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Cytosolic Ca^{2+} evaluation in rabbit parietal cells: a novel method to screen gastrin receptor antagonists

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Abstract

We have evaluated the application of the fura-2 method to detect cytosolic Ca^{2+} increase in gastric cells expressing CCK_B/gastrin receptors, in order to screen gastrin receptor antagonists, as an alternative to functional studies. We have characterized the receptors on parietal cell suspension from rabbit gastric mucosa and validated the method using both the CCK_B and CCK_A receptor agonists and antagonists. Human gastrin I (gastrin) (0.1 nM–4 μM) and sulfated cholecystokinin 26–33 (CCK-8) (0.01 nM–2 μM) dose-dependently augmented cytosolic Ca^{2+}. The efficacies of the two agonists were similar, but the potency of CCK-8 (EC_{50} 1.03 nM) was about 10-fold greater than that of gastrin (11 nM). Response to a submaximal dose of gastrin (50 nM) was dose-dependently blocked by the CCK_B-receptor antagonists CAM-1028 (4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1α,2β[*S*′(*S*′)4α]]-butanoate-*N*-methyl-D-glucamine) (IC_{50} 1.9 nM), L-365,260 (3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea) (IC_{50} 10 nM) and spiroglumide ((*R*)-4-(3,5-dichlorobenzamido)-5-(8-azaspiro[4.5]decan-8-yl)-5-oxopentanoic acid) (IC_{50} 2 μM). The results were in agreement with those obtained from binding studies in guinea-pig cortical membranes. The model was employed to optimize the synthesis of a new class of spiroglumide analogues which led to a new molecule, (*S*)-4-{(*R*)-4′-(3,5-dichlorobenzoylamino)-5′-(8-azaspiro[4.5]decan-8-yl)-5′-oxo}-pentanoylamino-5-(1-naphthylamino)-5-oxopentanoic acid (CR 2622), whose potency was about 100-fold greater than that of spiroglumide. CR 2622, as well as the other CCK_B receptor antagonists tested, exhibited no effect on basal $[Ca^{2+}]_i$. The simplicity and the reproducibility of this method suggest that it is a useful model to screen gastrin and antigastrin activity in parallel or as an alternative to binding studies.

Keywords: Ca^{2+}; Cytosolic; Fura-2; Parietal cell, rabbit; Elutriation; CCK_B/gastrin receptor antagonist

1. Introduction

A gastric acid stimulating hormone, 'gastrin', produced by the gastric antral mucosa was isolated and characterized by Gregory and Tracy (1964). Gastrin stimulates gastric acid secretion (Stening and Grossman, 1969) and exhibits trophic effects on target tissues (Nilsson, 1980).

Cholecystokinin (CCK), another gastrointestinal polypeptide hormone, is closely related to gastrin, carrying the same pentapeptide amide sequence on its biologically active C-terminal (Rehfeld, 1981).

In recent years, two CCK receptor subtypes (CCK_A and CCK_B) have been classified on a pharmacological basis using agonist and antagonist selectivity profiles. The CCK_A receptor is present both in peripheral tissues and in discrete brain areas; in the gallbladder, it regulates muscle motility, while in pancreatic acinar cells it stimulates enzyme secretion. Moreover, the CCK_A receptor present in gastric mucosa is coupled with pepsinogen secretion. The CCK_B/gastrin receptor predominates on mucosal cells of the stomach fundus, in gastrointestinal smooth muscle and in the central nervous system.

Several studies, carried out on different animal species, have shown that gastrin exerts a direct stimulatory effect on acid secretion from parietal cells. This functional response in isolated gastric glands, as well as in parietal cells, cannot be directly measured by titration; therefore, intracellular accumulation of weak bases such as [^{14}C]aminopyrine has been used as an indirect probe of H^+ secretion (Berglindh et al., 1976). Aminopyrine is a probe for acidification of the tubulovescicular and canalicular spaces and it must be stressed that accumulation of aminopyrine is an index of the amount of acid sequestered

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within parietal cells rather than of the total amount of acid secreted.

The uptake of [^{14}C]aminopyrine induced by gastrin in parietal cells is very weak (Soll, 1980; Chew and Hersey, 1982). Moreover, different cell preparations strongly varied in both basal and maximal aminopyrine accumulation, as well as in sensitivity to various secretagogues. For these reasons, it seems difficult to use aminopyrine uptake as a model for testing chemicals and therefore, the application of a new method to measure parietal gastrin receptor activation may serve as a useful tool to screen the antigastrin activity of the compounds tested.

Recent studies have demonstrated that gastrin receptor activation is linked to cytosolic Ca^{2+} variation. Indeed, previous reports have shown that specific agonists enhance intracellular Ca^{2+} concentration in gastrointestinal cells expressing cholecystokinin and/or gastrin receptors (Muallem et al., 1986; Tsunoda et al., 1988; Chew and Brown, 1986; Delvalle et al., 1992) as well as in the GH_3 rat anterior pituitary cell line (Takamiya et al., 1991; Saita et al., 1994; Smith et al., 1994).

The aim of this study was to evaluate the application of the fura-2 method, which is able to detect cytosolic Ca^{2+} increase, in order to screen gastrin antagonists as an alternative to previous functional studies, such as aminopyrine uptake.

For this purpose we investigated the antigastrin activity of glutamic acid derivatives, spiroglumide, formerly coded CR 2194 (Makovec et al., 1992; Revel et al., 1992) and its new analogue CR 2622 (Makovec et al., 1996). Their effect was compared to that exhibited by other potent and specific established gastrin antagonists, such as the benzodiazepine derivative L-365,260 (Lotti and Chang, 1989) and CAM-1028, a close analogue of the dipeptoid CI-988 (Hughes et al., 1990).

In addition, we have compared the potency of these compounds on peripheral gastrin receptor activation, with the affinity they displayed on central CCK_B receptor in radioligand binding studies.

2. Materials and methods

2.1. Intracellular Ca^{2+} monitoring

2.1.1. Preparation of isolated parietal cells from rabbit stomach

Cell isolation was carried out following the collagenase/EDTA procedure previously described (Soll, 1978) with minor modifications. Male New Zealand White rabbits weighing 2 kg were killed by intravenous injection of 2 ml of penthotal 50 mg/ml. The stomach was excised, opened along the smaller curvature and rinsed with ice cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) (composition in mM: NaCl 150, K_2HPO_4 3, NaH_2PO_4 0.6, pH 7.4).

The mucosa was scraped off, washed 3–4 times with PBS, and minced with scissors. About 7.5 g of mucosa were placed into 37.5 ml of medium A (composition in mM: NaCl 132, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1, Na_2HPO_4 5, $CaCl_2$ 1, Hepes 25, plus 0.2% glucose and 0.2% bovine serum albumin, 50 μg/ml gentamicin, pH 7.4) containing 0.25 mg/ml collagenase (300–350 U/mg) and 0.3 mg/ml pronase. The incubation was carried out at 37°C for 15 min in medium gassed with 95% O_2/5% CO_2. The supernatant was then discarded and the fragments were rinsed twice in 25 ml Ca^{2+}/Mg^{2+}-free medium A with 2 mM EDTA and incubated in 25 ml of the same medium at room temperature. After 10 min incubation, the supernatant was removed and discarded. The second digestion was done in medium A containing collagenase 0.25 mg/ml at 37°C under continuous gassing (95% O_2/5% CO_2) for 15 min. The third and final digestion was performed under the same conditions for 20 min; the enzyme digestion was supported with mechanical dispersion by repetitive pipetting.

After allowing the undigested fragments to settle, the supernatant was recovered, diluted with 4 vols. of medium A, filtered through a 200 μm nylon mesh, and centrifuged at 200 $\times$ g for 5 min. The pellet was resuspended in 40 ml of Earle's medium (containing in mM: NaCl 116, KCl 5.36, $MgSO_4$ 0.8, NaH_2PO_4 1, $CaCl_2$ 1.8, Hepes 10, plus 0.1% glucose and 0.2% bovine serum albumin, pH 7.4), filtered through a 100 μm nylon mesh, centrifuged, resuspended again in the same medium and filtered through a 62 μm nylon mesh. Cell suspension was centrifuged at 200 $\times$ g for 5 min and the pellet was resuspended in 10 ml Earle's medium. The final cell suspension consisted of approximately 20% parietal cells.

Enrichment in parietal cells was obtained by the counterflow centrifugal elutriation technique (Soll, 1978). The apparatus for continuous-flow elutriation consists of a centrifuge containing a rotor with four separation chambers (Curame 3000, Haereus) and a peristaltic pump fitted with loading equipment (Cole Parmer Masterflex easy-load). The cell suspension (5 ml/chamber) was injected into the elutriator chambers and the cells were separated on the basis of varying sedimentation velocity in counterflow (McEwen et al., 1968). Cells were purified in four fractions (the rotor was set at 2000 rpm, the flow rate at 26, 37 and 41 ml/min for the first three fractions, respectively), the fourth fraction, collected at 1800 rpm and 59 ml/min of counterflow rate, was enriched in parietal cells. Purity was 81 $\pm$ 6% (n = 26), and viability was > 95%, evaluated by exclusion of trypan blue. Normally 60–80 $\times$ 10^6 cells were obtained from about 7.5 g of gastric mucosa.

2.1.2. Ca^{2+} measurement

Cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, was monitored with the fluorescent Ca^{2+} indicator fura-2, as described by Grynkiewicz et al. (1985). Cells were incubated

with fura-2/AM (final concentration 4 μM) for 20 min at 37°C in Earle's medium.

After loading, the cell suspension was diluted with 10 vols. of Earle's medium, centrifuged for 5 min at $200 \times g$, finally resuspended in 10 ml of Hepes-buffered saline (HBS composition in mM: NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, Hepes 10, glucose 10, pH 7.4) at $5-10 \times 10^6$ cells/ml concentration, and kept at room temperature in the dark until use. The suspension (0.8×10^6 cells/1.5 ml) was added to a glass cuvette thermostated at 37°C under continuous stirring. Antagonists were added 1 min prior to agonists; controls received either Ca^{2+}-free HBS or dimethyl sulfoxide (for L-365,260 or devazepide) as vehicle.

Fluorescence recording was performed by a dual excitation fluorimetry in a fluorescence spectrometer (Perkin Elmer LS50B) using the Fast filter software package. Wavelengths were set at 340 nm and 380 nm for excitation, 505 nm for emission; data points were collected at 0.2–0.4 s intervals.

$[Ca^{2+}]_i$ was estimated from the ratios of 340 and 380 nm signals according to the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times \beta \times \frac{R - R_{min}}{R_{max} - R}$$

where K_d is the dissociation constant for fura-2-Ca^{2+} complex (224 nM), $\beta = F(380)_{min}/F(380)_{max}$, R is the ratio of fluorescence at 340 over 380 nm; R_{max} and R_{min} (fluorescence ratios at saturating and zero Ca^{2+} concentration respectively) were obtained in separate experiments by adding digitonin at 50 μM followed by 2 mM EGTA, and Tris and adjusted to pH 8.3.

Data were expressed as $[Ca^{2+}]_i$, or $\Delta\%[Ca^{2+}]_i$ i.e. the value obtained by subtracting the basal pre-agonist value from the peak post-agonist value.

2.2. Radioligand binding studies

Binding assays to central CCK_B receptor were carried out on membranes of guinea-pig cerebral cortex as previously described (Knapp et al., 1990). The tissue (about 1.5 g) was homogenized in 10 mM ice-cold Hepes (pH 7.4) 1 : 10 (w/v) and centrifuged at 4°C for 15 min at $48\,000 \times g$. The pellet was suspended in 20 vols. of tissue buffer (10 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 5.0 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4) and centrifuged as above. The final membrane pellet was suspended in tissue buffer to obtain the desired protein concentration.

Competition experiments were performed using an incubation volume of 1 ml containing tissue buffer supplemented with 1 mg/ml bovine serum albumin, 0.1 mg/ml bacitracin and 50 μM bestatin, membrane suspension (0.3–0.4 mg protein), 0.5 nM [^{3}H][*N*-methyl-Nle^{28,31}]-CCK-8 in the presence of different concentrations of test compounds. The nonspecific binding, representing 35–40% of total binding, was determined in the presence of 1 μM CCK-8. Incubation (150 min at 25°C) was terminated by rapid filtration through Whatman GF/B filters, pre-treated with buffer assay. Filter discs were rinsed with 4 ml of ice cold 0.9% NaCl, and counted with 8 ml of Ultima Gold MV scintillation cocktail in a B1900 Tricarb (Canberra Packard) β liquid scintillator, with 56% efficiency.

Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

2.3. Data analysis

In $[Ca^{2+}]_i$ response experiments, concentration-response curves were analysed by means of a computerized curve fitting technique (ALLFIT) using the four-parameter logistic equation to obtain EC_{50} or IC_{50} values (De Lean et al., 1978). The EC_{50} was defined as the agonist concentration producing a half-maximal response. The IC_{50} was defined as the antagonist concentration producing a 50% of inhibition of gastrin effect. The statistical significance of differences in upper plateaux between control group and CR 2622 groups was determined by performing one-way analysis of variance (ANOVA) and Duncan's multiple range test. The accepted level of significance was $P < 0.05$. Data are expressed as the means ± standard deviation (S.D.) of the given numbers (n) of cell preparations, each obtained from a different rabbit.

Inhibition binding constants were determined by a non-linear curve fitting program (EBDA version 4, for IBM PC; McPherson, 1985). Data are expressed as mean with 95% confidence limits of the number (n) of separate experiments.

2.4. Materials

Sulfated cholecystokinin 26–33 (CCK-8) and human gastrin I (gastrin) were obtained from Novabiochem (Laufelfingen, Switzerland). Stock solutions, 1 mg/ml CCK-8 in water and 1 mg/ml gastrin in 50 mM $NaHCO_3$, were stored at −20°C. L-365,260 (3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-(3-methylphenyl)urea) was obtained from Merck Sharp & Dohme Research Laboratories (Harlow, Essex, UK). CAM-1028 (4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1α,2β-[*S'*(*S'*)4α]]-butanoate-*N*-methyl-D-glucamine) was a gift of Dr. Hughes (Parke-Davis Neuroscience Research Center, Cambridge, UK). Devazepide ((*S*)-1*H*-indole-2-carboxamide-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)), spiroglumide ((*R*)-4-(3,5-dichlorobenzamido)-5-(8-azaspiro[4.5]decan-8-yl)-5-oxopentanoic acid) and CR 2622 ((*S*)-4-{(*R*)-4'-(3,5-dichlorobenzoylamino)-5'-(8-azaspiro[4.5]decan-8-yl)-5'-oxo}-pentanoyl-

amino-5-(1-naphthylamino)-5-oxopentanoic acid) were synthesized in our laboratories.

[*N*-methyl-Nle^{28,31}]CCK-8 was a gift of Dr. Hruby (University of Arizona, Tucson, USA). [^{3}H][*N*-methyl-Nle^{28,31}]CCK-8 (55 Ci/mmol) was from NEN (Boston, MA, USA). Hepes (acid form), digitonin, EGTA, Tris, collagenase type 1A and trypan blue were purchased from Sigma (St. Louis, MO, USA). Gentamicin sulfate was from BioWhittaker (Walkersville, MD, USA). Bovine serum albumin fraction V, bestatin and pronase were from Boehringer (Mannheim, Germany). Bacitracin was from Aldrich (Steinheim, Germany). Fura-2/AM was from Calbiochem (La Jolla, CA, USA). Ultima Gold MV scintillation cocktail was from Canberra Packard (Meriden, CT, USA).

3. Results

3.1. Characterization of CCK_B/gastrin receptors in a preparation enriched in parietal cells: functional response

3.1.1. Studies with agonists

Gastrin (0.1 nM–4 μM) and CCK-8 (0.01 nM–2 μM) dose dependently augmented cytosolic Ca^{2+}. $[Ca^{2+}]_i$ at basal conditions was 179 ± 49 nM (n = 29).

The normal onset of gastrin response was slower and its action longer lasting if compared to CCK-8 response. A maximally effective concentration of gastrin (4 μM) produced an average 1.7-fold increase over basal $[Ca^{2+}]_i$ and maximal activation induced by CCK-8 was achieved at about 200 nM with an average 2-fold increase. Thus the efficacies of the two agonists were similar, but the potency of CCK-8 (EC_{50} = 1.03 ± 0.3 nM, n = 4) was 10-fold greater than that of gastrin (EC_{50} = 11 ± 4.8 nM, n = 11).

Among the gastrin series peptides, CCK-4 (tetragastrin) is the smallest active peptide (Steigerwalt and Williams, 1984). CCK-4 (1 nM–10 μM) was able to induce Ca^{2+} elevation related to gastrin receptor interaction. CCK-4 was shown to be a weak gastrin receptor activator, in agreement with published data (EC_{50} = 200 ± 47 nM, n = 2).

As cell preparation (see Section 2) was always contaminated with a variable percentage of chief cells bearing CCK_A receptor, we tested the effect of CCK-8 added after gastrin stimulation. CCK-8 at 40 nM was able to induce an extra fluorescence transient, positively correlated to the percentage of chief cells. Fig. 1 shows the effect of the stimulation evoked by 50 nM gastrin followed by 40 nM CCK-8 in two different cell preparations; in panel A the enrichment in parietal cells was high (87%), while it was lower (64%) in panel B. Repeated doses of 50 nM gastrin did not induce a further fluorescence increase (data not shown).

To verify the responsiveness of our model, we tested the effect of carbachol and histamine, two agonists of

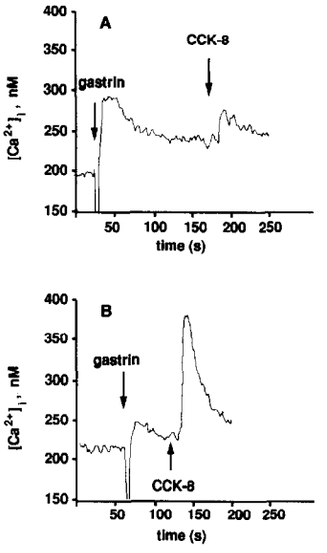

Fig. 1. Cell responsiveness to human gastrin I and CCK-8 in two separate sets of experiments. A shows the effect of 50 nM human gastrin I and 40 nM CCK-8 in a cell suspension containing 87% of rabbit parietal cells. B shows the effect induced by agonists in a cell suspension containing 64% of parietal cells. Data are expressed as nM of $[Ca^{2+}]_i$.

gastric acid secretion interacting with their own specific receptors. Carbachol at 60 μM induced a rapid and marked Ca^{2+} rise; basal $[Ca^{2+}]_i$ value was increased by 200–300%; in contrast, 100 μM histamine elicited no detectable response, in agreement with findings reported by Cabero et al. (1992) (data not shown).

3.1.2. Studies with antagonists

Antagonism studies were performed in a suspension enriched in parietal cells in which gastrin evoked at least 50% increase in $[Ca^{2+}]_i$ over resting levels. Among physiological CCK_B receptor agonists, we chose human gastrin because of its potency, clear effect and selectivity versus CCK_B receptor, thus overcoming the troubles linked to the presence of variable percentage of chief cells. Gastrin concentration was 50 nM (unless otherwise stated). Gastrin-induced Ca^{2+} elevation was variable among the experiments (ranging from 40 to 140% increase). To normalize and compare the data, we expressed the effect of antagonists as percentage of Ca^{2+} increase, taking the increase induced by 50 nM gastrin alone as 100%. In experiments carried out to evaluate the anti-CCK_A activity, 40 nM CCK-8 was added after gastrin stimulation.

As CAM-1028 was reported to be a potent and selective CCK_B receptor antagonist (Hughes et al., 1990), the experiment illustrated in Fig. 2 was designed to determine whether CAM-1028 antagonizes only Ca^{2+} increase induced by gastrin (CCK_B receptor activation) or is able to antagonize also the extra Ca^{2+} increase elicited by CCK-8 added after gastrin stimulation (CCK_A receptor activation) (Fig. 2A). CAM-1028, 30 nM, completely inhibited the effect of 50 nM gastrin, but left unchanged the subsequent

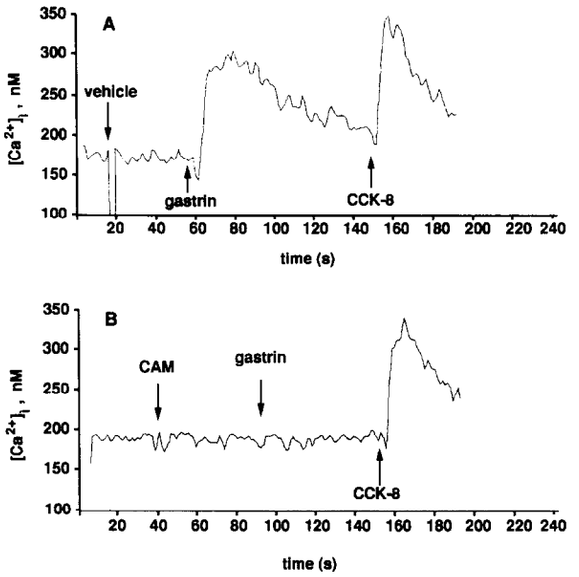

Fig. 2. Representative tracings of $[Ca^{2+}]_i$ elevation induced by 50 nM human gastrin I and 40 nM CCK-8 in control parietal cell suspension (A), or in parietal cells pre-exposed to 30 nM of CAM-1028 (B).

response elicited by 40 nM CCK-8 (Fig. 2B). The inhibitory effect of CAM-1028 was concentration-dependent with an IC_{50} value of 1.9 ± 0.6 nM (Fig. 3 and Table 1).

To assess whether antagonists with different chemical structures were effective, we tested the benzodiazepine derivatives L-365,260 and devazepide, and a glutamic acid derivative spiroglumide.

L-365,260 antagonized the gastrin response in a dose dependent manner with an IC_{50} value of 10 nM. Devazepide, a potent CCK_A receptor antagonist, also displayed some antagonism, but only higher doses blocked

Table 1
Effect of CCK receptor antagonists on gastrin-induced $[Ca^{2+}]_i$ elevation

	IC_{50} (nM ± S.D.)	n
CAM-1028	1.9 ± 0.6	3
L-365,260	10.4 ± 0.9	2
Devazepide	900 ± 570	2
Spiroglumide	2000 ± 1300	6
CR 2622	7.4 ± 4.3	9

Data were obtained using parietal-enriched cell suspension, loaded with 4 μM fura-2/AM. Cells were stimulated with 50 nM human gastrin I. Antagonists were added about 1 min prior to agonist. Data were expressed as $\Delta\%[Ca^{2+}]_i$. The values presented (mean ± S.D.) were obtained by non-linear regression analysis (ALLFIT program); n = number of separate experiments.

the gastrin-induced Ca^{2+} increase ($IC_{50} = \sim 1$ μM) (Fig. 3 and Table 1). When the experiment included both the agonists gastrin and CCK-8, devazepide at 30 nM was ineffective on gastrin stimulation, but strongly antagonized the subsequent CCK-8-induced Ca^{2+} increase (40% of inhibition) (data not shown). The resulting relative potency (1 : 30) between CCK_B receptor antagonist (L-365,260) and CCK_A receptor antagonist (devazepide) on inhibiting gastrin-induced $[Ca^{2+}]_i$ increase was in agreement with previously reported data (Roche et al., 1991). Spiroglumide, a compound already known to antagonize gastrin receptor interaction and its functional effect (Revel et al., 1992), was able to inhibit the Ca^{2+} rise induced by 50 nM gastrin; computer assisted curve analysis revealed an IC_{50} value of 2 ± 1.3 μM (Fig. 3 and Table 1).

In order to verify the selectivity of this experimental model with respect to gastrin antagonists, we evaluated the effect of antimuscarinic or antihistaminic drugs. Atropine, 30 nM, strongly inhibited the effect induced by 60 μM carbachol (70–80% of inhibition) but was practically inef-

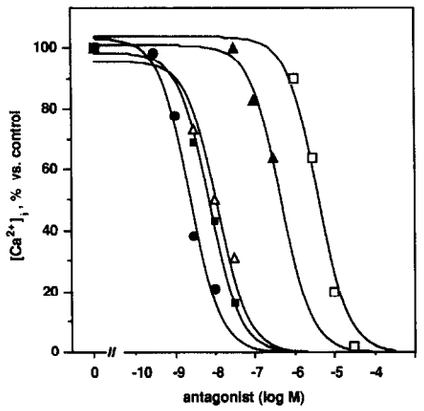

Fig. 3. Concentration-response curves of CCK antagonists on $[Ca^{2+}]_i$ increased by 50 nM human gastrin I in rabbit parietal cells. Curves were obtained by non-linear regression analysis (ALLFIT program). Data are percentage of response versus human gastrin I effect, taken as 100%. The figures are representative of 2–9 experiments with different cell preparations; in each experiment the values were determined in duplicate; CAM-1028 (●), CR 2622 (■), L-365,260 (△), devazepide (▲), spiroglumide (□).

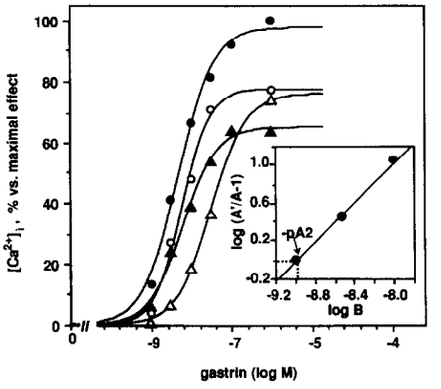

Fig. 4. Antagonism displayed by CR 2622 on $[Ca^{2+}]_i$ increased by human gastrin I in rabbit parietal cells. Curves, representative of two experiments, were obtained by non-linear regression analysis (ALLFIT program). Gastrin alone (●), +CR 2622 (○, 1 nM), (▲, 3 nM) and (△, 10 nM). Inset illustrates Schild plot, where A'/A is the ratio between the gastrin concentration with or without CR 2622, provoking the same effect; B is the concentration of CR 2622, and $-pA_2$ is the log of the concentration of B that produces a dose ratio (A'/A) of 2.

fective on Ca^{2+} elevation elicited by 2 μM gastrin. Cimetidine, a selective anti-H_2 drug, even at 100 μM induced no change on gastrin stimulation (data not shown).

The model, after being characterized, was exploited to optimize the synthesis of a new class of spiroglumide analogues. The information emerged from the screening led to a new molecule, coded CR 2622 (Makovec et al., 1996), whose potency was almost 300-fold greater than spiroglumide; curve analysis revealed an IC_{50} value of 7.4 ± 4.3 nM (Fig. 3 and Table 1). Gastrin dose-response curves were also performed to verify the nature of functional antagonism of CR 2622 (Fig. 4). CR 2622, in the range of 1–10 nM, caused a rightward parallel shift of the gastrin curve. Nevertheless, the upper plateau was not achieved even in the presence of 1 nM CR 2622, a concentration that only slightly blocked gastrin stimulation. Schild plot analysis (Fig. 4, inset) revealed a pA_2 value of 8.98. The calculated slope was close to unity (1.04 ± 0.02), consistent with competitive antagonism, although the maximal responses to gastrin stimulation were depressed. Duncan test analysis applied to the upper plateaux indicated a significant difference between control group and CR 2622 groups (one-way ANOVA $(3,11) = 20.17$, $P = 0.0004$), hence incompatible with the hypothesis of competitive antagonism.

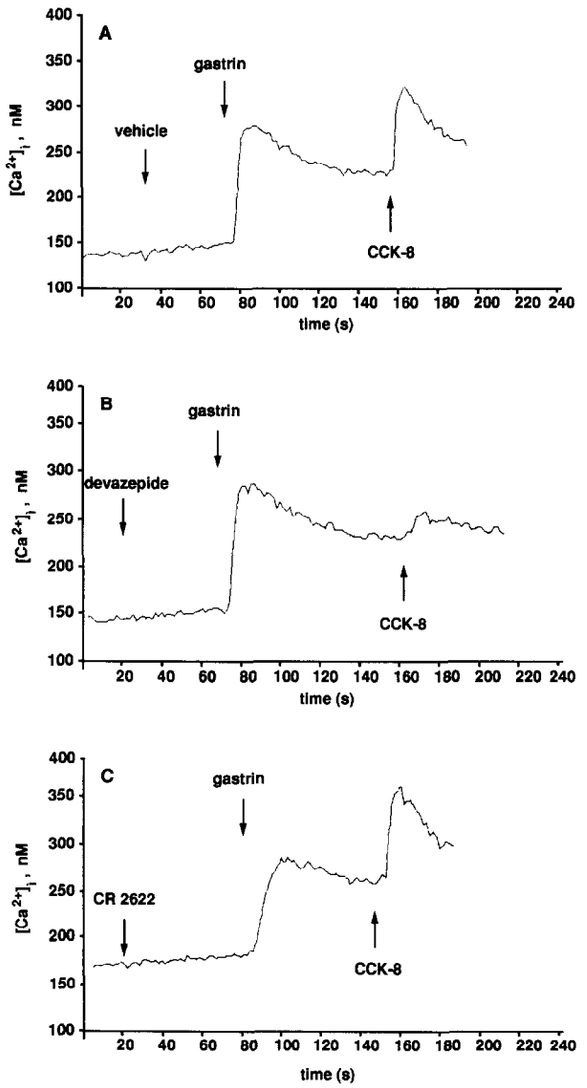

Fig. 5. The effect of CCK_A receptor antagonist in rabbit parietal cells. A: Ca^{2+} elevation induced by 50 nM human gastrin I and 40 nM CCK-8 in control cell suspension. B: Pre-exposure to devazepide (100 nM) resulted in an abatement of Ca^{2+} elevation elicited by CCK-8 without affecting human gastrin I stimulation. C: Pre-exposure to CR 2622 (3 nM) diminished the gastrin-induced Ca^{2+} elevation only.

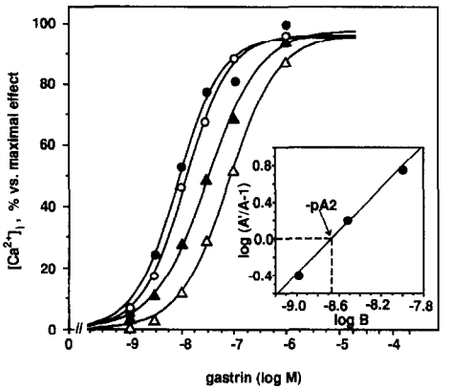

Fig. 6. Antagonism displayed by CR 2622 on cytosolic Ca^{2+} increased by human gastrin I in rabbit parietal cells pre-exposed to 100 nM devazepide. Curves, representative of two experiments, were obtained by non-linear regression analysis (ALLFIT program). Gastrin alone (●), +CR 2622 (○, 1 nM), (▲, 3 nM) and (△, 10 nM). Inset illustrates Schild plot, where A'/A is the ratio between the gastrin concentration with or without CR 2622, provoking the same effect; B is the concentration of CR 2622, and $-pA_2$ is the log of the concentration of B that produces a dose ratio (A'/A) of 2.

Moreover, results arising from binding studies were consistent with a competitive antagonism (data not shown). Therefore, we postulated that the presence of heterogeneous receptor populations in our cell preparation, due mainly to chief cells bearing CCK_A receptor, could be responsible for these discrepancies. To verify this hypothesis, we first checked the selective inhibition displayed by devazepide towards CCK-8-induced Ca^{2+} increase. Devazepide at 100 nM was ineffective versus gastrin-induced Ca^{2+} increase, but was able to inhibit the effect elicited by CCK-8 added after gastrin (Fig. 5A and B), whereas 3 nM CR 2622 (Fig. 5C) reduced the gastrin-induced Ca^{2+} increase and left the CCK-8-induced Ca^{2+} increase unchanged. When we repeated the concentration-response study with CR 2622 in the presence of 100 nM devazepide (Fig. 6), CR 2622 (1, 3 or 10 nM) shifted rightward the inhibition curves of gastrin to an extent similar to that obtained previously in the absence of devazepide. Schild plot analysis (Fig. 6, inset) indicated a pA_2 value of 8.66 and the slope was close to unity (1.17 ± 0.08). In this case, the inhibition by CR 2622 was overcome by gastrin; the

Table 2
Affinity of cholecystokinin receptor antagonists and reference agonists on specific binding of [^{3}H][*N*-methyl-Nle^{28,31}]CCK-8 to guinea-pig cortical membranes

Ligand	K_i (nM)
CCK-8	0.50 (0.2–0.8)
Human gastrin I	12.9 (9.2–16.6)
Spiroglumide	1 500 (1000–1900)
CR 2622	10.4 (6.5–14.3)
L-365,260	6.3 (2.4–10.2)
CAM-1028	3.7 (3.3–4.1)

Data reported are the mean and 95% confidence limits of three separate experiments, each performed in triplicate.

difference among the four plateaux was not significant (Duncan test significance level 0.05).

All gastrin/CCK receptor antagonists evaluated during the study were devoid of any effect on basal cytosolic Ca^{2+} levels.

3.2. Binding studies

Spiroglumide and CR 2622 exhibited a stereoselective and concentration dependent inhibition of [^{3}H][*N*-methyl-Nle^{28,31}]CCK-8 binding to gastrin/CCK_B receptors in guinea-pig cortical membranes (Makovec et al., 1996). The K_i for spiroglumide and CR 2622 was estimated to be 1.5 μM and 10.4 nM, respectively. The Hill coefficient of two compounds was not significantly different from unity ($P > 0.05$). The affinity of reference agents, agonists and CCK_B receptor antagonists, CAM-1028 and L-365,260, reflected the published data (Hughes et al., 1990; Knapp et al., 1990) (Table 2).

4. Discussion

In the present study we have described the application of a method to assay $[Ca^{2+}]_i$ variations by fura-2 to screen compounds with affinity for CCK_B/gastrin receptor using a suspension enriched in parietal cells from rabbit gastric mucosa. The method was validated by using both the CCK_B and CCK_A receptor agonists and antagonists. Human gastrin, CCK-8 and CCK-4, were all able to induce a dose-dependent Ca^{2+} increase in enriched parietal cells suspension. The relative potencies, CCK-8 > gastrin > CCK-4 were consistent with those described in the literature (Chang et al., 1989).

The main limitation of this gastric cell model is the variability in parietal cell enrichment from preparation to preparation, which is responsible for the irregular $[Ca^{2+}]_i$ increase induced by gastrin in terms of fold-increase over basal. For this reason, we decided to normalize the effect induced by stimuli in order to compare the results (see Section 3.1.2).

Another disadvantage is the presence of different cell types (parietal cells, mucosal cells and chief cells), because each of them, carrying a different receptor subpopulation, could contribute to Ca^{2+} increase, leading to erroneous results. The use of CCK/gastrin antagonists, different in chemical structure and subtype receptor affinity, helped to clarify the pattern of CCK/gastrin receptor activation. On the one hand, antagonists such as CAM-1028 and L-365,260 exhibited antigastrin activity, in agreement with previously published data, thus confirming that this experimental model was correctly responsive to gastrin receptor antagonists. On the other hand, the use of devazepide indicated that the stimulation elicited by gastrin and CCK-8 in sequence could reasonably be ascribed to the activation of two receptors (CCK_B and CCK_A respectively), which could be controlled separately.

The results obtained suggest that the study of intracellular Ca^{2+} mobilization with the fura-2 method may be a very useful model to screen gastrin antagonists: the potency ratios we obtained with CR 2622 and its analogues in this method are similar to those obtained in binding inhibition studies in guinea-pig cortical membranes (Makovec et al., 1996). Moreover, our results provide evidence that the Ca^{2+} signal evoked by gastrin on CCK_A receptor interfered with gastrin-receptor antagonism displayed by CR 2622 and that it is possible to overcome this interference using a potent and specific CCK_A receptor antagonist such as devazepide.

The gastrin receptor activation described here is not affected by anti-histaminergic or anti-cholinergic compounds, as occurs, for example, when the stomach perfused model according to Lai (1964) is used.

In our experience, the $[Ca^{2+}]_i$ assay also seems to devoid of problems arising from the aminopyrine accumulation test. Although [^{14}C]aminopyrine accumulation is a well-characterized method (Soll, 1980), few data, if any, are available on the use of this experimental approach to screen antigastrin activity. Preliminary experiments performed with histamine or carbachol, as acid secretion stimulants, gave good results. The data were reproducible and in agreement with those of other authors. In contrast, we obtained unsatisfactory results when stimulation by gastrin was performed. Indeed, gastrin-induced acid secretion monitored with aminopyrine-uptake was very weak when it occurred, despite the responsiveness of the cell preparation to gastrin, as verified with parallel Ca^{2+} measurement.

Gastrin increases cytosolic Ca^{2+} in parietal cells with a dose dependency similar to that obtained by this secretagogue on parietal cell function and on membrane inositol phospholipid turnover (Chiba et al., 1988; Roche et al., 1991). This observation suggests that intracellular Ca^{2+} mobilization is a functional consequence of CCK_B receptor activation, even though the exact correlation between this effect and acid secretion has not been established. However, the main purpose of this study was to use the variation of $[Ca^{2+}]_i$ concentration as a sensitive indicator

of receptor-ligand interaction and not to correlate the $[Ca^{2+}]_i$ elevation with acid secretion induced by gastrin. Moreover, this model is able to discriminate between receptor-agonists and receptor-antagonists, which does not occur in radioligand binding studies.

Results emerging from our studies on central CCK_B and peripheral gastrin receptors did not point out a difference between the two receptors. At present, the difference between central and peripheral CCK_B/gastrin receptor could not be definitely ruled out, because of the lack of functional studies performed on central CCK_B receptor. The only available data were from experiments carried out on immortalized line cells, such as GH_3 cells (Takamiya et al., 1991; Saita et al., 1994; Smith et al., 1994), or on a model that consists of a non-physiological CCK_B receptor-bearing cells (Kopin et al., 1992). Nevertheless, receptor cloning does not provide, by itself, a definite proof of the identity of CCK_B receptors, as the difference could be expressed at the functional level.

In conclusion, we have characterized a useful model for screening gastrin and antigastrin activity, in parallel or alternative to binding studies. In our experience, this model represents the only reproducible way to evaluate in vitro CCK_B receptor function, thus overcoming serious problems we had with [^{14}C]aminopyrine uptake. In addition, this experimental approach is simple, inexpensive and avoids using radiolabelled markers.

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