Muscarinic stimulation of inositol phosphate accumulation and acid secretion in gastric fundic mucosal cells

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The muscarinic agonist, carbachol (CCh), was shown to stimulate the production of inositol phosphates (IP) in isolated cells from rabbit fundic mucosa. This stimulatory effect was time- and dose-dependent: EC_{50} values for IP_1, IP_2 and IP_3 accumulation were not statistically different. The mean value was 30 ± 8 μM ($n=6$). The corresponding maximal stimulation (% of basal value) observed after 20 min incubation in the presence of 100 μM CCh was 160 ± 15%. CCh-induced IP accumulation was abolished by atropine (K_i = 0.32 ± 0.18 nM ($n=3$)). The CCh concentrations leading to half-maximal inhibition of *N*-[^{3}H]methylscopolamine binding and half-maximal IP accumulation were similar. The half-maximal value for CCh-induced aminopyrine accumulation was 8-times lower. These results indicate that IP_3-mediated mobilization of intracellular Ca^{2+} might be involved in CCh-induced acid secretion by parietal cells.

Muscarinic receptor *Gastric acid secretion* *Ca^{2+}* *Inositol phosphate* *(Fundic mucosal cell)*

1. INTRODUCTION

Stimulation of acid secretion from parietal cell is mediated by acetylcholine acting through muscarinic receptors. This stimulation is markedly potentiated by histamine acting through histamine-H2 receptors. Previous investigations demonstrated the involvement of cyclic AMP as a second messenger of the histamine-H2 effect (review [1]). Ca^{2+} is very likely involved in CCh-induced acid secretion. However, the origin of Ca^{2+} responsible for the rise in cytosolic free Ca^{2+} following muscarinic receptor activation in parietal cells is controversial [2–4].

Abbreviations: IP, inositol phosphates; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; CCh, carbamylcholine (carbachol); NMS, *N*-methylscopolamine; AP, aminopyrine

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Current experimental evidence (review [5]) indicates that receptor-mediated PIP_2 breakdown is part of a common mechanism by which several hormones or neurotransmitters (including acetylcholine acting through a muscarinic receptor [6]) increase free cytosolic Ca^{2+} within their target cells. In this study, we investigate the possible involvement of this mechanism in CCh-induced acid secretion by parietal cells [7].

2. MATERIALS AND METHODS

2.1. *Products*

myo-[2-^{3}H]Inositol (16.5 Ci/mmol) was purchased from New England Nuclear (Dreieich); *N*-[*methyl*-^{3}H]scopolamine methyl chloride (80 Ci/mmol) and [*dimethylamine*-^{14}C]aminopyrine (118 mCi/mmol) from Amersham (Bucks., England). CCh, scopolamine, atropine, Tris, Hepes and bovine serum albumin (BSA) (fraction V) were obtained from Sigma (St Louis, USA) and collagenase (0.75 U/mg) from Serva (Heidelberg).

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myo-[2-^{3}H]Inositol mono-, bis- and trisphosphate were gifts from Dr C.J. Kirk (Birmingham, England).

2.2. *Preparation of isolated gastric cells*

Cells from rabbit fundic mucosa were isolated using a collagenase-EDTA treatment as described in [7]. This procedure gave about 30×10^6 cells per g wet mucosa with 95% viability (trypan blue exclusion test). The mixed cell population contained 45–55% parietal cells, as determined by electron microscopy.

All incubations were performed in a standard medium (modified Earle's medium) with the following composition: 116 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Hepes, 0.1% glucose, 0.2% BSA, pH 7.4.

2.3. *Aminopyrine accumulation test*

Cells suspended in the standard medium (1.5×10^6 cells per ml) were incubated with 0.05 μCi per ml of [^{14}C]AP (3 μM) and various CCh concentrations at 37°C for 20 min under continuous gassing (95% O_2/5% CO_2) and stirring (final volume, 1.5 ml). Triplicate samples (0.4 ml of each tube) were mixed with 0.9 ml ice-cold standard medium and centrifuged for 1 min (Eppendorf microfuge). The cell pellet was suspended in 0.1 ml of 10% $HClO_4$ and the radioactivity was measured in a β liquid scintillation counter (Beckman LS 7500). AP accumulation was measured by the radioactivity recovered in the cell pellet expressed as percentage of the radioactivity present in the cell suspension.

2.4. *Measurement of inositol phosphate accumulation*

Gastric mucosal cells were incubated for 3 h at 37°C under continuous gassing (O_2/CO_2) in 5 ml standard medium containing 180 μCi *myo*-[2-^{3}H]-inositol (2×10^7 cells per ml). After two washings with 5 ml standard medium, cells were equilibrated for 20 min at 37°C in the presence of 10 mM LiCl. Cells were further incubated for 20 min at 37°C under continous stirring (300 strokes/min) and gassing (O_2/CO_2) in the absence (control) or presence of CCh. The reaction was stopped by rapid addition of 1 ml of 10% $HClO_4$ (PCA). The pH of the extracts was adjusted to 7.0 by adding 0.1 M KOH. After centrifugation of PCA extracts, IP_1, IP_2 and IP_3 were separated by chromatography on Dowex 1×10 (100–200 mesh formate form) columns (4.0×0.6 cm) as described by Berridge and Irvine [8] with minor modifications [9]. This protocol was established using purified labeled IP_1, IP_2 and IP_3 as standards. Radioactivity present in the IP_1, IP_2 and IP_3 fractions was measured by liquid scintillation counting in the gel phase using 60% (v/v) liquid scintillation cocktail ACS II. Labeled inositol lipids were determined after chloroform/methanol/HCl (100:100:1) extraction and deacylation in the presence of NaOH according to Creba et al. [10]. All results were corrected for quenching and expressed in dpm. Each value represents the mean ± SE of 3 separate experiments.

2.5. *[^{3}H]NMS binding assay*

All assays with intact isolated cells were performed in triplicates in the same experimental conditions as those used for IP measurements. The inhibition of [^{3}H]NMS binding was measured by incubating cells suspended in standard medium (7×10^6 cells per ml) with 0.5 nM [^{3}H]NMS, 10 mM LiCl, and various concentrations of CCh (final volume, 0.2 ml). After 20 min incubation at 37°C, under O_2/CO_2 gassing and continuous stirring, samples from the cell suspension were rapidly filtered on Whatman GF/B filters. The filters were rinsed with 3×5 ml of ice-cold filter washing medium (20 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). The radioactivity of the filter was measured in 4 ml of ACS II (Amersham) in a liquid scintillation counter. Nonspecific binding was determined in parallel by incubating cells with [^{3}H]NMS in the presence of an excess of unlabeled scopolamine (10 μM).

3. RESULTS AND DISCUSSION

Fig.1 shows the time courses of IP accumulation and of inositol lipid breakdown in *myo*-[2-^{3}H]-inositol prelabeled fundic mucosal cells. CCh (100 μM) stimulated IP_1, IP_2 and IP_3 accumulation in a similar time-dependent manner (fig.1, left panel). After a 5 min incubation period, the IP contents, expressed as % of basal value, were 120 ± 6; 128 ± 6 and 134 ± 10% for IP_1, IP_2 and IP_3, respectively. Up to 45 min, the IP content increased linearly with time, suggesting that the rate of CCh-stimu-

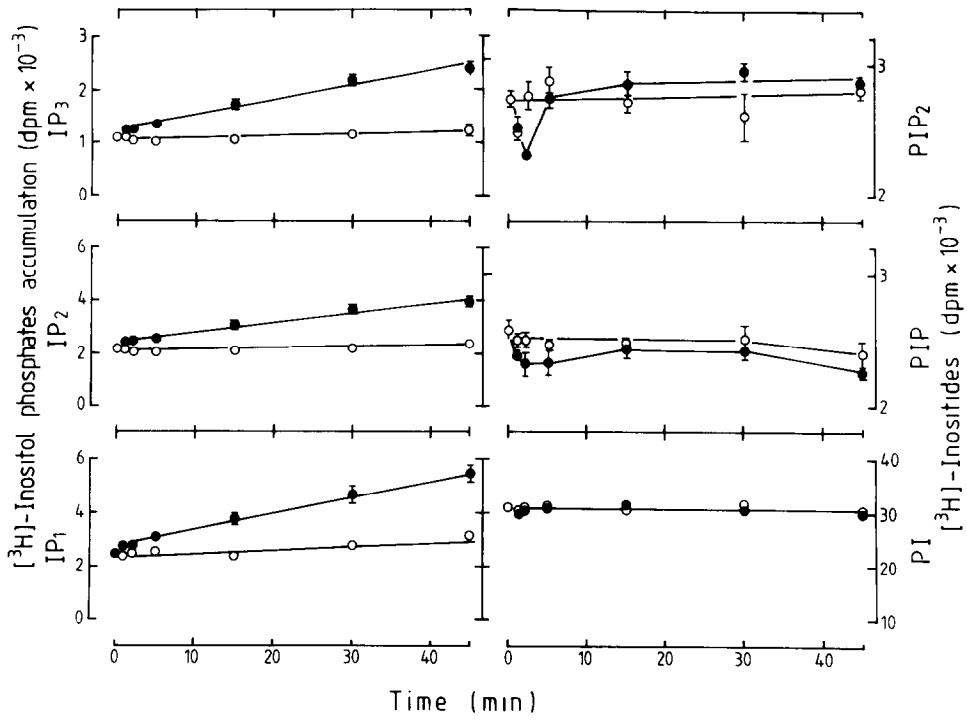

Fig.1. Time courses of IP production and of breakdown of phosphoinositol lipids in fundic mucosal cells. Cells isolated from rabbit fundic mucosa (10^8 cells) were labeled with *myo*-[2-^{3}H]inositol as described in section 2. (Left) IP contents as a function of time, expressed as dpm per sample ± SE of 3 separate experiments, in control (○) and CCh-stimulated cells (●). (Right) Amounts of phosphoinositol lipid substrates, expressed as dpm per sample ± SE of 3 separate determinations, as a function of time in the absence (○) or presence of CCh (100 μM) (●). Where no error bars are shown, the SE is contained within the symbol.

lated inositol lipid breakdown reached a steady-state value. Previous studies showed that, after a 20 min incubation period, both CCh binding and CCh-induced AP accumulation also reach a steady-state value [7].

As in many other systems (review [8]), PI represented the major inositol lipid (85% of total inositol lipids). Under CCh stimulation (100 μM), the PI pool remained fairly stable (fig.1, right panel). A transient and small decrease in the sizes of PIP and PIP_2 pools was observed. PIP and PIP_2 pools were restored after approx. 10 min, suggesting that PIP and PIP_2 were resynthesized by PI phosphorylation. As compared to other tissues, such as isolated rat hepatocytes or WRK_1 cells [11], the observed reduction in the size of the PIP_2 pool was relatively small. This observation might reflect the poor efficacy of CCh to induce IP accumulation.

Fig.2 shows that CCh produced dose-dependent IP accumulation in isolated gastric, fundic mucosal cells. IP_1 was produced in higher amounts than IP_2 or IP_3 (60, 30 and 10% of total IP for IP_1, IP_2 and IP_3, respectively). This seems to indicate that in fundic mucosal cells, as in other cell types [12], 10 mM LiCl predominantly inhibited IP_1 phosphatase. The EC_{50} values for CCh-induced IP_1, IP_2 and IP_3 accumulation were similar and the mean value was 30 ± 6 μM ($n = 6$). CCh-stimulated IP accumulation in *myo*-[2-^{3}H]inositol prelabeled fundic mucosal cells is clearly receptor mediated. Atropine inhibited the CCh effect in a dose-dependent manner (fig.3) and, at 1 μM, completely inhibited the response induced by 100 μM CCh. The inhibition constant K_i was 0.32 ± 0.18 nM ($n = 3$), indicating that the cholinergic receptor involved is of the muscarinic type.

The dose dependencies for CCh binding to muscarinic receptors, CCh-induced IP and AP accumulations, determined under identical experi-

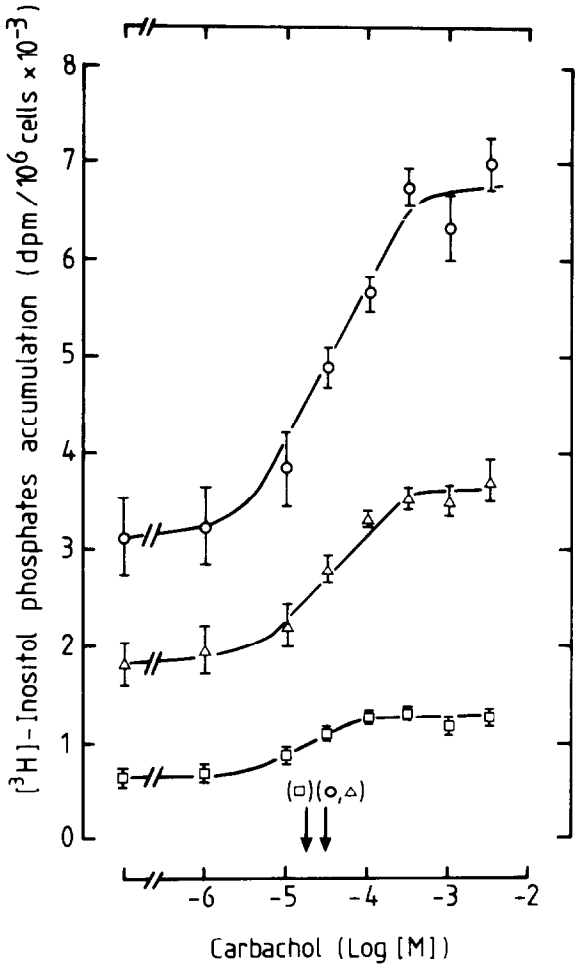

Fig.2. Dose-response curve of IP production in fundic mucosal cells after CCh stimulation. *myo*-[2-^{3}H]Inositol prelabeled cells were incubated for 20 min at 37°C with increasing amounts of CCh. Labeled inositol phosphates, IP_1, IP_2 and IP_3, were separated chromatographically and their radioactivity measured and expressed as dpm per 10^6 cells ± SE for 3 separate experiments (○) IP_1, (△) IP_2, (□) IP_3.

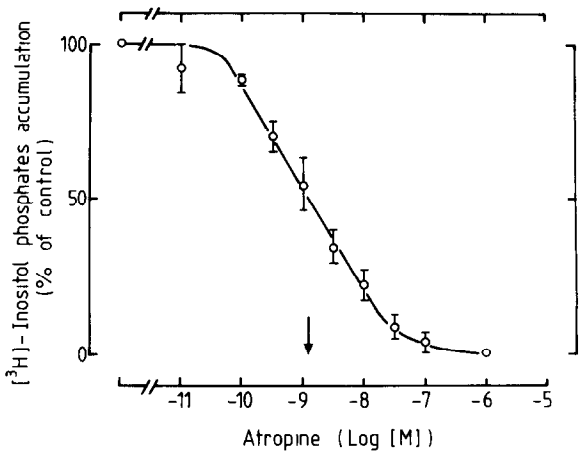

Fig.3. Effect of atropine on CCh-stimulated IP production. *myo*-[2-^{3}H]Inositol prelabeled cells were incubated for 20 min at 37°C with 100 μM CCh in the presence of various atropine concentrations. Labeled IP were separated and measured. Results ± SE (3 determinations) were expressed as percentage of control (cells incubated without atropine).

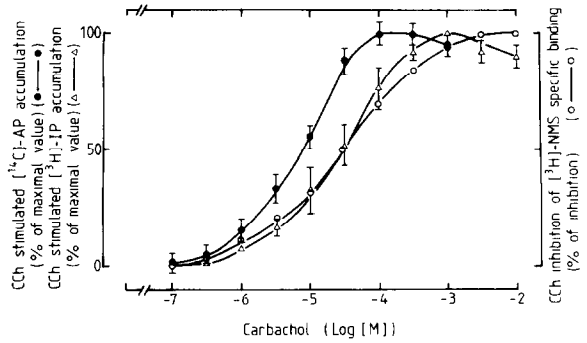

Fig.4. Dose-response curves of CCh inhibition of [^{3}H] NMS specific binding, of CCh-stimulated IP production and of CCh-stimulated AP accumulation in isolated fundic mucosal cells. Results for AP accumulation (●——●) and IP production (△——△) are normalized and expressed as percentages of the maximal value measured for the different CCh molar concentrations. Binding data are represented as percentages of inhibition of maximal [^{3}H]NMS specific binding by different CCh concentrations (○——○) after correction for the radioligand-induced shift.

mental conditions, are shown in fig.4. The apparent K_d for CCh binding, as deduced from the determination of dose-dependent inhibition of [^{3}H]NMS binding by CCh, was 50 ± 5 μM ($n = 3$). The Hill coefficient was 0.74 suggesting the presence of multiple affinity states of the receptor for agonists, as described by Birdsall et al. [13]. CCh induced acid secretion from parietal cells, as estimated by AP accumulation, with an apparent K_{act} of 6 ± 2 μM ($n = 6$). The apparent K_{act} for CCh-induced IP formation on the same cell preparation was 30 ± 8 μM ($n = 6$).

These results are compatible with the existence of a causal relationship between the three events: binding of agonist to its receptor, activation of the

phospholipase C and acid secretion from parietal cells. On the other hand, the differences in K_{act} for IP and AP accumulations might be explained by an amplification phenomenon.

As already demonstrated for muscarinic receptors from several other tissues (review [6]), we show here that activation of muscarinic receptors in isolated rabbit gastric mucosal cells leads to increased inositol lipid breakdown. Preliminary studies on partially purified parietal cells from this crude preparation (not shown) indicate that a CCh-induced inositol lipid breakdown can also be demonstrated. These results suggest that the well documented IP_3-mediated Ca^{2+} mobilization from intracellular stores might contribute to the CCh-induced acid secretion from parietal cells [5,8].

There are some controversies concerning the origin of Ca^{2+} involved in the control of acid secretion from parietal cells. Using EDTA-treated isolated dog parietal cells, Soll [2] and Muallem and Sachs [3] showed that the cholinergic effect is entirely dependent on the presence of Ca^{2+} in the extracellular medium. They concluded that an increase in membrane permeability is the main mechanism by which CCh increases free cytosolic Ca^{2+}. However, the possibility must be considered that, in the experimental conditions used by the authors, the EDTA treatment leads to partial depletion of intracellular Ca^{2+} stores. Conversely, Chew [14] reported that CCh-induced acid secretion from rabbit gastric glands is independent of the presence of extracellular Ca^{2+} suggesting that mobilization of intracellular Ca^{2+} might contribute predominantly to the putative rise in cytosolic free Ca^{2+} following cholinergic stimulation. Our observation that CCh increases inositol lipid breakdown supports such an interpretation. However, it must be pointed out that the CCh-induced IP accumulation was relatively small. In addition, we cannot evaluate the fraction of accumulated IP which originated from inositol 1,4,5-trisphosphate which is a second messenger of Ca^{2+} mobilization from endoplasmic reticulum [15]. Indeed, it was shown that in several cells, the production of inositol 1,3,4-trisphosphate which is inactive as a Ca^{2+} mobilizing agent, can also be activated following hormone stimulation. Under experimental conditions identical to those of this study, we previously showed that about 40% of CCh-induced acid secretion is independent of extracellular Ca^{2+} [4].

All together, these results suggest that, in isolated rabbit parietal cells, CCh-induced inositol lipid breakdown contributes to the muscarinic stimulation of acid secretion.

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