 0{\cdot}005$ when compared with the values of the control response (open column).

Discussion

Based on our results, we can hypothesize the existence of two different internal calcium pools mobilized by acetylcholine, one of them released mainly by activation of M_2 receptors and responsible for the first, stronger response to acetylcholine in Ca^{2+}-free medium and the other one depending on activation of M_3 receptors. This is consistent with previous observations that M_2 receptors can also couple to stimulation of phosphoinositide hydrolysis via G-proteins other than those used to couple to inhibition of adenyl cyclase (Hulme et al 1990). After depletion, the internal pool sensitive to M_2 receptors can not be refilled and the response to acetylcholine is not reproducible after loading the preparation in a Ca^{2+}-containing medium.

Another possible explanation for the present results excludes participation of M_2 receptors in releasing Ca^{2+} from intracellular pools and posits a mechanism related to inhibition of adenyl cyclase activity. In this case, the contractile response to acetylcholine in Ca^{2+}-free medium depends on Ca^{2+}-release from internal stores sensitive to M_3 receptor and activation of M_2 receptors by the agonist increases this response of ileum through a decrease in the cyclic AMP levels. Cyclic AMP has been shown to decrease internal free Ca^{2+} by different mechanisms related to inhibition of Ca^{2+} influx (Abe & Karaki 1988), increase in Ca^{2+} extrusion (Bülbring & den Hertog 1980) and increase in Ca^{2+} uptake by intracellular stores (Saida & Van Breemen 1984), but cyclic AMP also reduces the levels of IP_3 elevated by activation of muscarinic receptors (Prestwich & Bolton 1995). Therefore, a decrease in cyclic AMP levels results in an increase in the contractile response to acetylcholine. If we assume this hypothesis, no explanation can be given about the subtype of muscarinic receptor implicated in the first, non-reproducible contractile response to acetylcholine in Ca^{2+}-free medium, for in our experimental conditions, 4-DAMP did not inhibit it. This suggests that the M_3 receptor subtype is not involved or is only slightly involved. The involvement of other stimuli released by activation of M_2 receptors from neuronal elements present in smooth muscle strips can not be excluded (Barocelli et al 1993).

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