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L-365,260 inhibits *in vitro* acid secretion by interacting with a PKA pathway

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1 The aim of this study was to analyse the antisecretory mechanism of L-365,260 *in vitro* in isolated rabbit gastric glands.

2 We showed that compound L-365,260, described as a non-peptide specific competitive CCK-B receptor antagonist, was able to dose-dependently inhibit [^{14}C]-aminopyrine accumulation induced by histamine (10^{-4} M), carbachol (5×10^{-5} M), 3-isobutyl-1-methyl-xanthine (IBMX) (5×10^{-6} M) and forskolin (5×10^{-7} M) with similar IC_{50} values respectively of $1.1\pm0.6\times10^{-7}$ M, $1.9\pm1.2\times10^{-7}$ M, $4.2\pm2.0\times10^{-7}$ M and $4.0\pm2.8\times10^{-7}$ M.

3 We showed that L-365,260 acted beyond receptor activation and production of intracellular second messengers and that it had no action on the H^+/K^+-ATPase.

4 We found that L-365,260 inhibited cyclic AMP-induced [^{14}C]-aminopyrine accumulation in digitonin-permeabilized rabbit gastric glands, suggesting that this compound acted, at least in part, as an inhibitor of the cyclic AMP-dependent protein kinase (PKA) pathway.

Keywords: L-365,260; isolated rabbit gastric glands; [^{14}C]-aminopyrine accumulation; H^+/K^+-ATPase; protein kinase inhibitor

Abbreviations: H^+/K^+-ATPase, H^+/K^+-adenosinetriphosphatase; PKA, cyclic AMP-dependent protein kinase

Introduction

Acetylcholine, gastrin and histamine are the three most important mediators that stimulate gastric acid secretion by interacting with specific receptors located at the basolateral membrane of the parietal cells. Acetylcholine interacts with a muscarinic M_3 receptor (Kajimura *et al*., 1992), gastrin with a CCK-B/gastrin receptor (Kopin *et al*., 1992) and histamine with a histamine H_2 receptor (Black *et al*., 1972). It has been established that the gastric histamine H_2 receptor is coupled to the formation of cyclic AMP (Chew *et al*., 1980). Activation of the parietal cell M_3 cholinoceptor or the CCK-B/gastrin receptor leads to an elevation of $[Ca^{2+}]_i$ (Chew *et al*., 1992). The observation that cholinergic or gastrin stimulation of acid secretion is potentiated by histamine (Berglindh *et al*., 1976; Soll, 1978; Chew & Hersey, 1982; Oiry *et al*., 1995) suggests that cyclic AMP and $[Ca^{2+}]_i$ interact at some level. Whatever the complexity of the mechanism of acid secretion, the binding of these ligands to the receptors on the basolateral membrane of the parietal cell generates changes in second messengers and activation of protein kinase cascades that lead to final activation of the H^+/K^+-adenosine triphosphatase (H^+/K^+-ATPase) (EC 3.6.1.36) (Sachs *et al*., 1976; Forte & Soll, 1989). One role of cyclic AMP is the activation of cyclic AMP-dependent protein kinase (PKA) (Chew, 1985). PKA has specific functional targets and also initiates a phosphorylation cascade through activation of other downstream protein kinases the actions of which cause proton pump activation and cytoskeletal rearrangements which are characteristic of the stimulated parietal cell. It has been suggested that Ca^{2+}/calmodulin-dependent kinase may play an important regulatory role in the modulation of cytoskeletal structure and function. Recently, Tsunoda *et al*. (1992) reported that Ca^{2+}/calmodulin-dependent kinase II (CaMK II) mediates cholinergic-stimulated parietal cell secretion. In resting parietal cells, the H^+/K^+-ATPase is sequestered in the cytoplasmic membrane compartments of low K^+ permeability: the tubulovesicles. Stimulation of the parietal cells causes a cytoskeletal reorganization and a fusion of the tubulovesicles with the apical plasma membrane, transferring the proton pump to that surface (Forte & Soll, 1989). The H^+/K^+-ATPase constitutes the final step common to each secretagogue in the production of acid by parietal cells and so provides a target for inhibitors used therapeutically as anti-ulcer agents such as omeprazole (Fellenius *et al*., 1981).

In a previous study in which we compared the acid secretion induced by the C-terminal octapeptide of cholecystokinin (CCK-8) and [Leu^{11}]gastrin(5–17) in isolated rabbit gastric glands (Oiry *et al*., 1995), we confirmed the ability of histamine to potentiate the action of CCK-8 (or [Leu^{11}]gastrin(5–17)). To better understand the cooperativity between CCK and histamine, we tested the potency of various classes of CCK receptor antagonists (L-365,260; PD-135,158; YM-022; JMV-180 and L-364,718) as inhibitors of histamine-induced [^{14}C]-aminopyrine accumulation. We found that PD-135,158; YM-022; JMV-180 and L-364,718 had no effect on histamine-induced [^{14}C]-aminopyrine accumulation. Surprisingly, only compound L-365,260 appeared active and even more potent than cimetidine to inhibit histamine-induced [^{14}C]-aminopyrine accumulation. Compound L-365,260, already described in the literature as a competitive and specific CCK-B/gastrin receptor antagonist (Lotti & Chang, 1989), presented a different and specific behaviour compared to other CCK-B/gastrin antagonists (PD-135,158, YM-022 and JMV-180).

It has been mentioned that in the rat *in vivo*, low doses of L-365,260 inhibited pentagastrin-stimulated secretion (Hirst *et al*., 1991), whereas higher doses inhibited basal-, histamine- and carbachol-stimulated secretion (Hirst *et al*., 1991; Nishida

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et al., 1992; Pendley *et al*., 1993). In order to provide a better understanding of the role of L-365,260 in acid secretion *in vitro*, we decided to investigate further its antisecretory mechanism in isolated rabbit gastric glands.

Methods

Drugs

Collagenase EC 3.4.24.3 was obtained from Serva (Heidelberg, Germany). NaCl, KCl, Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $CaCl_2$, K_2CO_3, LiCl, ammonium formate, adenosine 3′:5′-cyclic monophosphate (cyclic AMP), adenosine-5′-triphosphate (ATP), glucose, sucrose, mannitol, $MgCl_2$, ethylene diamine tetraacetic acid (EDTA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), Tris[hydroxy-methyl]amino-methane (TRIS), piperazine-N,N′-bis[2-ethanesulphonic acid] (PIPES), trichloroacetic acid (TCA), succinic acid, pyruvic acid, digitonin, histamine dihydrochloride, cimetidine, acetyl-choline chloride (Ach), carbamylcholine chloride (carbachol), 3-isobutyl-1-methylxanthine (IBMX), forskolin, atropine sulphate, valinomycin and choline chloride were from Sigma (St Louis, MO, U.S.A.). Bovine serum albumin (BSA) fraction V was from Euromedex (France). The protein concentration was evaluated using the Bio-Rad protein assay. Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad too (Richmond, CA, U.S.A.). Alumina was from ICN Biomedicals. Sulphated CCK-8 (H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2), JMV-180 (Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-O-CH_2-CH_2-C_6H_5) and YM-022 [(R)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea] were synthesized in our Laboratory. [^{14}C]-Dimethylamine aminopyrine (60–120 Ci mmol^{-1}) was purchased from NEN (Le Blanc Mesnil). [2,5-^{3}H]-Histamine dihydrochloride (51.0 Ci mmol^{-1}), [γ-^{32}P]-ATP (5000 Ci mmol^{-1}), [8-^{3}H]-adenine (20–25 Ci mmol^{-1}), *myo*-[2-^{3}H]-inositol (16–20 Ci mmol^{-1}) and Complete Phase Combining System for liquid scintillation counting (PCS) were purchased from Amersham (Buckinghamshire, U.K.). Omeprazole was a gift from Astra Laboratories. A stock solution was prepared at a 10^{-2} M concentration in 50% (v v^{-1}) dimethylsulphoxide (DMSO) and 50% (v v^{-1}) H_2O and was stored at −20°C.

L-365,260 [3R-(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N-(3-methylphenyl) urea] and L-364,718 (MK-329 or Devazepide)[3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4 benzodiazepin-3-yl)-1H-indole-2-carboximide] were a gift from Dr P. Anderson, Merck Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.). PD-135,158 [4([2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7-trimethyl-bicyclo [2.2.1] hept-2-yl)oxy]carbonyl]amino] propyl] amino]-1-phenylethyl] amino -4-oxo-[1S-α.2β[S*(S*)]4α]]-butanoate N-methyl-D-glucamine (bicyclo system 1S-endo) was a gift from Dr D. Horwell, Parke-Davis Neuroscience Research Centre (Cambridge, U.K.). A stock solution of each CCK antagonist was prepared in pure DMSO at a 10^{-3} M concentration and stored at −20°C. Dilutions were made with incubation medium (see below). The maximal final concentration of DMSO without any effect on basal [^{14}C]-aminopyrine accumulation experiments was 0.5%.

Isolated rabbit gastric gland preparations

Gastric glands were isolated according to the method previously described by Berglindh & Öbrink (1976) with some modifications. New Zealand White rabbits (2.5 kg, INRA-Montpellier) were killed by cervical dislocation and exsanguination. The stomachs were rapidly removed, the antral portion was cut away and discarded, and the fundus was cut open along the smaller curvature. The stomach contents were emptied out and the mucosa was rinsed with water at room temperature and wiped with disposable towels to eliminate excess mucus and food particles. The mucosal layer was gently scraped off the muscle layer with a blunt spatula and collected in cold buffer ('AP buffer') containing (in mM) NaCl 132.4, KCl 5.4, Na_2HPO_4 5, NaH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1, 2 g l^{-1} glucose and 2 g l^{-1} BSA adjusted to pH 7.4. The mucosal pieces were suspended and decanted in cold buffer several times until the supernatant remained clear (about three times). The tissue was rapidly minced into small pieces. As emphasized by Berglindh & Öbrink (1976), this step is crucial and must be carried out very quickly. The minced mucosa was resuspended and allowed to settle in cold buffer, after which excess buffer was decanted. This operation was repeated three times. Mucosal tissue (p gram) was transferred into a round-bottom flask and 2 p ml of collagenase solution in buffer (0.2 mg ml^{-1}) was added. Tissue was incubated in a water bath for 50 min at 37°C with gentle agitation and the suspension was continuously bubbled with 95% O_2–5% CO_2. Incubation was stopped by dilution with 250 ml of cold buffer. Glands were purified by successive decanting/resuspension steps in cold buffer and were resuspended at the adequate dilution in the incubation medium (10 p ml). The protein concentration was evaluated using an assay based on the Bradford dye-binding procedure. Under these dilution conditions, the protein concentration was 6 mg ml^{-1}.

Gastric gland permeabilization

Gastric gland permeabilization was achieved as previously described by Thibodeau *et al*. (1994). Before treatment with permeabilizing agents, freshly isolated glands were washed once in a K^+ rich permeabilization medium ('K medium') containing (in mM) KCl 100, NaCl 20, $MgSO_4$ 1.2, NaH_2PO_4 1, mannitol 40, HEPES 20, Tris (pH 7.4) 10. Glands were resuspended in K medium at a 10–20% cytocrit and incubated in the presence of digitonin at the concentration of 30 μg ml^{-1} for 20 min at 37°C. During the permeabilization procedure, 10 μM cimetidine was included to antagonize the effect of any endogenously released histamine. After permeabilization, the gland suspension was diluted to a 5% cytocrit in K medium in which 10 mM succinate and 1 mM pyruvate were added as oxidative substrates. Glands were maintained in this medium and used within 30 min for the aminopyrine accumulation assay.

[^{14}C]-Aminopyrine accumulation

Rabbit gastric glands were incubated in AP buffer (intact glands) or K buffer supplemented with succinate and pyruvate (permeabilized glands) at 37°C with various test compounds and 0.1 μCi ml^{-1} [^{14}C]-aminopyrine in a final volume of 1.5 ml under magnetic agitation in 24-welled tissue culture plates (Multiwell, Falcon) kept under 95% O_2–5% CO_2. In the case of digitonin-permeabilized glands, cimetidine was always present to avoid an action of endogenous histamine on any remaining intact parietal cells. After 20 min incubation with the various compounds as indicated for individual experiments (agonists, antagonists, activators, substrates), glands were transferred to Eppendorf vials and centrifuged at 12,000 r.p.m. for 2 min. The supernatants were discarded and the pellets

were dissolved in 100 μl 10% v v^{-1} $HClO_4$ and added to scintillation liquid PCS before counting. Incubations were performed in duplicate and mean values were used for calculations. The background count was obtained after incubation of glands under the same conditions but without any secretagogue and was substracted from all counts.

Binding experiments in isolated rabbit gastric glands

Binding experiments were carried out in the AP buffer described previously. We tested the abilities of various CCK receptor antagonists (JMV-180, L-365,260 and PD-135,158) to interact with histamine receptors expressed in the rabbit gastric glands. [2,5-^{3}H]-Histamine dihydrochloride was used as radioligand. For displacement experiments, gastric glands (1.2 mg) were incubated with 12.3 nM of radioligand for 50 min at 37°C in a final volume of 250 μl with various concentrations of agonists and antagonists as appropriate. Non-specific binding was determined in the presence of 1 mM histamine. Incubation was terminated by adding 1 ml of incubation medium at 4°C supplemented with BSA (20 g l^{-1}). Aliquots were centrifuged at 12,000 r.p.m. for 2 min. The supernatants were discarded, the pellets were dissolved in 100 μl 10% v v^{-1} $HClO_4$ and added to scintillation liquid PCS before counting. Incubations were performed in duplicate and mean values were used for calculations.

Measurement of inositol phosphates in isolated rabbit gastric glands

Intracellular inositol phosphates were determined as previously described by Qian *et al.* (1993) with some modifications. Gastric glands (13 mg protein ml^{-1}) were incubated with 400 μCi *myo*-[2-^{3}H]-inositol for 2 h at 37°C. The glands were washed three times in AP buffer and incubated (15 min, 37°C) in 20 ml of the same buffer containing 20 mM LiCl. Aliquots of [^{3}H]-inositol-loaded glands (0.4 ml) were incubated (15 min, 37°C) with buffer or with various compounds as described in each individual experiment in a final volume of 0.5 ml. The incubation was terminated by adding 500 μl $HClO_4$ (5% v v^{-1}). Each tube subsequently received 155 μl K_2CO_3 2 M and 25 μl HEPES 0.4 M, and the contents were vortexed and centrifuged (3000 r.p.m., 10 min). Nine-hundred μl of each tube were collected and each pellet was washed with 500 μl H_2O. After centrifugation (3000 r.p.m., 10 min), 500 μl of each supernatant was pooled with the previous 900 μl and the mixture was applied to a column containing 1.6 ml of a 1:4 (pp^{-1}) Dowex AG-1-X8 anion-exchange resin in distilled water. The columns were washed in the following manner: 10 ml distilled water, 4 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 5 ml of 1 M ammonium formate. The eluates were then assayed for their radioactivity after the addition of 10 ml PCS solution to each vial.

Measurement of cyclic AMP in isolated rabbit gastric glands

Intracellular cyclic AMP was determined by measuring the formation of cyclic [^{3}H]-AMP from [^{3}H]-adenine as described previously (Weiss *et al.*, 1985) with some modifications. Briefly, gastric glands (13 mg protein ml^{-1}) were incubated with 400 μCi [^{3}H]-adenine for 2 h at 37°C. The glands were washed three times in AP buffer and incubated (15 min, 37°C) in 25 ml of the same buffer containing 1 mM IBMX. Aliquots of [^{3}H]-adenine-loaded glands (0.4 ml) were incubated (15 min, 37°C) with buffer or with various compounds as described in each individual experiment in a final volume of 0.5 ml. The incubation was terminated by adding 1 ml of ice-cold 5% (v v^{-1}) TCA. 100 μl of cold 5 mM ATP/5 mM cyclic AMP were added and the mixture was centrifuged at 3000 r.p.m. for 10 min. Each supernatant (1 ml) was eluted through sequential chromatography on Dowex and alumina columns as already described. The eluates were then assayed for their radioactivity after the addition of 10 ml PCS solution to each vial.

Preparation of gastric vesicles enriched in gastric H^+/K^+-ATPase activity

Rabbit gastric mucosal membrane vesicles containing H^+/K^+-ATPase were prepared according to the method previously described by Reenstra & Forte (1990). All subsequent steps were performed with ice-cold solutions. The rabbit stomach was washed in PBS (NaCl 150 mM; phosphate, pH 7.4, 5 mM), the mucosal layer was gently scraped off the muscle layer with a blunt spatula, weighed, covered with a hypotonic homogenizing buffer (MSEP (mM): mannitol 125, sucrose 40, EDTA 1, PIPES-Tris, (pH 6.7) 5) and minced with scissors. The mucosal pieces were suspended and decanted in MSEP several times until the supernatant remained clear. MSEP was added (25 ml g^{-1} scraped tissue) and the tissue triturated in a Potter-Elvehjem homogenizer with 12 passes at 200 r.p.m. The homogenate was spun at 600 r.p.m. for 10 min. The supernatant was decanted and saved while the pellet was resuspended in MSEP, rehomogenized and spun as before. The two supernatants were combined and spun at 6000 r.p.m. for 10 min. The supernatant was respun at 11,000 r.p.m. for 10 min and the resulting supernatant was recentrifuged at 19,500 r.p.m. for 90 min. The pellet was resuspended in isotonic suspending medium (SM (mM): sucrose 300, EDTA 0.2, Tris-HCl, (pH 7.3) 5) and the vesicle-rich fraction was further purified by density gradient centrifugation. Vesicles were placed on the top of a cushion of 5% p v^{-1} Ficoll (in SM) and spun at 32,000 r.p.m. for 2 h. Membranes were removed from the interface with a syringe, diluted 5 fold in SM and recentrifuged. The membrane pellet, used as the gastric vesicular fraction containing gastric H^+/K^+-ATPase, was resuspended in a minimal volume of the same buffer. The material was aliquoted and stored at −80°C until use. Membrane protein concentration was determined by the Bradford method using the Bio-Rad protein assay.

H^+/K^+-ATPase assay

H^+/K^+-ATPase activity was determined by measuring the release of $^{32}P_i$ from [γ-^{32}P]-ATP according to a method previously described by Fellenius *et al.* (1981) with some modifications. The membrane protein (10 μg) was preincubated 15 min at 37°C with the test compound (L-365,260 or omeprazole) in two assay media containing (in mM) $MgCl_2$ 2, Tris/acetate, (pH 7.4) 40, KCl 20 and 10 μM valinomycin (buffer 1: dosage of the H^+/K^+-ATPase activity in the presence of Mg^{2+} and K^+) and (in mM) $MgCl_2$ 2, Tris/acetate, (pH 7.4) 40, choline chloride 20 (buffer 2: dosage of the H^+/K^+-ATPase activity in the presence of Mg^{2+}) in a total volume of incubation of 1 ml. The enzyme reaction was started by adding 50 μl of 2 mM [γ-^{32}P]-ATP. After incubation at 37°C for 4 min, the reaction was terminated by adding 1 ml of 10% v v^{-1} trichloroacetic acid. $^{32}P_i$ released was separated by centrifugation on activated charcoal (a 20% w v^{-1} solution in HCl 0.1 N, 20 min at 3000 r.p.m.) and 1 ml supernatant was measured by liquid scintillation counting. The H^+/K^+-ATPase activity was calculated by substracting the basal rate (in the

absence of K^+) from the rate of hydrolysis of ATP in the presence of K^+. Per cent inhibition was calculated as follows: [(mean value of control activity − mean value of test activity)/mean value of control activity] × 100. The doses inhibiting H^+/K^+-ATPase activity by 50% (IC_{50}) were calculated by linear regression analysis.

Results

Antisecretory effect of L-365,260 in isolated rabbit gastric glands

In isolated rabbit gastric glands, CCK-8, histamine, acetylcholine and carbachol stimulated acid secretion evaluated by [^{14}C]-aminopyrine accumulation in a dose-dependent manner. Compared with the basal value, 10^{-8} M CCK-8 produced a 25% increase in [^{14}C]-aminopyrine accumulation, while 10^{-4} M histamine and 10^{-4} M Ach (or carbachol) produced respectively a 50% and a 35% increase. In Figure 1, results are expressed as percentage of the maximal response obtained with 10^{-8} M CCK-8, 10^{-4} M histamine and 10^{-4} M Ach or carbachol. The effective concentrations producing 50% of the maximal response (EC_{50}) were respectively 0.41 ± 0.24 nM, 4.0 ± 1.1 μM, 1.2 ± 0.8 μM and 1.6 ± 1.2 μM (mean ± s.d. from at least three independent experiments performed in duplicate). In the same model, we showed that the phosphodiesterase inhibitor IBMX (5×10^{-6} M) and the adenylyl cyclase activator forskolin (5×10^{-7} M), two compounds known to enhance cyclic AMP levels in parietal cells, induced an increase in [^{14}C]-aminopyrine accumulation with the same efficacy as histamine (three independent experiments performed in duplicate).

We tested the potency of atropine to inhibit the [^{14}C]-aminopyrine accumulation induced by 5×10^{-5} M carbachol (Figure 2). Atropine inhibited such a stimulation with an IC_{50} of $3.6 \pm 2.8 \times 10^{-8}$ M (mean ± s.d. from four independent experiments performed in duplicate).

In a previous work (Oiry *et al*., 1995), we observed a potentiation of the CCK-8-induced [^{14}C]-aminopyrine accumulation in the presence of histamine, in accordance with the results obtained by other authors (Berglindh *et al*., 1976; Soll, 1978; Chew & Hersey, 1982). While 10^{-4} M histamine-induced [^{14}C]-aminopyrine accumulation was about 2.5 times higher than 10^{-8} M CCK-8-induced response, [^{14}C]-aminopyrine accumulation induced by 10^{-4} M histamine + 10^{-8} M CCK-8 was about eight times higher than the 10^{-8} M CCK-8 response. To better understand this potentiation phenomenon between CCK-8 and histamine, we tested the ability of various classes of CCK receptor antagonists to inhibit [^{14}C]-aminopyrine accumulation induced by 10^{-4} M histamine. Four CCK-B/gastrin receptor antagonists including the two benzodiazepine analogues L-365,260 (Lotti & Chang, 1989) and YM-022 (Nishida *et al*., 1994), the dipeptide mimicking compound PD-135,158 (Hughes *et al*., 1990), the modified C-terminal fragment of CCK JMV-180 (Galas *et al*., 1988; Amblard *et al*., 1994) as well as the CCK-A receptor antagonist L-364,718 (Chang & Lotti, 1986), a benzodiazepine analogue, were used in a concentration range from 10^{-10}–10^{-6} M. While YM-022, PD-135,158, JMV-180 and L-364,718 failed to inhibit histamine-induced [^{14}C]-aminopyrine accumulation, L-365,260 dose-dependently inhibited 10^{-4} M histamine-induced [^{14}C]-aminopyrine accumulation with an IC_{50} of $1.1 \pm 0.6 \times 10^{-7}$ M (mean ± s.d. from four independent experiments performed in duplicate) (Figure 3). The same kind of experiment was carried out on the activated muscarinic receptor. Whereas YM-022, PD-135,158, JMV-180 and L-364,718 failed to inhibit 5×10^{-5} M carbachol- induced [^{14}C]-

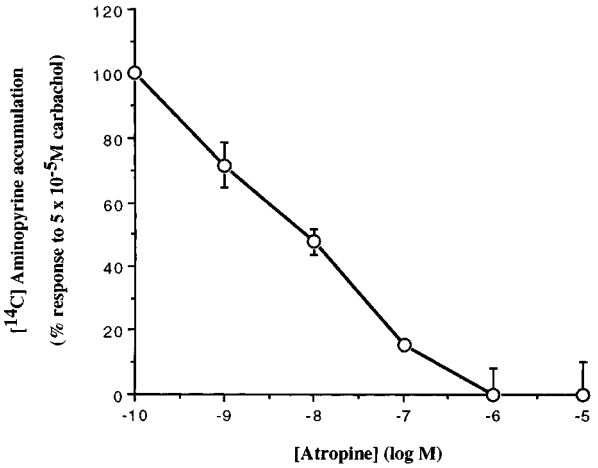

Figure 2 Effect of atropine on [^{14}C]-aminopyrine accumulation induced by 5×10^{-5} M carbachol in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 5×10^{-5} M carbachol. Results are the means ± s.d. of four experiments each performed in duplicate.

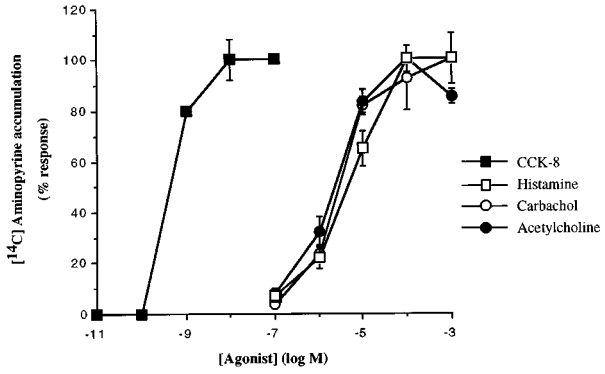

Figure 1 Dose-response curves for CCK-8, histamine, carbachol and acetylcholine on [^{14}C]-aminopyrine accumulation in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained respectively with 10^{-8} M CCK-8, 10^{-4} M histamine and 10^{-4} M carbachol or acetylcholine. Results are the means ± s.d. of three experiments each performed in duplicate.

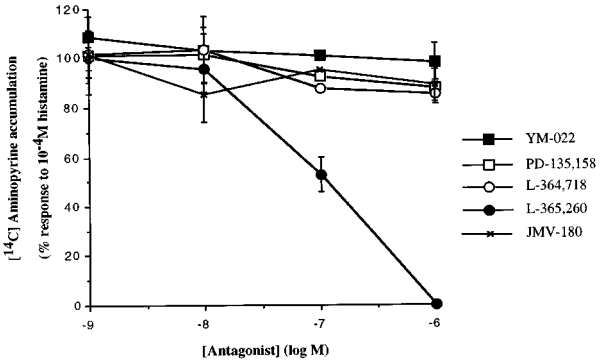

Figure 3 Effect of YM-022, PD-135,158, L-364,718, L-365,260 and JMV-180 on [^{14}C]-aminopyrine accumulation induced by 10^{-4} M histamine in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 10^{-4} M histamine. Results are the means ± s.d. of four experiments each performed in duplicate.

aminopyrine accumulation, L-365,260 dose-dependently inhibited carbachol-induced [^{14}C]-aminopyrine accumulation with an IC_{50} of $1.9 \pm 1.2 \times 10^{-7}$ M (mean ± s.d. from three independent experiments performed in duplicate) (Figure 4). The same results were obtained with [^{14}C]-aminopyrine accumulation induced by 5×10^{-5} M Ach (three independent experiments performed in duplicate) (not shown).

Finally, we have tested the ability of the CCK receptor antagonists to inhibit [^{14}C]-aminopyrine accumulation induced by 5×10^{-6} M IBMX (Figure 5) and 5×10^{-7} M forskolin (Figure 6), two compounds known to enhance the cyclic AMP content in the parietal cell. Only compound L-365,260 was able to inhibit such stimulations in a dose-dependent manner with IC_{50} of $4.2 \pm 2.0 \times 10^{-7}$ M and $4.0 \pm 2.8 \times 10^{-7}$ M, respectively (mean ± s.d. from at least three independent experiments performed in duplicate).

To estimate the role of CCK-B/gastrin receptors in such inhibitions, we tested the potency of L-365,260 to inhibit [^{14}C]-aminopyrine accumulation induced by 10^{-4} M histamine plus 10^{-8} M CCK-8 (potentiation of the acid secretion) in the presence or in the absence of 10^{-6} M PD-135,158 (a saturating concentration for CCK-B/gastrin receptors). L-365,260 inhibited in a dose-dependent manner [^{14}C]-aminopyrine accumulation induced by histamine plus CCK-8 with IC_{50} values of $4.1 \pm 1.7 \times 10^{-7}$ M (absence of PD-135,158) and $2.4 \pm 1.4 \times 10^{-7}$ M (presence of PD-135,158) (means ± s.d. from three independent experiments performed in duplicate) (Figure 7).

These results clearly demonstrated that in isolated rabbit gastric glands, compound L-365,260 inhibited acid secretion whatever the stimulatory pathway, by interacting with a site different from the CCK-B/gastrin receptor. Finally, the CCK antagonists were tested on basal [^{14}C]-aminopyrine accumulation, in a concentration range from 10^{-9} M to 5×10^{-6} M (a maximal concentration of antagonist in which the presence of DMSO had no effect on basal [^{14}C]-aminopyrine accumulation). While YM-022, PD-135,158, JMV-180 and L-364,718 had no effect on basal [^{14}C]-aminopyrine accumulation, we showed that L-365,260 inhibited in a dose-dependent manner the basal [^{14}C]-aminopyrine accumulation (Figure 8).

Antisecretory effect of omeprazole in isolated rabbit gastric glands

To test the H^+/K^+-ATPase as a potent target for L-365,260 activity, we compared the antisecretory behaviour of L-365,260 and omeprazole in isolated rabbit gastric glands. Omeprazole inhibited in a dose-dependent manner [^{14}C]-

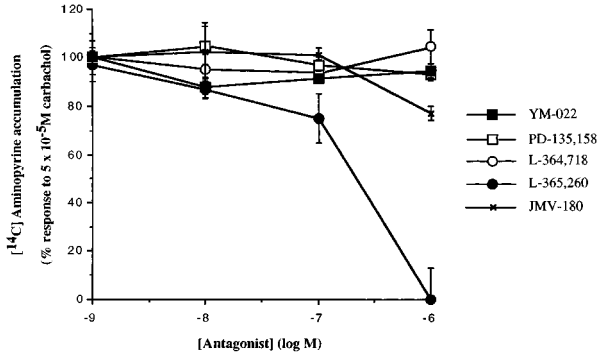

Figure 4 Effect of YM-022, PD-135,158, L-364,718, L-365,260 and JMV-180 on [^{14}C]-aminopyrine accumulation induced by 5×10^{-5} M carbachol in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 5×10^{-5} M carbachol. Results are the means ± s.d. of three experiments each performed in duplicate.

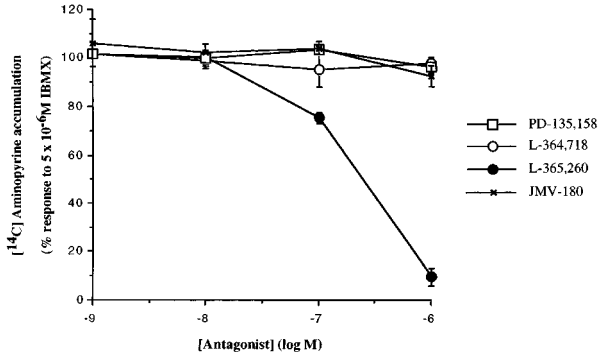

Figure 5 Effect of PD-135,158, L-364,718, L-365,260 and JMV-180 on [^{14}C]-aminopyrine accumulation induced by 5×10^{-6} M IBMX in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 5×10^{-6} M IBMX. Results are the means ± s.d. of four experiments each performed in duplicate.

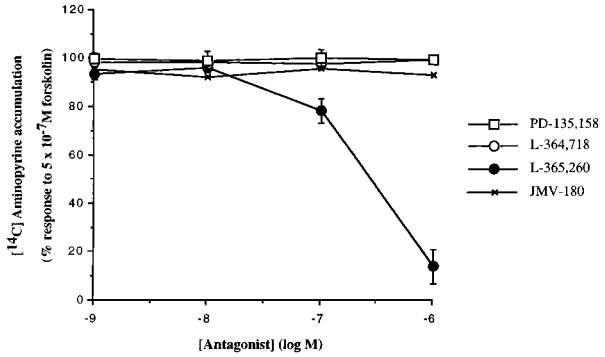

Figure 6 Effect of PD-135,158, L-364,718, L-365,260 and JMV-180 on [^{14}C]-aminopyrine accumulation induced by 5×10^{-7} M forskolin in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 5×10^{-7} M forskolin. Results are the means ± s.d. of four experiments each performed in duplicate.

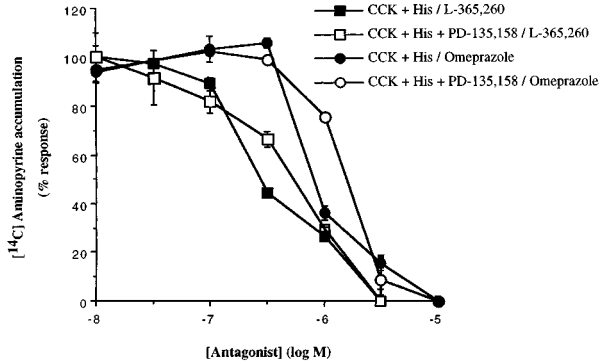

Figure 7 Effect of L-365,260 and omeprazole on [^{14}C]-aminopyrine accumulation induced by 10^{-8} M CCK-8 plus 10^{-4} M histamine in the absence (closed symbols) or in the presence (open symbols) of 10^{-6} M PD-135,158 in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 10^{-8} M CCK-8 plus 10^{-4} M histamine with or without 10^{-6} M PD-135,158. Results are the means ± s.d. of three experiments each performed in duplicate.

aminopyrine accumulation induced by 10^{-8} M CCK-8, 10^{-4} M histamine and 5×10^{-5} M carbachol with IC_{50} values respectively of $7.7\pm2.2\times10^{-7}$ M, $5.2\pm0.9\times10^{-7}$ M and $8.2\pm4.7\times10^{-7}$ M (mean ± s.d. from four independent experiments performed in duplicate) (Figure 9).

We showed that omeprazole inhibited [^{14}C]-aminopyrine accumulation induced by 5×10^{-6} M IBMX and 5×10^{-7} M forskolin in a dose-dependent manner with IC_{50} of $1.1\pm0.3\times10^{-6}$ M and $1.2\pm0.2\times10^{-6}$ M respectively (mean ± s.d. from four independent experiments performed in duplicate) (Figure 9).

The potency of omeprazole to inhibit [^{14}C]-aminopyrine accumulation induced by 10^{-4} M histamine plus 10^{-8} M CCK-8 was tested in the presence or in the absence of 10^{-6} M PD-135,158 to selectively block the CCK-B/gastrin receptors. Even in the presence of a saturating concentration of PD-135,158 (10^{-6} M), omeprazole inhibited in a dose-dependent manner [^{14}C]-aminopyrine accumulation with IC_{50} values of $8.0\pm2.0\times10^{-7}$ M (absence of PD-135,158) and $1.3\pm0.7\times10^{-6}$ M (presence of PD-135,158) (means ± s.d. from three independent experiments performed in duplicate) (Figure 7).

Omeprazole alone was tested on basal [^{14}C]-aminopyrine accumulation in a concentration range from $10^{-8}-10^{-4}$ M (a maximal concentration of omeprazole in which the presence of DMSO had no effect on basal [^{14}C]-aminopyrine accumulation). We found that omeprazole inhibited in a dose-dependent manner the basal [^{14}C]-aminopyrine accumulation (Figure 8).

These experiments indicated that L-365,260 and omeprazole presented similar antisecretory profiles.

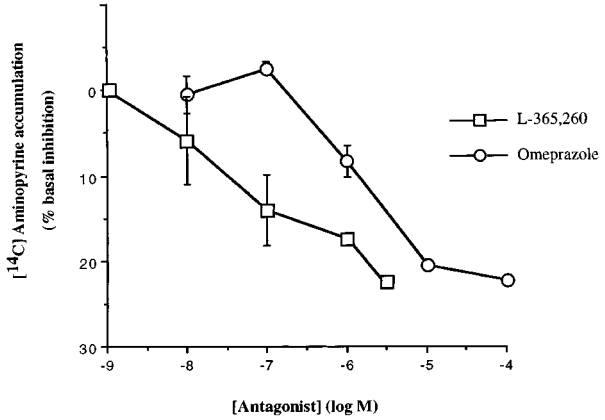

Figure 8 Effect of L-365,260 and omeprazole on basal [^{14}C]-aminopyrine accumulation in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the basal inhibition. Results are the means ± s.d. of three experiments each performed in duplicate.

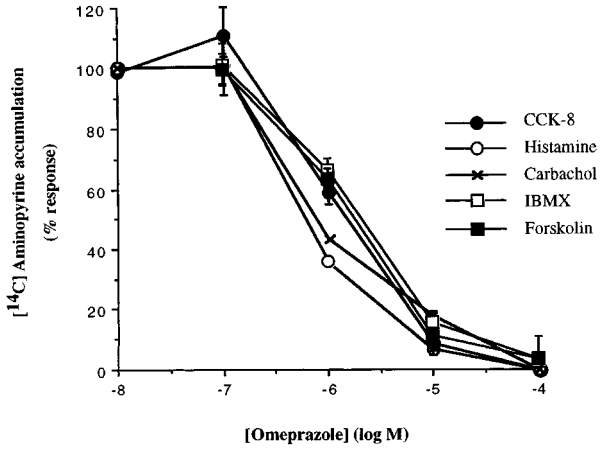

Figure 9 Effect of omeprazole on [^{14}C]-aminopyrine accumulation induced by 10^{-8} M CCK-8, 10^{-4} M histamine, 5×10^{-5} M carbachol, 5×10^{-6} M IBMX and 5×10^{-7} M forskolin in isolated rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 10^{-8} M CCK-8, 10^{-4} M histamine, 5×10^{-5} M carbachol, 5×10^{-6} M IBMX and 5×10^{-7} M forskolin. Results are the means ± s.d. of four experiments each performed in duplicate.

Interaction of CCK antagonists with H_2 receptors in isolated rabbit gastric glands

To test the putative interaction of L-365,260 with H_2 receptors, the effects of some CCK receptor antagonists on [2,5-^{3}H]-histamine dihydrochloride binding were investigated. PD-135,158, L-365,260 and JMV-180 were tested in a concentration range from $10^{-10}-10^{-6}$ M. As shown in Figure 10, none of these antagonists was able to inhibit [2,5-^{3}H]-histamine dihydrochloride binding. As a control, histamine and cimetidine (H_2 receptor antagonist) were tested for their potency to inhibit the specific binding of [2,5-^{3}H]-histamine dihydrochloride in a concentration range from $10^{-8}-10^{-3}$ M. We showed that histamine and cimetidine inhibited [2,5-^{3}H]-histamine dihydrochloride binding with affinities respectively of $1.4\pm1.1\times10^{-6}$ M and $2\pm1.1\times10^{-5}$ M (mean ± s.d. from three independent experiments performed in duplicate) (not shown).

Effect of L-365,260 on second messenger production in isolated rabbit gastric glands

The effect of L-365,260 was tested on intracellular cyclic AMP increase induced by histamine and on intracellular inositol phosphate increase induced by carbachol. L-365,260 (10^{-6} M) did not affect cyclic AMP production induced by 10^{-4} M histamine nor IP production induced by 5×10^{-5} M carbachol. As a control, we showed that histamine-induced cyclic AMP production was inhibited by 10^{-4} M cimetidine and that carbachol-induced IP production was inhibited by 10^{-4} M atropine (three independent experiments performed in triplicate) (not shown).

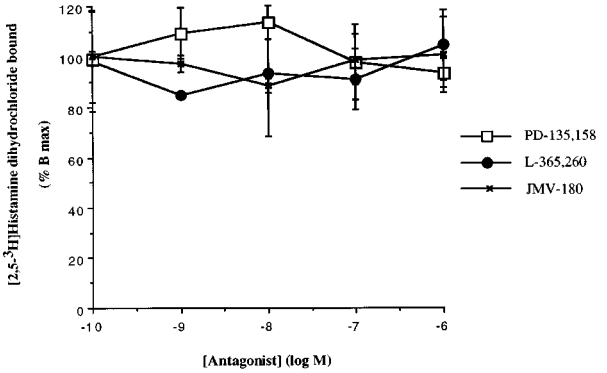

Figure 10 Binding experiments of [2,5-^{3}H]-Histamine dihydrochloride on isolated rabbit gastric glands. The specific binding of [2,5-^{3}H]-Histamine dihydrochloride was measured in the presence of various concentrations of PD-135,158, L-365,260 and JMV-180. Non specific binding was evaluated in the presence of 10^{-3} M histamine. Results are the means ± s.d. of three experiments each performed in duplicate.

Effect of L-365,260 on H^+/K^+-ATPase activity

The H^+/K^+-ATPase activity was assayed in the presence of L-365,260 in a concentration range from 10^{-7} to 5×10^{-5} M. Even a high concentration of L-365,260 (5×10^{-5} M) had no effect on the H^+/K^+-ATPase activity. As a control, we showed that omeprazole inhibited H^+/K^+-ATPase activity in a dose-dependent manner with an IC_{50} value of $4.3\pm2.0\times10^{-5}$ M (mean ± s.d. from four independent experiments performed in duplicate) (Figure 11).

Effect of L-365,260 in cyclic AMP dependent protein kinase (PKA) pathway

As already described by Yao *et al.* (1996), we observed that cyclic AMP produced a dose-dependent [^{14}C]-aminopyrine accumulation in digitonin-permeabilized gastric glands. The secretory activity reached a plateau at 100 μM cyclic AMP (not shown). To test whether L-365,260 interacted with PKA or its downstream signalling pathway, we tested a 5×10^{-6} M concentration of L-365,260 on [^{14}C]-aminopyrine accumulation induced by 100 μM cyclic AMP. We showed that L-365,260 completely inhibited cyclic AMP-induced [^{14}C]-aminopyrine accumulation (Figure 12).

Discussion

On the basis of our previous observation (Oiry *et al.*, 1995) showing that L-365,260, a specific CCK-B receptor antagonist (Lotti & Chang, 1989), inhibited histamine-induced [^{14}C]-aminopyrine accumulation, we decided to study in more detail the mechanism by which this compound promoted its antisecretory activity in isolated rabbit gastric glands.

In the present study, we found that this inhibition did not result from a direct interaction between L-365,260 and H_2 receptors.

In the absence or in the presence of the specific CCK-B receptor antagonist PD-135,158, compound L-365,260 inhibited in a dose-dependent manner [^{14}C]-aminopyrine accumulation induced by CCK-8 + histamine with comparable IC_{50} values. This result indicated that in addition to its effect at the CCK-B/gastrin receptor, the inhibitory effect of L-365,260 was mediated through a site different from the CCK-B/gastrin receptor.

When compounds L-365,260, YM-022, PD-135,158, JMV-180 and L-364,718 were tested for their ability to inhibit carbachol-induced [^{14}C]-aminopyrine accumulation, we found that only L-365,260 was active and inhibited such a stimulation. This inhibition does not result from a direct interaction between L-365,260 and the M_3 receptor (Lotti & Chang, 1989).

The fact that elevation of the intracellular cyclic AMP level induced by histamine or accumulation of IP_S induced by carbachol were not inhibited by L-365,260 indicated that this compound did not act as an adenylate cyclase inhibitor or a phospholipase C inhibitor. The inhibitory action of L-365,260 seemed to be located downstream these two effectors.

L-365,260 inhibited in a dose-dependent manner IBMX- and forskolin- induced [^{14}C]-aminopyrine accumulation with comparable IC_{50} values whereas YM-022, PD-135,158, JMV-180 and L-364,718 were without effect. These results suggested again that the inhibitory action of L-365,260 seemed to be located downstream the second messenger production.

These different results led us to postulate that L-365,260 could act as an inhibitor of H^+/K^+-ATPase activity, the common late step in acid secretion. We decided to compare the dose-response profiles of L-365,260 and the proton pump inhibitor omeprazole on acid secretion in isolated rabbit gastric glands. In accordance with the results published in the literature (Wallmark *et al.*, 1983), we showed that omeprazole inhibited [^{14}C]-aminopyrine accumulation stimulated by CCK-8, histamine, carbachol, IBMX and forskolin with similar IC_{50} values. Omeprazole (like L-365,260) inhibited in a dose-dependent manner basal [^{14}C]-aminopyrine accumulation. Whereas Lotti & Chang (1989) failed to demonstrate that L-365,260 inhibits basal acid secretion *in vivo* in mice, our results are in accordance with some reports (Hirst *et al.*, 1991; Nishida *et al.*, 1992; Pendley *et al.*, 1993) showing that L-365,260 is able to inhibit *in vivo* basal acid secretion in the rat.

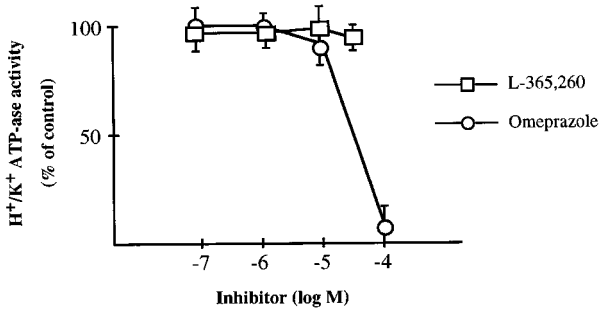

Figure 11 Effect of L-365,260 and omeprazole on H^+,K^+-ATPase activity. Ten μg of gastric vesicles were preincubated 15 min at 37°C with the test compound at indicated concentrations in two assay media containing (in mM) $MgCl_2$ 2, Tris/acetate, pH 7.4 40, KCl 20 and 10 μM valinomycin (buffer 1) and (in mM) $MgCl_2$ 2, Tris/acetate, pH 7.4 40, choline chloride 20 (buffer 2). The enzyme reaction was started by adding 2 mM [γ-^{32}P]-ATP. After incubation at 37°C for 4 min, the reaction was terminated by adding 1 ml of 10% trichloroacetic acid and $^{32}P_i$ released was separated by centrifugation and measured by liquid scintillation counting. Results are the means ± s.d. of four experiments each performed in duplicate.

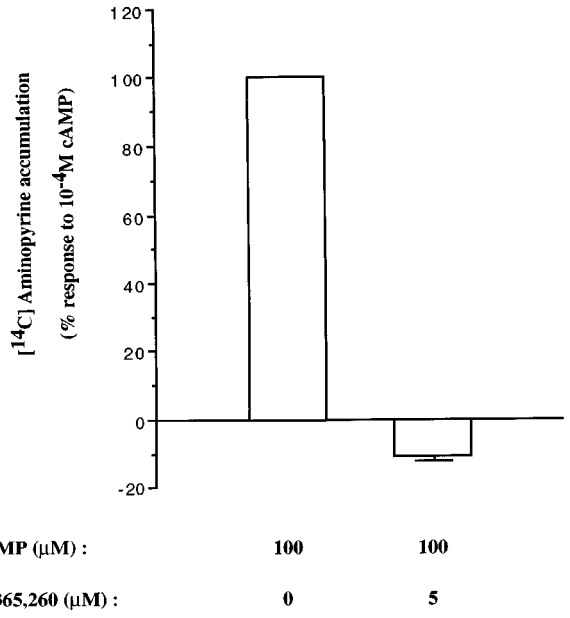

Figure 12 Effect of L-365,260 on [^{14}C]-aminopyrine accumulation induced by 10^{-4} M cyclic AMP in digitonin-permeabilized rabbit gastric glands. After subtraction of the basal [^{14}C]-aminopyrine accumulation, data were expressed as percentage of the response obtained with 10^{-4}M cyclic AMP. Results are the means ± s.d. of three experiments each performed in triplicate.

We finally tested L-365,260 on H^+/K^+-ATPase activity in rabbit gastric vesicles. While omeprazole inhibited H^+/K^+-ATPase activity in a dose-dependent manner, L-365,260 had no effect. It probably acted at targets more distal from the proton pump since 5×10^{-5} M L-365,260 did not affect the proton pump activity.

These results suggested that one site of action of L-365,260 influencing acid secretion was located between the second(s) messenger(s) system and the proton pump. To inhibit intracellular traffic, the most probable molecular target(s) of L-365,260 are likely to be kinase(s). The list of possible candidates is extensive, but among them, we excluded protein kinase C since it creates an inhibitory signal in rabbit parietal cells (Urushidani & Nagao, 1996). According to the study performed by Thibodeau *et al*. (1994) in permeabilized gastric glands, we tested L-365,260 as an inhibitor of the PKA pathway. As described by these authors for the specific PKA inhibitor H89, we showed that L-365,260 was able to inhibit [^{14}C]-aminopyrine accumulation induced by cyclic AMP in digitonin-permeabilized gastric glands. On the basis of the results obtained by Yao *et al*. (1996), this result supports the idea that one L-365,260 site of action for explaining its inhibitory effect on acid secretion resides, at least in part, in the PKA pathway. However, it is possible that other protein kinases may be inhibited by L-365,260. Recently, Urushidani *et al*. (1997) showed that compound ME-3407 was able to dose-dependently inhibit aminopyrine accumulation in rabbit gastric glands stimulated by any agonist, by inhibiting myosin light chain kinase (MLCK, a kinase responsible for the membrane recruitment events associated with parietal cell stimulation) and PKA activities. On the basis of this study, we can suggest that MLCK constitutes a putative target of L-365,260. On the other hand, according to the recent study of Urushidani & Forte (1997), some pharmacological agents such as KN-93 (a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) (Mamiya *et al*., 1993) or U73343 (a negative control of the PLC inhibitor U73122) (Muto *et al*., 1997) have potent antisecretory activities *in vitro* due to their protonophoretic action. So, it cannot be excluded that L-365,260 might alter the measured proton gradient *via* some action as a protonophore.

In summary, L-365,260 produced a dose-dependent inhibition of [^{14}C]-aminopyrine accumulation in isolated rabbit gastric glands stimulated by various agonists. We showed that L-365,260 was able to act beyond receptor activation and production of intracellular second messengers. We found that L-365,260 was not an inhibitor of the final effector of acid secretion, the H^+/K^+-ATPase. We showed that it interfered with the PKA pathway, suggesting that the target of L-365,260 was some protein(s) kinase(s).

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