Muscarinic M2-receptors enhance polyphosphoinositol release in rat gastric mucosal cells

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Muscarinic receptor types and their effects on the inositol phosphate second messenger system were studied in enzymatically dispersed rat gastric mucosal cells. Radioreceptor binding studies indicated the presence of a single class of binding sites (4860 ± 875 sites/cell) and affinities of 0.42 ± 0.12 nM, 176 ± 32 nM and 13 μM for *N*-methylscopolamine, pirenzepine and carbachol, respectively. In cells prelabelled with *myo*-[^{3}H]inositol carbachol induced a dose-dependent increase in inositol-1-phosphate in the presence of lithium with an ED_{50} of 10 μM which was antagonized by atropine and pirenzepine (IC_{50} 9 and 700 nM, respectively). Carbachol stimulated amino[^{14}C]pyrine uptake, used as a measure of acid secretion, with an ED_{50} of 10 μM. The good correlation between these responses suggests a role for inositol phosphates in the muscarinic M2-receptor mediated acid secretion.

Gastric mucosal cell *Acid secretion* *Muscarinic M2-receptor* *Pirenzepine* *Carbachol* *Inositol phosphate*

1. INTRODUCTION

The interactions of histamine and acetylcholine with their surface receptors on gastric mucosal parietal cells initiate the secretion of hydrogen ions [1,2]. The response to histamine occurs through receptor-mediated stimulation of cyclic adenosine monophosphate (cAMP). The second messenger of muscarinic cholinergic receptors has, until present, remained elusive. Available data indicate that the enzymes adenylate or guanylate cyclase are not involved [1–3]. The acid secretory response to cholinergic agonists was highly susceptible to changes in extracellular calcium concentration [1,2] thereby resembling the calcium dependence of some cholinergic receptors in other tissues which are known to enhance the turnover rates of phosphatidylinositols [4]. Recent work suggests that the increased turnover of phosphatidylinositols is secondary to a receptor-mediated activation of a polyphosphoinositol phosphodiesterase which cleaves the phospholipid into inositol phosphates and 1,2-diacylglycerol [4]. Inositol-1,4,5-trisphosphate appears to release calcium from nonmitochondrial intracellular stores [4], while 1,2-diacylglycerol activates the calcium- and phospholipid-dependent protein kinase C [5]. These mechanisms are likely to represent an important second messenger system involved in calcium-dependent signal transmission.

Recent evidence suggests that muscarinic receptors are not homogeneous [6–8]. A subclassification has been proposed based primarily on pharmacologic studies with the muscarinic antagonist pirenzepine [7]. The M1-receptors display a high affinity for pirenzepine while M2-receptors bind pirenzepine with an approx. 40–200-fold lower affinity [6,8–10]. To characterize the type of muscarinic receptor present in rat gastric mucosal cells we performed radioreceptor binding studies with muscarinic antagonists and an agonist. We then investigated whether gastric mucosal

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muscarinic receptors are coupled to the phosphoinositol second messenger system. Finally, stimulation of aminopyrine uptake (as a measure of hydrogen ion secretory response) by the muscarinic agonist carbachol was employed to assess a cellular response to receptor activation. Our results suggest that muscarinic receptors in rat gastric mucosal cells display a low affinity for pirenzepine (M2-receptors) and stimulate acid secretion by increasing intracellular concentrations of inositol phosphates.

2. MATERIALS AND METHODS

Rat gastric mucosal cells were isolated as described [11,12]. After isolation cells were washed in buffer C (composition in mM: 70 NaCl, 5 KCl, 1.5 sodium phosphate, 50 Hepes, 20 sodium bicarbonate, 1.0 calcium, 1.5 magnesium, 0.2% bovine serum albumin). Cell viability was greater than 90% as estimated by the trypan blue exclusion test.

2.1. *Binding studies*

The cells were diluted to 2×10^6 cells/ml and incubations with tritiated *N*-methylscopolamine (NMS) and drugs were performed in 1 ml buffer C for 20 min at 37°C. Incubations were terminated by rapid filtration through Whatman GFC glass fiber filters under reduced pressure. The filters were washed with 2×3 ml buffer C and radioactivity retained was determined. Specific binding, calculated by subtraction of binding in the presence of 0.1 μM NMS was 60–80% of the total radioactivity retained on the filters.

2.2. *Phosphoinositol experiments*

The isolated cells were incubated at 10^7 cells/ml with 10 μCi/ml of tritiated *myo*-inositol (17 Ci/mmol) in oxygenated buffer C for 2 h and washed 3 times in buffer C supplemented with 0.1 mM unlabeled *myo*-inositol. After final resuspension in buffer C incubations were performed in 0.4 ml and terminated by adding 1 ml of ice-cold methanol. The phosphoinositol assays were performed as described in [13]. Lipids were extracted by addition of 0.5 ml chloroform and vortex-mixing for 10 s. Another 0.5 ml chloroform and 0.5 ml water were added and the tubes were centrifuged. The upper phase was applied to 1 ml columns of Dowex 1-X8 in the formate form (Bio Rad) and washed with 10 ml water and 15 ml of 60 mM sodium formate/5 mM sodium tetraborate. Inositol-1-phosphate (IP) was eluted with 15 ml of 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was determined in the first 5 ml of each eluate by liquid scintillation spectrophotometry. The elution of IP was controlled with a ^{14}C-labelled standard.

The accumulation of IP varied between 2- and 6-fold basal levels during 30 min in 10 individual experiments. Parietal cells accumulated 3–6-times more IP than the smaller and metabolically [1,2] less active nonparietal cells when compared by cell number. For these experiments parietal cells were enriched to a content of 70–90% by isopycnic centrifugation in percoll [11].

2.3. *Aminopyrine uptake*

The accumulation of amino[^{14}C]pyrine as described by Sonnenberg et al. [11] and modified by Ecknauer et al. [14] was used as an indirect measure of hydrogen ion production. Incubations ($1–2 \times 10^6$ cells/ml in buffer C) performed in the presence of 10 μM dibutyryl-cAMP [14] were terminated by adding 0.4 ml of the cell suspension to 1 ml cold buffer C and centrifugation for 1 min. The supernatant was removed, the pellet washed once with 1 ml cold buffer C and then dissolved in 100 μl of 1 M NaOH for determination of radioactivity. The maximal stimulation of aminopyrine accumulation varied between 2- and 10-fold in individual experiments without affecting dose response relations.

2.4. *Statistical methods*

Data are reported as means ± SE from triplicate determinations from representative experiments. All experiments were repeated at least twice with reproducible results. ED_{50} and IC_{50} values were determined by the log-probit method.

2.5. *Materials*

Reagents were of analytical grade and were purchased from Merck, Darmstadt, unless indicated otherwise. Hepes (Serva, Heidelberg), bovine serum albumin and NMS (Sigma, Taufkirchen, FRG), tritiated *N*-methylscopolamine, *myo*-inositol, amino[^{14}C]pyrine and [^{14}C]IP (Amersham Buchler, Dreieich, FRG).

3. RESULTS

The interaction of carbachol and of the muscarinic antagonist pirenzepine with muscarinic receptors was investigated by heterologous competition studies in which tritiated *N*-methylscopolamine was displaced by the respective unlabeled ligand. The data were analysed by use of the computerized nonlinear least squares curve fitting program 'Ligand' [15] to derive kinetic parameters from equilibrium binding studies (fig.1). The binding capacity was 4850 ± 875 sites/cell. The data were best represented by a model assuming a single binding site to which NMS had an affinity of K_d = 0.43 ± 0.12 nM, pirenzepine K_d = 176 ± 32 nM. The goodness-of-fit was not improved by fitting the data to a two-binding site model. Carbachol displayed an affinity of K_d = 12.8 ± 5.5 μM to 63% and an affinity of K_d = 1.4 ± 0.9 mM to 37% of the binding sites. The two-site model fitted the data significantly better than a 1-site model (p = 0.01).

The effect of the muscarinic agonist carbachol on the release of inositol phosphates was investigated in isolated cells prelabeled with tritiated inositol for 2 h. The subsequent addition of

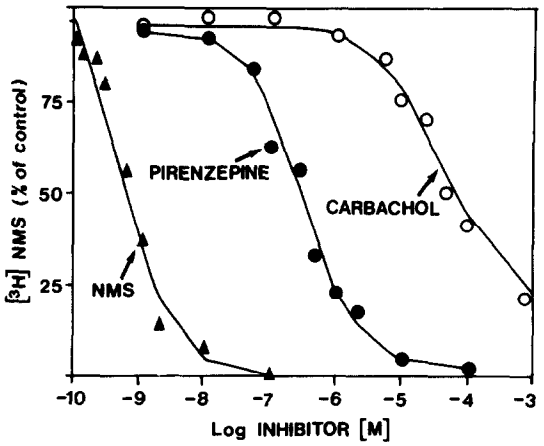

Fig.1. Displacement of tritiated NMS by unlabeled NMS, pirenzepine and carbachol in isolated rat gastric mucosal cells. The concentration of β(H)NMS was 0.25 nM. The curves for NMS and pirenzepine were best fitted to models assuming a single binding site. The displacement curve of carbachol was significantly better fitted by a model assuming 2 binding sites (data from 3 experiments, D.F.28, F = 40.3). The affinities are stated in the text.

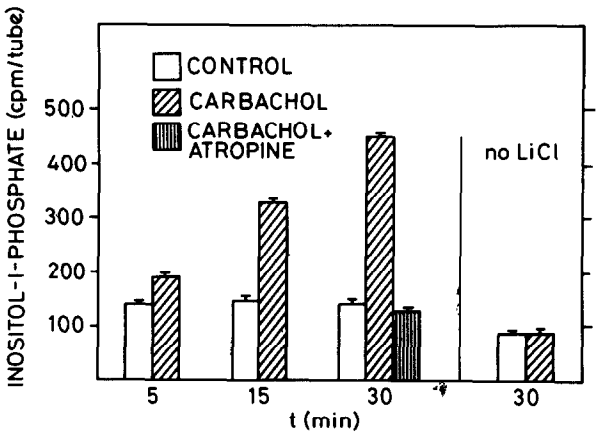

Fig.2. Time course of inositol phosphate accumulation stimulated by 0.1 mM carbachol in isolated gastric mucosal cells in the presence or absence of 10 mM LiCl.

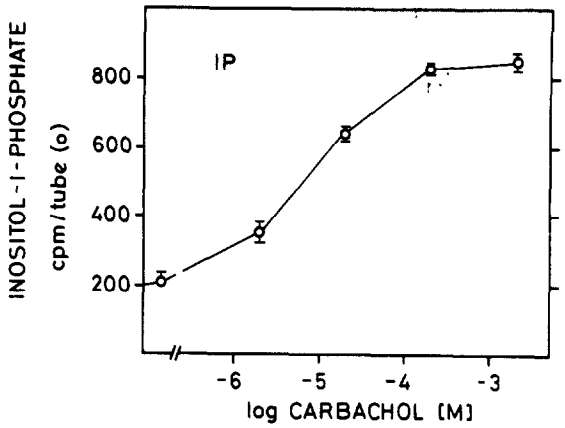

Fig.3. Effect of carbachol on the accumulation of inositol phosphates during 30 min in isolated rat gastric mucosal cells.

0.1 mM carbachol caused a time-dependent increase in IP in the presence of 10 mM LiCl (fig.2) a known inhibitor of IP phosphatase [17] which was used to amplify the response. The maximal increase in IP varied between 2- and 4-fold of control levels in 10 independent experiments. The increase in IP was blocked in the presence of 1 μM atropine (fig.2). In a dose-response study, half-maximal stimulation of IP was observed in the presence of 12 ± 4 μM carbachol (fig.3). In further experiments the potencies of atropine and pirenzepine to inhibit the inositol phosphate response to 0.1 mM carbachol were evaluated. Half-maximal inhibition was observed in the

presence of 9 ± 3 nM atropine or 700 ± 200 nM pirenzepine (fig.4).

The accumulation of amino[^{14}C]pyrine was used to obtain an index of acid secretion by parietal cells in response to carbachol. The amino[^{14}C]pyrine accumulation proved to be rather small when carbachol was added as the only stimulant. Ecknauer et al. [14] reported that larger stimulation was obtained when carbachol was added in the presence of dibutyryl-cAMP which enhanced the secretory response to carbachol without affecting dose-response relations [14]. We therefore adopted this method which exploits the synergism between cAMP- and inositol phosphate-dependent acid secretion. Dibutyryl-cAMP (10 μM) enhanced amino[^{14}C]pyrine accumulation by 100%. Carbachol caused a further dose-dependent accumulation and half-maximal stimulation was observed at 8 ± 4 μM carbachol (n = 3) (fig.5).

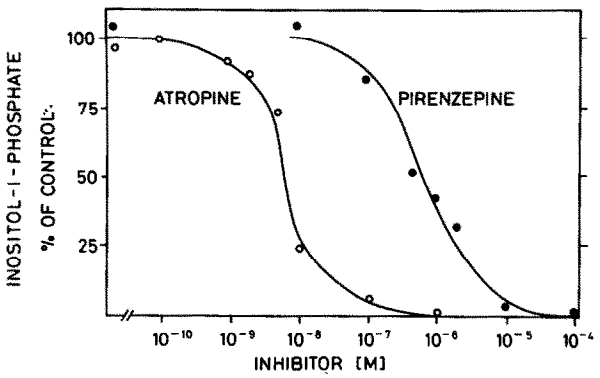

Fig.4. Inhibition of inositol phosphate accumulation in response to carbachol (0.1 mM) by the muscarinic antagonists atropine or pirenzepine. It should be noted that the inhibition curves were carried out in the presence of 0.1 mM carbachol and hence IC_{50} values will be greater than K_d values.

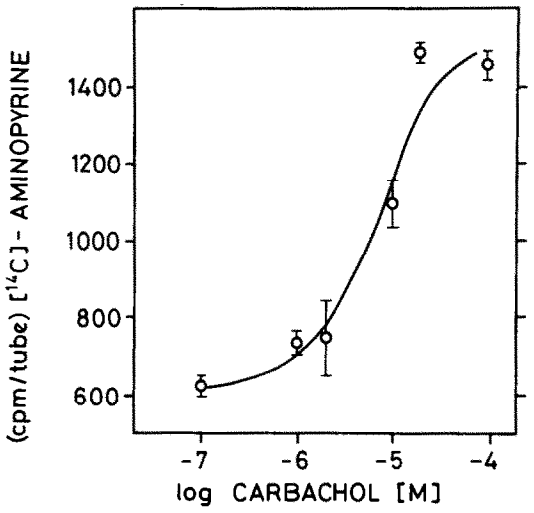

Fig.5. Accumulation of amino[^{14}C]pyrine in response to carbachol in the presence of 0.01 mM dibutyryl-cAMP.

4. DISCUSSION

The present study correlates muscarinic receptor binding to the stimulation of inositol phosphate formation and acid secretion in rat gastric mucosal cells. The muscarinic antagonist NMS labeled a single apparent population of binding sites with high affinity in agreement with previous data obtained with this ligand [8,9]. In contrast to other muscarinic antagonists pirenzepine interacts with different populations of muscarinic binding sites with affinities ranging from 40 to 3000 nM [6–10]. This has permitted a distinction of muscarinic receptors to which pirenzepine displays a high affinity, termed M1-sites, from such to which pirenzepine displays a low affinity, termed M2-sites. In rat gastric mucosal cells pirenzepine bound to a single population of binding sites with an affinity of 0.2 μM which agrees with the presence of M2-sites. Similar results have been described by Hammer [10] in homogenates of canine gastric mucosal membranes. The affinity (K_d) of carbachol of 13 μM also agrees with previous data obtained in rat gastric mucosa [14]. The interaction of muscarinic agonists with different affinity states of the muscarinic receptors is a well-known phenomenon [6–10,18]. Our results suggest that the higher-affinity state of the receptor is involved in mediating muscarinic responses.

Carbachol stimulated the release of inositol phosphates as indicated by the time-dependent accumulation of IP in the presence of lithium. The inhibition of inositol-1-phosphatase by lithium [13] led to an accumulation of IP over time. This product probably derived from the degradation of inositol triphosphate to inositol diphosphate and IP as indicated by previous studies [4,13]. Half-maximal stimulation of IP was observed in the presence of 10 μM carbachol which represents a concentration very close to the one required for half-maximal occupation of muscarinic receptors

and therefore suggests the absence of spare receptors.

Parietal cells accounted for more than 50% of the IP accumulated although they represented only approx. 20% of the cell population. This may reflect the greater cell volume or the greater responsiveness of parietal cells to carbachol. Further studies are needed to investigate this point.

A comparison of the inhibitory potencies of atropine and pirenzepine in a given experimental system may be used as an additional parameter to distinguish M1- from M2-receptors [6,9,10,16]. Pirenzepine was about 5-fold less potent than atropine at M1-receptors in inhibiting muscarinic responses while 100–200-fold lower affinities were observed for the M2-receptors [16]. Thus, the relatively low potency of pirenzepine in inhibiting the muscarinic response suggests that M2-receptors are involved in inositol phosphate generation in rat gastric mucosal cells. Similar results have been reported by Rosenfeld [17] who compared the inhibition of carbachol-stimulated aminopyrine accumulation by pirenzepine and atropine in rat mucosal cells.

Our data contrast with the previously made proposal that M2-receptors are involved in the inhibition of adenylate cyclase while M1-receptors mediate the inositol phosphate response [18]. Our observations agree, however, with a recent study [9] which indicated that M2-receptors in chick heart cells mediate inositol phosphate release while M1-receptors are coupled to the inhibition of adenylate cyclase.

In agreement with previous studies in isolated rat gastric cells [14,17] carbachol stimulated acid secretion half-maximally at a concentration of 10 μM. The dose response for acid secretion thus closely paralleled the response for inositol phosphate generation. Our data therefore support the view that muscarinic receptors on rat parietal cells stimulate acid secretion by activating a phospholipase C-type phosphodiesterase which results in the release of phosphoinositols.

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