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Gastrin and the neuropeptide PACAP evoke secretion from rat stomach histamine-containing (ECL) cells by stimulating influx of Ca^{2+} through different Ca^{2+} channels

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1. Gastrin and PACAP stimulate secretion of histamine and pancreastatin from isolated rat stomach ECL cells. We have examined whether or not secretion depends on the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the pathways by which gastrin and PACAP elevate $[Ca^{2+}]_i$. Secretion was monitored by radioimmunoassay of pancreastatin and changes in $[Ca^{2+}]_i$ by video imaging. The patch clamp technique was used to record whole-cell currents and membrane capacitance (reflecting exocytosis).

2. In the presence of 2 mM extracellular Ca^{2+}, gastrin and PACAP induced secretion and raised $[Ca^{2+}]_i$. Without extracellular Ca^{2+} (or in the presence of La^{3+}) no secretion occurred. The extracellular Ca^{2+} concentration required to stimulate secretion was 10 times higher for gastrin than for PACAP. Depletion of intracellular Ca^{2+} pools by thapsigargin had no effect on the capacity of gastrin and PACAP to stimulate secretion.

3. Gastrin-evoked secretion was inhibited 60–80 % by L-type channel blockers and 40 % by the N-type channel blocker ω-conotoxin GVIA. Combining L-type and N-type channel blockers did not result in greater inhibition than L-type channel blockers alone. Whole-cell patch clamp measurements confirmed that the ECL cells are equipped with voltage-dependent inward Ca^{2+} currents. A 500 ms depolarising pulse from −60 mV to +10 mV which maximally opened these channels resulted in an increase in membrane capacitance of 100 fF reflecting exocytosis of secretory vesicles.

4. PACAP-evoked secretion was reduced 40 % by L-type channel blockers but was not influenced by inhibition of N-type channels. SKF 96365, a blocker of both L-type and receptor-operated Ca^{2+} channels, inhibited PACAP-evoked secretion by 85 %. Combining L-type channel blockade with SKF 96365 abolished PACAP-evoked secretion.

5. The results indicate that gastrin- and PACAP-evoked secretion depends on Ca^{2+} entry and not on mobilisation of intracellular Ca^{2+}. While gastrin stimulates secretion via voltage-dependent L-type and N-type Ca^{2+} channels, PACAP acts via L-type and receptor-operated Ca^{2+} channels.

ECL cells are histamine-containing endocrine/paracrine cells in the oxyntic mucosa of the stomach. They play a key role in the control of acid secretion because of their ability to secrete histamine, which stimulates adjacent acid-producing cells (for reviews see Håkanson *et al.* 1994, 1998). Like most peptide hormone-producing cells, ECL cells contain chromogranin A(CGA) and/or CGA-derived peptides. One of these peptides is pancreastatin, which is co-secreted with histamine (Chen *et al.* 1994, 1996; Lindström *et al.* 1997). The two products are stored together in the same secretory organelles (Zhao *et al.* 1999). Unlike histamine, which is synthesised in the cytosol prior to accumulation in secretory organelles, pancreastatin is located in secretory organelles only (Zhao *et al.* 1999). Thus, pancreastatin may be considered a better marker for ECL cell exocytosis than histamine.

From studies of isolated ECL cells, it has become clear that secretion of histamine and pancreastatin can be stimulated by the peptide hormone gastrin (Prinz *et al.* 1993; Lindström *et al.* 1997). The neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) and its chemical relatives vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) likewise are powerful activators of ECL cell secretion *in vitro* (Lindström *et al.* 1997; Zeng *et al.* 1998, 1999*a*; Lindström & Håkanson, 2001).

Gastrin stimulates secretion from the ECL cells by binding to cholecystokinin (CCK_2) receptors (Sandvik &

Waldum, 1991; Prinz *et al.* 1993; Lindström *et al.* 1997, 1999). Binding of gastrin to its receptor causes phosphatidylinositol bisphosphate breakdown by phospholipase C (Seva *et al.* 1994; Kinoshita *et al.* 1998). This leads to the production of inositol trisphosphate (IP_3), which can be expected to mobilise Ca^{2+} from intracellular stores. Indeed, it has been demonstrated that gastrin elicits IP_3 formation (Kinoshita *et al.* 1998) and induces an initial transient peak followed by sustained elevation in free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in ECL cells (Prinz *et al.* 1993, 1994). The transient peak was thought to reflect mobilisation of Ca^{2+} from intracellular stores while the subsequent plateau phase depended on the influx of extracellular Ca^{2+}. PACAP was likewise found to induce biphasic $[Ca^{2+}]_i$ responses in isolated ECL cells (Zeng *et al.* 1999*a*). Although Ca^{2+}-induced histamine secretion has been demonstrated in permeabilised ECL cells (Höhne-Zell *et al.* 1997) and the Ca^{2+} ionophore A23187 has been shown to stimulate histamine secretion from isolated ECL cells (Sakai *et al.* 1995), it is not yet clear to which extent different sources of Ca^{2+} contribute to exocytosis. Ca^{2+} channels are of three major types: voltage-operated, receptor-operated and store-operated channels (or capacitative; for a review see Berridge, 1997). Electrophysiological characterisation of ECL cells has suggested the involvement of voltage-operated Ca^{2+} channels of both L-type and N-type (Bufler *et al.* 1998; reviewed by Prinz *et al.* 1999).

The aim of the work is to evaluate the Ca^{2+} dependence of exocytosis in ECL cells and to explore the differential regulation of secretion by gastrin and PACAP.

METHODS

Chemicals

Rat gastrin-17 was obtained from Research Plus (Bayonne, NJ, USA). Rat PACAP-27 was from Peninsula Europe (St Helens, Merseyside, UK). Thapsigargin and all the voltage-operated Ca^{2+} channel blockers were supplied by Almone Labs (Jerusalem, Israel). The receptor- and voltage-operated Ca^{2+} channel blocker SKF 96365, the L-type channel activator Bay K 8644 and the neurotoxic Na^+ channel inhibitor tetrodotoxin (TTX) were purchased from Calbiochem (La Jolla, CA, USA). Fura-2 AM was purchased from Molecular Probes (Eugene, OR, USA). Matrigel was from Collaborative Biomedical Products (Bedford, MA, USA). Cadmium-, barium- and lanthanum chloride, fetal calf serum and DMEM–Ham's F12 medium were from Sigma (St Louis, MO, USA). Bovine serum albumin (BSA, fraction V) was from ICN (Aurora, OH, USA). Pronase was from Boehringer Mannheim (Mannheim, Germany). Iodixanol (Optiprep) was from Nycomed Pharma (Oslo, Norway).

Isolation of ECL cells

The stomachs from four male Sprague-Dawley rats (300–400 g) were used for each cell preparation. The rats were killed by a blow to the head followed by decapitation. All experiments were carried out according to the guidelines of our institution's animal welfare committee. Anaesthesia was avoided due to its suppressive effects on ECL cell function (Norlén *et al.* 2000). ECL cells were purified as described earlier (Lindström *et al.* 1997) with a few modifications. Briefly, oxyntic mucosal cells were dispersed using pronase digestion (0.9 mg ml^{-1}) and Ca^{2+} chelation (1 mM EDTA). The ECL cells were enriched by repeated counterflow elutriation using first a standard chamber and then a Sanderson chamber (Beckman, Palo Alto, CA, USA). The enriched cells from the standard chamber were collected at 25 ml min^{-1} and at a speed of 2000 r.p.m. (380–560 *g*). They were purified further in a Sanderson chamber and collected at 18 ml min^{-1} and 2000 r.p.m. This cell preparation consisted of approximately 2 million cells, about 80% being ECL cells (verified by histamine immunocytochemistry, see below).

The ECL cell preparation was then subjected to density gradient centrifugation. A stock solution of 60% iodixanol was enriched with Hepes (final concentration 15 mM, pH 7.4) and 10 mg ml^{-1} BSA. This solution was diluted to 10.8% iodixanol with a medium (medium C) consisting of (mM): 140 NaCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 15 Hepes at pH 7.4, 11 glucose, 0.5 dithiothreitol and 10 mg ml^{-1} BSA. In a 15 ml centrifuge tube, 10 ml of the 10.8% iodixanol solution was overlaid with the enriched ECL cells (2×10^6 in 1 ml medium C) and centrifuged (Spinchron R centrifuge, Beckman). A slow acceleration period (400 r.p.m.) was followed by 5 min centrifugation at 1000 r.p.m. Deacceleration lasted 5 min. The cells in the light density fraction (above the 10.8% cushion) were collected. This fraction consisted of 90–95% ECL cells determined by immunocytochemistry using an anti-histamine antiserum (1:1000) (Håkanson *et al.* 1986) with 4% 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide as fixative (Panula *et al.* 1988).

Primary cell culture

For secretion experiments, purified ECL cells were cultured in 96-well plates pre-coated with Matrigel (diluted 1:10 in DMEM–Ham's F12 medium, 10 000 cells per well, 100 μl vol). The cells were cultured on cover slips (diameter: 25 mm) or in Petri dishes (diameter: 40 mm) pre-coated with diluted Matrigel for video-image analysis experiments or for patch-clamp studies, respectively. All cell cultures were incubated in a humid atmosphere with 5% CO_2–95% air at 37 °C for 24–48 h until the start of the experiments. The culture medium consisted of DMEM–Ham's F12 medium (1:1) supplemented with 2% fetal calf serum, 2 mM glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 250 ng ml^{-1} amphotericin B, 10 μg ml^{-1} insulin, 5.5 μg ml^{-1} transferrin, 5 ng ml^{-1} selenious acid, 0.5 μg ml^{-1} BSA, 15 mM Hepes, 10 μM pyridoxal-5-phosphate, 10 nM hydrocortisone and 100 pM gastrin-17.

Secretion experiments

After 48 h, the cells were washed with serum-free and gastrin-free culture medium. After equilibration for about 2 h, the medium was again aspirated and replaced with secretion medium consisting of (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$ (unless otherwise stated), 10 Hepes at pH 7.0, containing the various stimulatory or inhibitory compounds. Maximally effective concentrations (10 nM) of gastrin and PACAP were used to challenge the cells (Lindström *et al.* 1997). To analyse the effects of Ca^{2+} channel blockade, the cells were pretreated with the respective blocker for 30 min, while control cells were pretreated with secretion medium only. Voltage-operated Ca^{2+} channels can be classified based on their pharmacological and/or electrophysiological properties (for recent reviews see Jones, 1998; Davila, 1999). High voltage-activated (HVA) Ca^{2+} channels are of the L, N, P, Q or R type. L-type Ca^{2+} channels mediate dihydropyridine (e.g. nifedipine)-sensitive currents, while N-type Ca^{2+} channels are blocked irreversibly by ω-conotoxin GVIA. P-type Ca^{2+} channels are resistant to dihydropyridine and ω-conotoxin GVIA but sensitive to ω-agatoxin IVA and funnel spider venom toxin. This is also the case for Q-type Ca^{2+} channels, but they can also be effectively blocked by ω-agatoxin MVIIC. R-type currents are HVA Ca^{2+} currents resistant to the all-above mentioned blockers. T-type Ca^{2+} channels on the other hand mediate a low-threshold Ca^{2+} current with fast inactivation. The Ca^{2+} channel blockers tested and the concentrations used are listed in Table 2. The concentrations were chosen from the literature references

in the table. To explore the consequences of depletion of intracellular Ca^{2+}, the cells were pretreated for 4 h with various concentrations of thapsigargin, which is an irreversible inhibitor of the Ca^{2+} pumps operating in the sarcoplasmic and endoplasmic reticulum (for a review see Treiman *et al.* 1998). Secretion studies lasted for 30 min at 37 °C and were interrupted by centrifuging the plates at 220 *g* for 1 min. The supernatants were collected and stored at −20 °C until measurement of pancreastatin.

Video image analysis of free cytosolic Ca^{2+}

After 24–48 h, ECL cells cultured on Matrigel-coated glass cover slips were loaded with 0.5 μM of the Ca^{2+} fluorophore fura-2 AM for 30 min at 37 °C in 2 ml of medium consisting of (mM): 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 25 Hepes, 3 glucose (pH 7.4). This solution was used as the standard medium throughout the experiment. Following 30 min incubation, extracellular fluorophore was removed by washing the cells with 2 ml of the medium. The cover slip was mounted in a perfusion chamber (37 °C) placed over the objective of an inverted microscope (Nikon Diaphot 200, Tokyo, Japan). The light was generated by a xenon arc lamp and filtered through two narrow band-pass filters (340 and 380 nm). The filters alternated by the use of a computer-controlled filter wheel. The light reached the cells via a dichroic reflector. Fluorescence emission (at 510 nm) from individual cells was monitored using an intensified videocamera (PTI 100) and the fluorescence intensity ratio (340/380 nm) was calculated using PTI software (Imagemaster, South Brunswick, NJ, USA). The 340/380 nm ratios were used to obtain estimates of the free Ca^{2+} concentrations (Grynkiewicz *et al.* 1985) using fura-2 after calibration with known Ca^{2+} concentrations (Calcium Imaging Calibration Kit; Molecular Probes). The peak cytosolic Ca^{2+} level attained for each cell is referred to as peak $[Ca^{2+}]_i$. The sustained response was calculated by integrating the curve during the 3 min (180 s) stimulation period ($\int\Delta[Ca^{2+}]_i$) and expressed in micromolar seconds (μM s).

Electrophysiological measurements

Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard (Sekema, Stockholm, Sweden) and fire polished before use. The pipette resistance ranged between 3–6 MΩ when filled with pipette-filling solution specified below. Whole-cell currents were recorded using an EPC-9 patch clamp amplifier and the software Pulse (Heka Elektronik, Lambrecht/Pfalz, Germany). Exocytosis was detected as changes in the cell membrane capacitance (Neher & Marty, 1982) by using the 'lock-in'(which adds a 500 Hz sinus wave to the holding potential) or the 'captrack' functions of the EPC-9 amplifier. The zero-current potential was adjusted with the pipette in the bath before establishing the seal and the holding potential was −60 mV. All recordings were performed using the standard whole-cell configuration, in which the pipette solution is washed into the cell replacing the cytosol. Exocytosis was elicited either by voltage clamp depolarisation (from −60 to +10 mV) or by infusion of Ca^{2+}–EGTA buffers.

The standard extracellular (bath) solution used for the electrophysiological measurements consisted of (mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 Hepes and 3 glucose. When exocytosis and/or Ca^{2+} currents were elicited with voltage-clamp depolarisation, 20 mM NaCl were equimolarly replaced by tetraethylammonium (TEA) chloride which blocks voltage-gated K^+ currents. To study the pharmacology of the Ca^{2+} currents, Ba^{2+} was used as a charge carrier with the concentration of the divalent ion increased to 10 mM. This increases the Ca^{2+} current amplitude 3-fold above that seen at physiological Ca^{2+}. The pH of the extracellular solutions was adjusted to 7.4 with addition of NaOH.

For the infusion experiments, the intracellular solution contained (mM): 125 potassium glutamate, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 Hepes (pH 7.15 with KOH), 5 EGTA and 0, 2.5, 3.5 or 4.5 $CaCl_2$, yielding a free Ca^{2+} concentration of either < 0.0001, 0.16, 0.36 or 1.5 μM. The free Ca^{2+} concentration was calculated using the constants of Martell & Smith (1971). When exocytosis was initiated by voltage-clamp depolarisation the intracellular medium contained (mM): 125 caesium glutamate, 10 CsCl, 10 NaCl, 1 $MgCl_2$, 3 Mg-ATP, 0.05 EGTA and 5 Hepes. Measurements of Ca^{2+} currents were performed with (mM): 125 CsCl, 1 $MgCl_2$, 10 EGTA, 3 Mg-ATP, 10 Hepes (pH 7.15 with CsOH) and 0.1 cAMP in the pipette solution. Adjustment of the pH of the pipette solution was made by adding either KOH or CsOH.

All electrophysiological measurements were peformed at a temperature of 31–34°C. In the infusion experiments, the rate of capacitance increase ($\Delta C_m/\Delta t$) was measured during the linear phase, usually during the first 2 min.

Determination of pancreastatin

The pancreastatin-like immunoreactivity was measured by radioimmunoassay using rat pancreastatin as standard (Håkanson *et al.* 1995). The amount of pancreastatin released during the 30 min incubation period was expressed as femtomolar equivalents of rat pancreastatin per well.

Data analysis

Statistical analysis was performed using Student's unpaired *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

Effects of gastrin and PACAP on cytosolic Ca^{2+}

Cytosolic Ca^{2+} levels were 171 ± 3 nM ($n = 98$ cells) in resting ECL cells. In a Ca^{2+}-free medium (0 Ca^{2+} + 1 mM EGTA), gastrin and PACAP (10 nM) produced a transient increase in $[Ca^{2+}]_i$ peaking at 286 ± 25 nM and 249 ± 19 nM, respectively (Fig. 1*A* and *B*). Presumably, this increase reflects mobilisation of Ca^{2+} from intracellular stores. With Ca^{2+} present in the extracellular medium (1.28 mM) the peak $[Ca^{2+}]_i$ increased to 441 ± 21 nM ($n = 63$) in response to gastrin and 492 ± 20 nM ($n = 43$) in response to PACAP. Also, under these circumstances, the increases in $[Ca^{2+}]_i$ measured over 3 min were sustained rather than transient (Fig. 1*A* and *B*). The integrated Ca^{2+} responses (3 min), illustrating the magnitude and duration of the increase in $[Ca^{2+}]_i$, were 29.4 ± 2.0 μM after stimulation by gastrin and 35.5 ± 2.5 μM after PACAP. Without Ca^{2+} in the medium the corresponding values were 7.0 ± 1.3 μM and 7.0 ± 0.9 μM, respectively. The results are in agreement with previous reports (Prinz *et al.* 1994, 1999; Zeng *et al.* 1999*a*).

Effects of intracellular Ca^{2+}–EGTA buffers on exocytosis

Secretion from many endocrine cells depends on increases in $[Ca^{2+}]_i$ (Burgoyne, 1991). Since PACAP and gastrin increase $[Ca^{2+}]_i$, we were interested in investigating the correlation between $[Ca^{2+}]_i$ and exocytosis in ECL cells. To this end we used the whole-cell configuration of the patch-clamp technique in combination with measurement of cell capacitance increase as a single-cell indicator of exocytosis (i.e. fusion of secretory vesicles with the

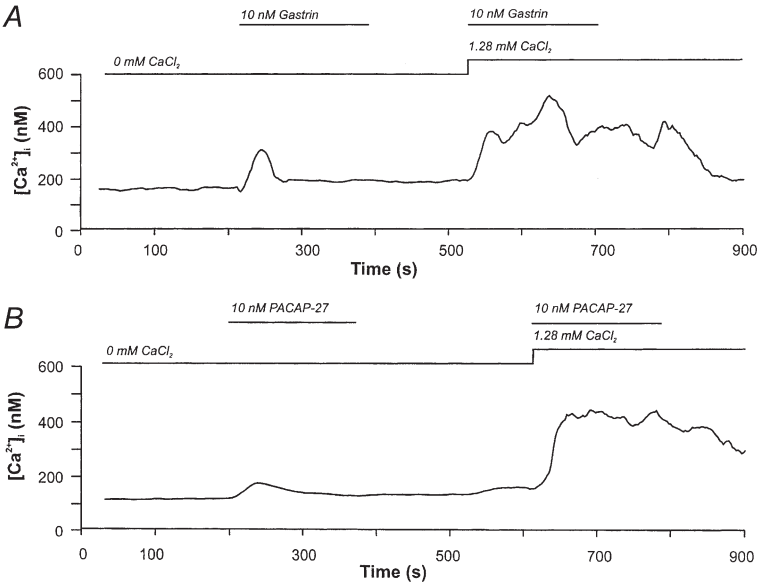


Figure 1. Increased cytosolic Ca^{2+} in ECL cells upon PACAP and gastrin stimulation

Stimulation with 10 nM gastrin (*A*) or PACAP (*B*) for 3 min in a Ca^{2+}-free medium resulted in a transient increase in $[Ca^{2+}]_i$. The peak rapidly returned back to basal levels. Stimulation with 10 nM gastrin (*A*) or PACAP (*B*) for 3 min in a Ca^{2+}-containing medium (1.28 mM $CaCl_2$) resulted in sustained increases in $[Ca^{2+}]_i$.

plasma membrane; Neher & Marty, 1982). This has the additional advantage that it is possible to control the intracellular environment since the pipette solution replaces the cytosol when the cell is dialysed. The membrane potential was clamped at −60 mV to avoid activation of voltage-dependent Ca^{2+} channels; the resting membrane potential in the ECL cells has been measured to be between −60 and −70 mV (Loo *et al.* 1995; Bufler *et al.* 1998). Figure 2*A* shows two representative curves demonstrating the increase in cell capacitance (exocytosis) upon infusion of a solution containing a high concentration (1.5 μM) of Ca^{2+} or a medium devoid of the cation (< 1 nM). In both traces, step changes of 100–200 fF were observed suggesting compound exocytosis and endocytosis as in other endocrine cells (Eliasson *et al.* 1996). In the absence of $[Ca^{2+}]_i$, exocytosis did not occur. The rate of exocytosis increased in response to increasing intracellular Ca^{2+} levels in a linear fashion up to 1.5 μM $[Ca^{2+}]_i$ (Fig. 2*B*).

Effect of extracellular Ca^{2+} on secretion

To investigate the source of Ca^{2+} initiating exocytosis in reponse to gastrin and PACAP, we first studied the effect of varying the extracellular Ca^{2+} concentration. In previous studies of ECL cell secretion, we used a medium containing 2 mM $CaCl_2$ (Lindström *et al.* 1999). Here we studied the influence of extracellular Ca^{2+} on pancreastatin secretion by varying the extracellular Ca^{2+} concentration between 0 and 30 mM Ca^{2+}. In the absence of gastrin or PACAP the ECL cells did not respond to variations of extracellular Ca^{2+} (Fig. 3*A* and *B*). In the absence of extracellular Ca^{2+} (0 Ca^{2+} + 1 mM EGTA), the ECL cells failed to respond to either gastrin or PACAP (although there was a clear but transient rise in $[Ca^{2+}]_i$, see Fig. 1). Gastrin was likewise without stimulatory effect in a medium containing 0.3 mM Ca^{2+} (Fig. 3*A*); in fact, a response was not obtained until Ca^{2+} was increased to 1 mM. In contrast, PACAP induced secretion at external Ca^{2+} concentrations as low as 0.1 mM, but the efficacy was

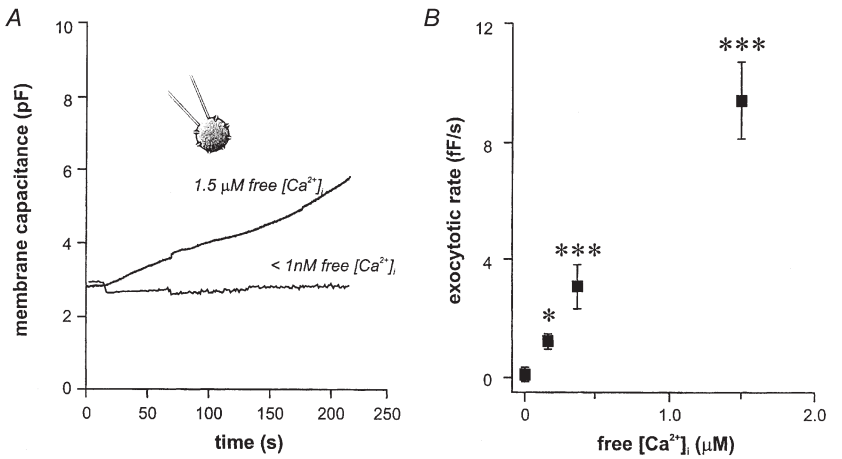


Figure 2. Increase in membrane capacitance upon infusion of Ca^{2+}–EGTA buffers

A, infusion of a Ca^{2+}–EGTA buffer containing 1.5 μM free Ca^{2+} or zero (< 1 nM) Ca^{2+} into single ECL cells caused an increase in membrane capacitance of 12 fF and 0.1 fF s^{-1}, respectively. *B*, the exocytotic rate as a function of $[Ca^{2+}]_i$. The data points are means of 7, 3, 3 and 12 experiments, respectively, performed with < 1 nM, 160 nM, 365 nM and 1.5 μM free Ca^{2+} in the pipette solution.

only 45% of that obtained at 2 mM (Fig. 3*B*). Increasing external Ca^{2+} to 10 mM resulted in similar gastrin-evoked pancreastatin secretion as with 2 mM Ca^{2+}, while it reduced PACAP-evoked secretion (Fig. 3*C* and *D*). Increasing external Ca^{2+} to 30 mM reduced both gastrin- and PACAP-stimulated secretion compared to that observed at 2 mM Ca^{2+}. This is probably due to a shift in the activation of the voltage-dependent Ca^{2+} channels towards a more positive membrane potential due to surface screening effects (Hille, 1991).

Effect of intracellular Ca^{2+} depletion

The contribution of intracellularly stored Ca^{2+} was studied using thapsigargin. Pretreatment with thapsigargin (0.1 nM for 4 h) depleted intracellular stores of Ca^{2+}, as indicated by the fact that PACAP and gastrin failed to produce the expected transient $[Ca^{2+}]_i$ response in a Ca^{2+}-free medium (see Fig. 4*A*). In a medium containing 1.28 mM Ca^{2+}, neither PACAP- nor gastrin-stimulated secretion of pancreastatin was affected by pretreatment with thapsigargin (Fig. 4*B*). Higher concentrations of thapsigargin than 0.1 μM were not tested because of the risk of inducing Ca^{2+} entry blockade (see review by Taylor & Broad, 1998). Thapsigargin *per se* (30 min incubation) did not increase $[Ca^{2+}]_i$ (data not shown) or pancreastatin secretion (Fig. 4*C*).

Effect of Ca^{2+} entry blockers on secretion and cytosolic Ca^{2+} response

Since extracellular Ca^{2+} proved crucial for secretion, we used a variety of Ca^{2+} entry blockers to determine which entry pathways were involved. Lanthanum (La^{3+}), a non-selective Ca^{2+} channel blocker, completely blocked gastrin- and PACAP-evoked secretion (Fig. 5*A*), confirming the importance of Ca^{2+} entry. Nifedipine (10 μM) and nimodipine (10 μM), blockers of voltage-operated L-type Ca^{2+} channels, inhibited gastrin-evoked secretion by 75–80% and PACAP-evoked secretion by 40–45% (Fig. 5*B* and *C*). Nifedipine also reduced the peak (35–40% decrease)

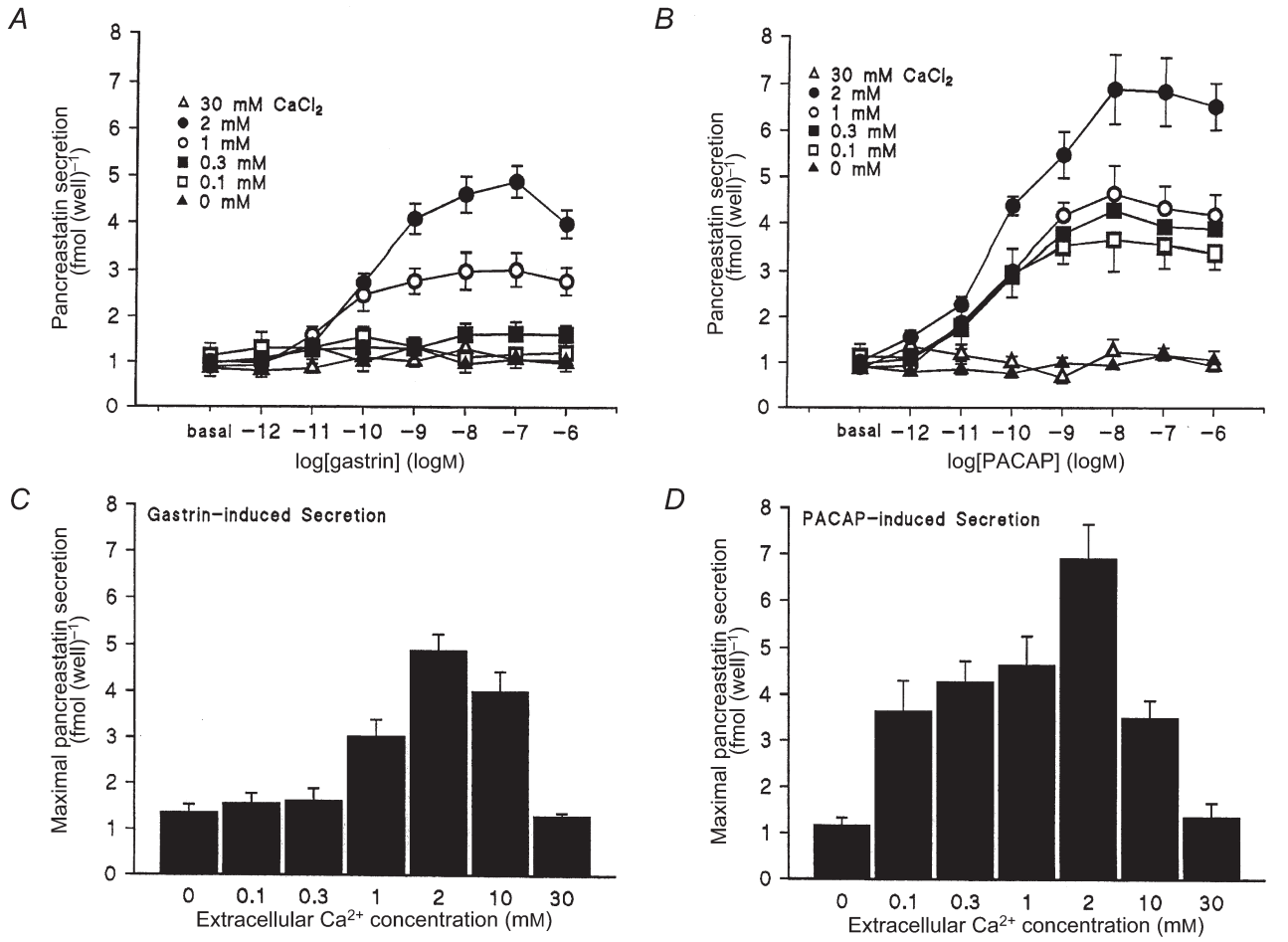


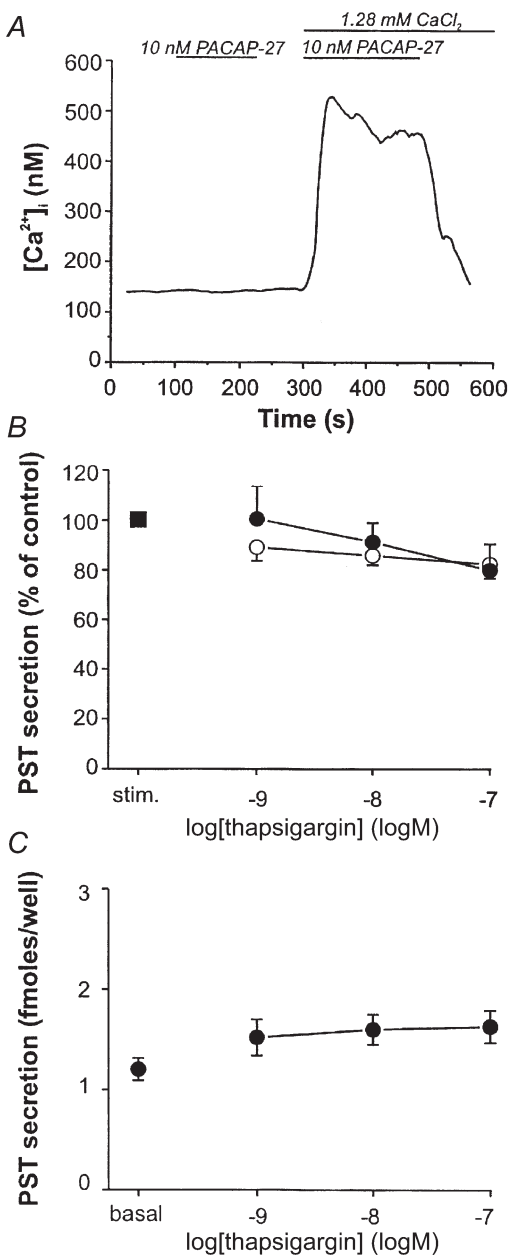
Figure 3. Effect of the extracellular Ca^{2+} concentration on PACAP- and gastrin-induced pancreastatin secretion

Concentration–reponse curves for gastrin (*A*) and PACAP (*B*) in the presence of different extracellular Ca^{2+} concentrations on pancreastatin secretion. Histograms demonstrating the effect of the extracellular Ca^{2+} concentration on the maximal pancreastatin secretion induced by 10 nM gastrin (*C*) or 10 nM PACAP (*D*). Means ± S.E.M.; $n = 8$–10.

Table 1. Effect of nifedipine or SKF 96365 on gastrin- and PACAP-evoked rise in $[Ca^{2+}]_i$

	Peak response (nM $[Ca^{2+}]_i$)	Sustained response (μM s)	n
PACAP	482 ± 25	37.0 ± 2.5	18
PACAP + 10 μM Nifedipine	372 ± 20**	18.9 ± 2.6***	18
Gastrin	378 ± 25	26.6 ± 2.7	27
Gastrin + 10 μM Nifedipine	308 ± 16*	11.8 ± 1.3***	27
PACAP	414 ± 16	31.8 ± 1.5	6
PACAP + 30 μM SKF 96365	337 ± 37	17.4 ± 3.4*	6
Gastrin	446 ± 22	34.7 ± 2.4	6
Gastrin + 30 μM SKF 96365	342 ± 14**	12.1 ± 3.0***	6

The response to each agonist is given with or without added channel blocker (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's unpaired t test). The Ca^{2+} response is monitored during 3 min.



as well as the sustained $[Ca^{2+}]_i$ response (50–55% decrease) to both gastrin and PACAP (see Table 1). The L-type channel blockers calcicludine and verapamil also inhibited secretion, but to a lesser degree than nifedipine and nimodipine (Table 2). ω-Conotoxin GVIA (1 μM), an effective blocker of voltage-operated N-type channels, reduced the gastrin-evoked secretion by 40% without affecting PACAP-evoked secretion (Fig. 5*D*). The P/Q-type and T-type channel blockers had little or no effect on stimulated secretion (Table 2). SKF 96365 (30 μM), an effective blocker of both receptor- and voltage-operated Ca^{2+} entry (Merritt *et al.* 1990), inhibited both gastrin- and PACAP-evoked secretion by 80% (Fig. 5*E*) and reduced peak and sustained $[Ca^{2+}]_i$ responses to both peptides by 30–65% (see Table 1).

Complete inhibition of secretion was not found with any individual blocker. We therefore examined possible additive effects by combining different types of blockers. Combining maximal concentrations of nifedipine and ω-conotoxin GVIA did not cause additive inhibition of either gastrin- or PACAP-stimulated secretion (Fig. 6*A*).

Figure 4. Effect of thapsigargin on pancreastatin secretion from the ECL cells

A, ECL cells were pretreated with 100 nM thapsigargin for 4 h. Stimulation with 10 nM PACAP in a Ca^{2+}-free extracellular media did not evoke an increase in $[Ca^{2+}]_i$; however, an increase was evident in Ca^{2+}-containing media. *B*, concentration–response curve illustrating the lack of effect of pretreatment (4 h) with increasing concentrations of thapsigargin on stimulated pancreastatin (PST) secretion. ●, gastrin-induced secretion, ○, PACAP-induced secretion. ■, PACAP- and gastrin-evoked PST secretion (stim) from controls (set to 100%). Means ± S.E.M.; $n = 6$–7. *C*, effect of thapsigargin *per se* on pancreastatin secretion from ECL cells. Basal, indicates basal secretion. Means ± S.E.M.; $n = 10$.

Interestingly, the combination of nifedipine and SKF 96365 abolished PACAP-evoked secretion but failed to cause additive inhibition of gastrin-stimulated secretion (Fig. 6*B*).

Further evidence for the involvement of L-type Ca^{2+}-channels in the secretion of pancreastatin was obtained using the L-type Ca^{2+}-channel activator Bay K 8644 (Triggle & Rampe, 1989). Bay K 8644 *per se* stimulated pancreastatin secretion in a concentration-dependent manner (Fig. 7), inducing a 2.6-fold increase at 3 μM. However, the same concentration of Bay K 8644 did not influence the secretion evoked by 10 nM PACAP or gastrin (data not shown). Also, at 3 μM, Bay K 8644 raised $[Ca^{2+}]_i$ (peak response: 332 $\pm$ 25 nM; integrated 3 min response: 15.9 $\pm$ 2.8 μM s, $n = 8$) compared to basal levels. These effects were prevented by 10 μM nifedipine (Fig. 7).

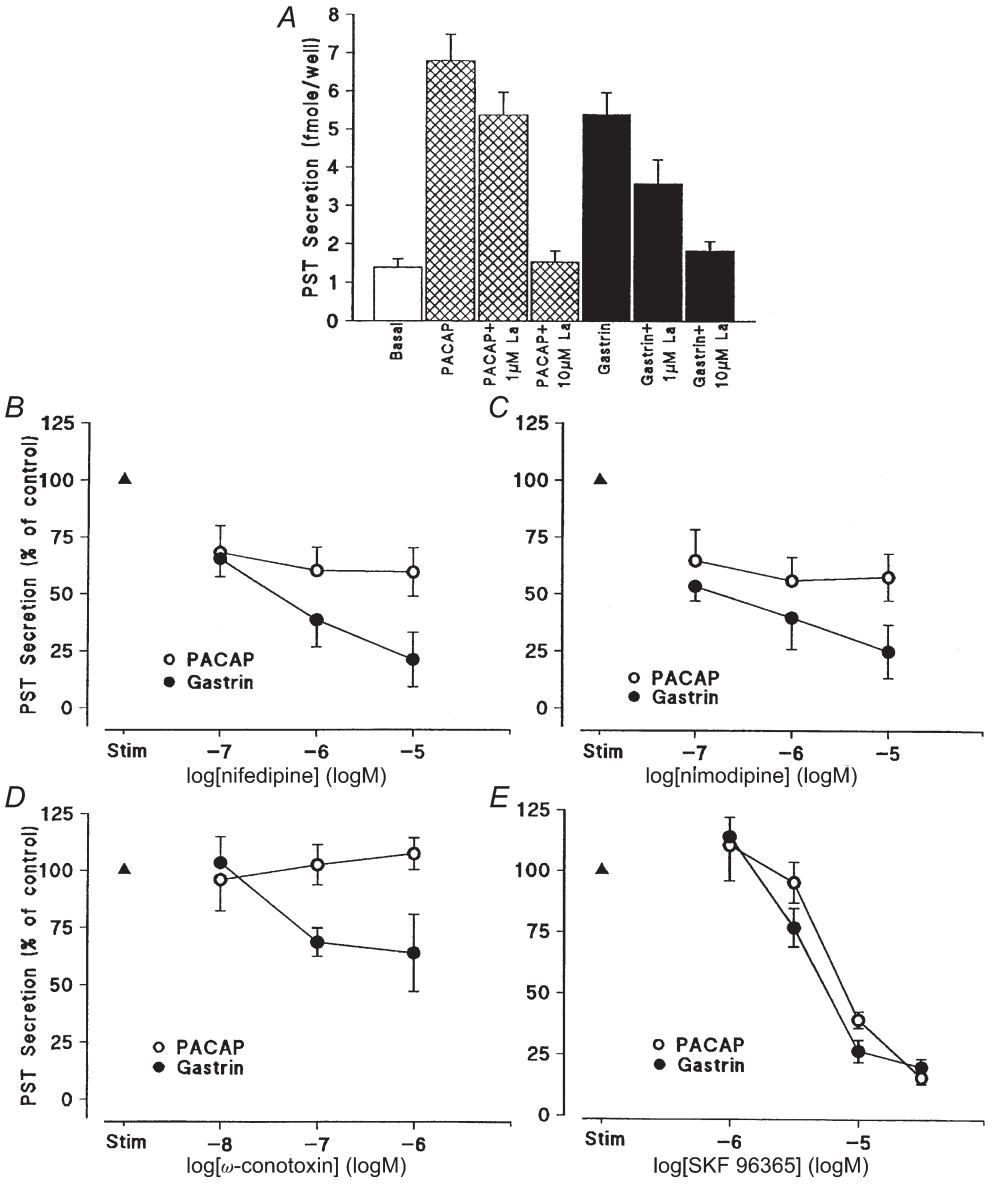


Figure 5. Effect of Ca^{2+} entry blockers on stimulated pancreastatin secretion from ECL cells

A, histogram illustrating the effect of $LaCl_3$ (La) on both gastrin- and PACAP-induced pancreastatin (PST) secretion. Concentration–response curves demonstrating the effect of the L-type Ca^{2+} channel blockers nifedipine (*B*) and nimodipine (*C*) and the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (*D*) on gastrin-induced (●) and PACAP-induced (○) pancreastatin (PST) secretion. ▲, PACAP- and gastrin-evoked secretion (Stim) from controls (secretion set at 100 %). *E*, concentration–response curve demonstrating the effect of the receptor-operated and L-type channel blocker SKF 96365 on gastrin-induced (●) and PACAP-induced (○) pancreastatin (PST) secretion. ▲, PACAP- and gastrin-evoked secretion (Stim) from controls (secretion set at 100 %). Means $\pm$ S.E.M.; $n = 6$–10.

Table 2. Effects of various Ca^{2+} channel blockers on gastrin- and PACAP-evoked secretion of pancreastatin from isolated ECL cells

Blocker	Channel selectivity	Maximally effective concentration (Reference)	Gastrin-stimulated secretion (% remaining)	PACAP-stimulated secretion (% remaining)
ω-Agatoxin	P-/Q-types	1 μM (Teramoto *et al.* 1995)	91.3 ± 17.5	113.4 ± 13.0
sFTX-3.3	P-/Q-/T-types	100 nM (Scott *et al.* 1992)	74.4 ± 14.0	111.5 ± 10.3
Calcicludin	Neuronal L-type	10 nM (Schweitz *et al.* 1994)	28.2 ± 8.9**	65.9 ± 19.5
Nifedipine	L-type	10 μM (Triggle & Janis, 1987)	20.9 ± 2.5**	59.3 ± 6.3*
Nimodipine	L-type	10 μM (Triggle & Janis, 1987)	24.7 ± 2.9**	55.7 ± 5.8*
Verapamil	L-type	50 μM (Triggle & Janis, 1987)	39.6 ± 12.2**	54.9 ± 15.3*
ω-Conotoxin MVIIA	N-type	1 μM (Olivera *et al.* 1987)	58.9 ± 8.5*	76.4 ± 7.2
ω-Conotoxin GVIA	N-type	1 μM (Olivera *et al.* 1985)	63.8 ± 6.8*	95.7 ± 16.9
SKF 96365	L-type and ROC	30 μM (Merritt *et al.* 1990)	20.2 ± 3.2**	15.9 ± 2.7**

The effect of each blocker is expressed as the percentage of the response to gastrin or PACAP that remains (* $P < 0.05$, ** $P < 0.01$; ANOVA followed by Dunnet's test for multiple comparisons). Means ± S.E.M.; $n = 6$–10. Secretion is measured after 30 min incubation with gastrin or PACAP. ROC, receptor-operated channels.

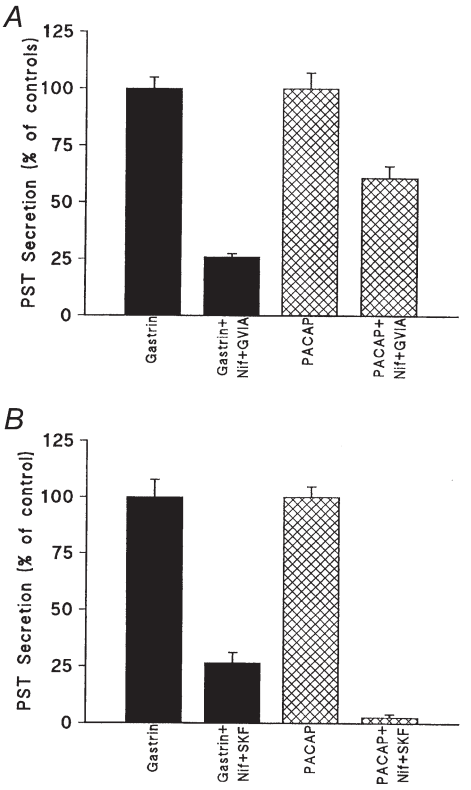


Figure 6. Effect of combining Ca^{2+} entry blockers on stimulated pancreastatin secretion from ECL cells.

A, effect of combining 10 μM nifedipine (Nif) and 1 μM ω-conotoxin GVIA (GVIA) on gastrin- and PACAP-stimulated pancreastatin (PST) secretion. *B*, effect of combining 10 μM nifedipine and 30 μM SKF 96365 (SKF) on gastrin- and PACAP-stimulated pancreastatin (PST) secretion. Means ± S.E.M.; $n = 6$–8.

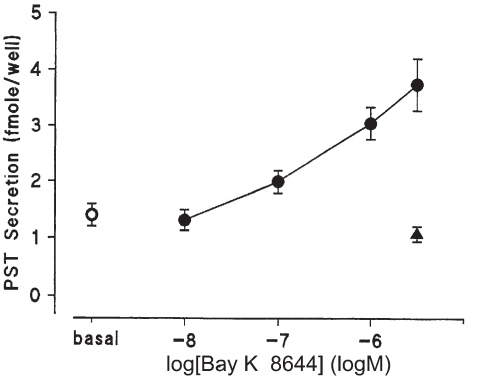


Figure 7. Effect of the L-type Ca^{2+} channel activator Bay K 8644 on pancreastatin secretion from ECL cells

Concentration–response curve demonstrating the stimulatory effect of the L-type Ca^{2+} channel activator Bay K 8644 on pancreastatin (PST) secretion. ○, basal secretion. ▲, effect of combining 3 μM Bay K 8644 with 10 μM nifedipine. Means ± S.E.M.; $n = 6$.

Exocytosis elicited by voltage-clamp depolarisation: demonstration of L-type and N-type Ca^{2+} channels

Since pancreastatin secretion was reduced by L-type channel blockers (Fig. 5*B* and *C*), we characterised voltage-gated Ca^{2+} channels in single ECL cells using the patch-clamp technique. First we measured the current(I)–voltage(V) relationship of the Ca^{2+} current using the whole-cell configuration in the presence of 2.6 mM extracellular $CaCl_2$ (Fig. 8*A* and *B*). Depolarising pulses from −60 mV to various membrane potentials ranging between −40 to +30 mV with 10 mV increments were applied. The summed responses from 10 cells are shown in Fig. 8*B*. The

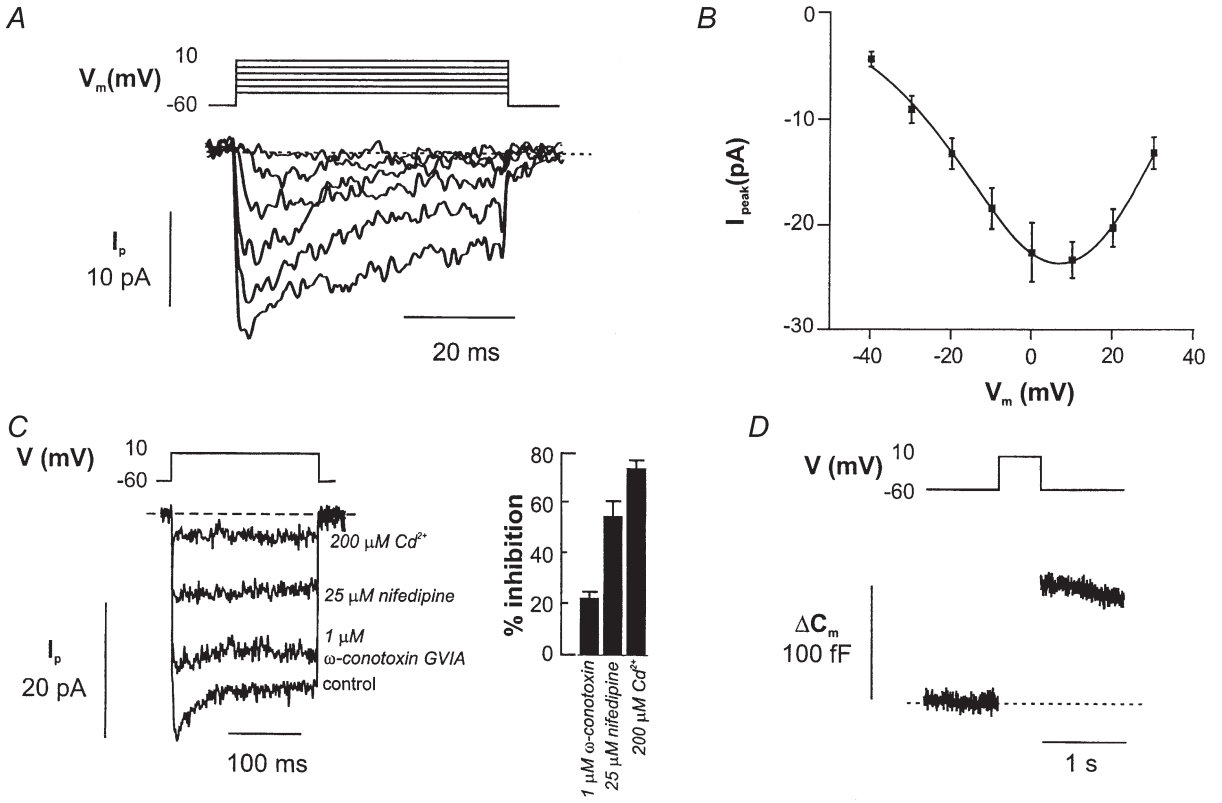


Figure 8. Electrophysiological measurements of Ca^{2+} currents in single ECL cells

A, ECL cells were depolarised from −60 mV to voltages between −40 and +30 mV in 10 mV increments. For clarity only the responses up to +10 mV are shown. The experiments were performed with 2.6 mM extracellular Ca^{2+} and in the presence of 0.1 μg ml^{-1} TTX. *B*, peak current (I)– voltage (V) relationship for the Ca^{2+} current. The curve is drawn according to eqn (1). *C* (left panel), to investigate the presence of L-type and N-type voltage-dependent Ca^{2+} channels on single ECL cells, inward currents were elicited by 200 ms voltage-clamp depolarisations from −60 to +10 mV and the Ca^{2+} channel blockers ω-conotoxin GVIA, nifidipine and Cd^{2+} were added after each other at concentrations of 1 μM, 25 μM and 200 μM, respectively. *C* (right panel), histogram demonstrating the effect of Ca^{2+} channel blockers on the peak current. Means ± S.E.M. of 3–8 experiments. *D*, increase in membrane capacitance upon a 500 ms depolarisation from −60 to 0 mV measured on a single ECL cell in the presence of NaCl.

activation properties of the Ca^{2+} current can be described by approximating the data points to a modified Boltzmann equation:

$$I = G \frac{V - V_r}{1 + \exp(-(V - V_h)/k)}, \quad (1)$$

where I is the measured current, G is the whole-cell conductance of the calcium current (estimated to be 0.9 ± 0.2 nS, $n = 10$), V is the measured membrane potential, V_r is the reversal potential (determined to be 53 ± 5 mV), V_h is the membrane potential at which the current is half-maximally activated (-3 ± 4 mV) and k is the slope coefficient (13 ± 1 mV). The maximal current was achieved when the cell was depolarised to +10 mV and amounted to -23 ± 2 pA. The results are comparable to those of previous studies on ECL cells using Ba^{2+} as charge carrier (Bufler *et al.* 1998).

The pharmacology of the Ca^{2+} current, induced by depolarising single ECL cells, was investigated using Ba^{2+} as charge carrier instead of Ca^{2+}. This generates currents larger in magnitude, which makes it easier to detect possible inhibition caused by the blockers. Fig. 8*C* (left panel) demonstrates that voltage-dependent Ca^{2+} channels are activated by depolarising a single ECL cell from −60 to +10 mV during 200 ms. The N-type channel blocker ω-conotoxin GVIA supressed the Ca^{2+} current and adding nifedipine (25 μM) caused a further inhibition. Combining ω-conotoxin GVIA and nifedipine did not result in total suppression of the current as indicated in Fig. 8*C*, suggesting that besides voltage-dependent L- and N-type Ca^{2+} channels, the ECL cells employ additional Ca^{2+} channels. Alternatively, the blockers were used at submaximal concentrations. Finally, the current was virtually abolished (~90 % inhibition) by the non-selective calcium channel blocker Cd^{2+}. The mean effect of each individual blocker is shown in Fig. 8*C* (right panel). We also studied exocytosis evoked by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels as shown in Fig. 8*D*. A 500 ms depolarisation from −60 to +10 mV evoked an increase in capacitance of 106 fF in this particular cell and the mean membrane capacitance increase amounted to 75 ± 19 fF ($n = 8$).

Na^+ channels are not involved in exocytosis

TTX-sensitive and voltage-dependent Na^+ currents could be detected in ECL cells as shown in Fig. 9*A* and *B*. The voltage dependence was investigated by application of 50 ms depolarising pulses from −60 mV to potentials ranging between −40 to +10 mV. The mean peak currents (Fig. 9*C*) were approximated with eqn (1) to yield the activation properties. The calculated value for

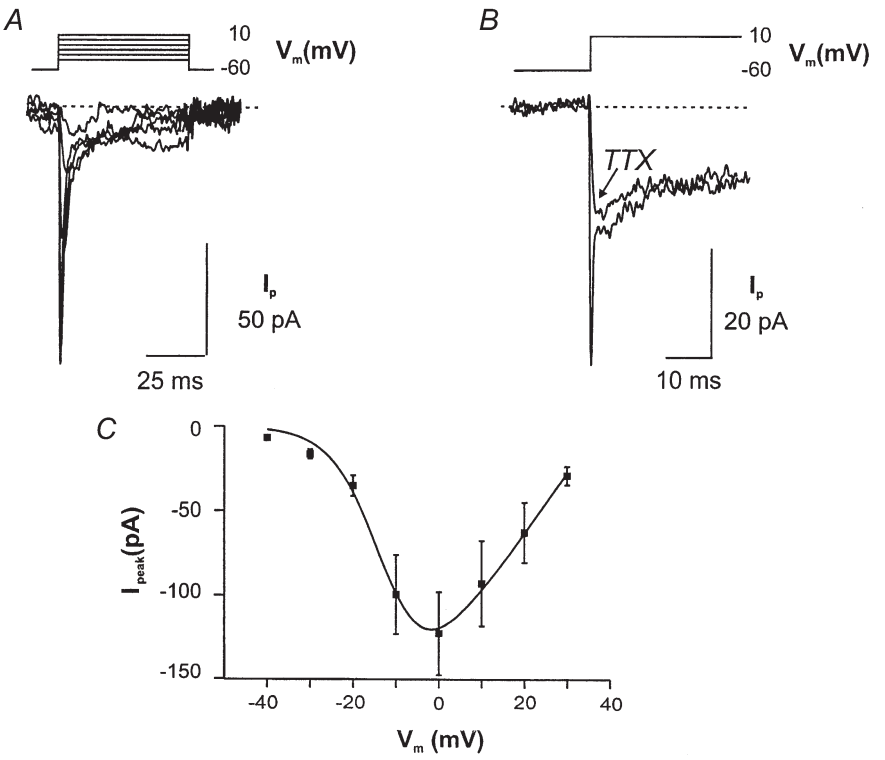


Figure 9. Electrophysiological measurements of Na^+ currents in single ECL cells

A, inward currents from a single ECL cell were elicited by depolarisation from −60 mV to varying voltages between −40 and +10 mV. *B*, the Na^+ peak (the fast transient part of the current) was blocked by application of 0.1 μg ml^{-1} TTX. *C*, peak $I-V$ relationship for the Na^+ current. The curve was drawn according to eqn (1). Means ± S.E.M. of 6–8 experiments.

conductance (G), the reversal potential (V_r), the membrane potential at which the current is half-maximally activated (V_h) and the slope coefficient (k) was 3.9 ± 1.4 nS, 41 ± 3 mV, −12 ± 3 mV and 5.7 ± 1.4 mV, respectively. The maximal current was achieved when a depolarising pulse from −60 to 0 mV was applied and amounted to −122 ± 25 pA ($n = 6$).

To investigate if the Na^+ current was important for exocytosis, NaCl was replaced with choline chloride (Fig. 10*A*). The Na^+ current did not seem to be coupled to exocytosis since the increase in membrane capacitance in the presence of choline chloride did not differ from that in the presence of NaCl (Fig. 10*B*). In accordance with the patch-clamp findings we found that pretreatment with 0.1 μg ml^{-1} TTX or replacement of extracellular Na^+ with choline failed to affect either gastrin- or PACAP-stimulated pancreastatin secretion (Fig. 10*C* and *D*).

DISCUSSION

Rat stomach ECL cells operate under the control of circulating gastrin. Local messengers/neurotransmitters also control ECL cell function but their physiological significance remains to be explored. Isolated ECL cells respond not only to gastrin (via an action on CCK_2 receptors) but also to the neuropeptides PACAP-27, -38 and VIP (Lindström *et al.* 1997; Zeng *et al.* 1998, 1999*a*; Läuffer *et al.* 1999). In fact, PACAP stimulates secretion with a greater efficacy than gastrin (Lindström *et al.* 1997; Läuffer *et al.* 1999; Lindström & Håkanson, 2001). However, the pathways behind the stimulus–secretion coupling evoked by either gastrin or PACAP have so far not been examined in detail.

Gastrin and PACAP evoke increases in cytosolic Ca^{2+}

Isolated ECL cells, incubated in a medium containing 1.28 mM Ca^{2+}, responded to both gastrin and PACAP with increases in $[Ca^{2+}]_i$ in agreement with previous reports (Prinz *et al.* 1993, 1994; Zeng *et al.* 1996, 1999*a*). The initial rapid response is most likely due to the formation of IP_3, which in turn acts on intracellular IP_3 receptors to mobilise Ca^{2+} from stores in the endoplasmic reticulum (Berridge & Irvine, 1984). On the other hand, the sustained response probably depends on Ca^{2+} influx as it is abolished in the absence of extracellular Ca^{2+} (Prinz *et al.* 1993, 1994; Zeng *et al.* 1996). Infusion of Ca^{2+} up to a free concentration of 1.5 μM into the cytosol stimulated exocytosis in a linear fashion. The concentrations of $[Ca^{2+}]_i$ required for exocytosis to occur in ECL cells (Fig. 2) were similar to those recorded after stimulating with

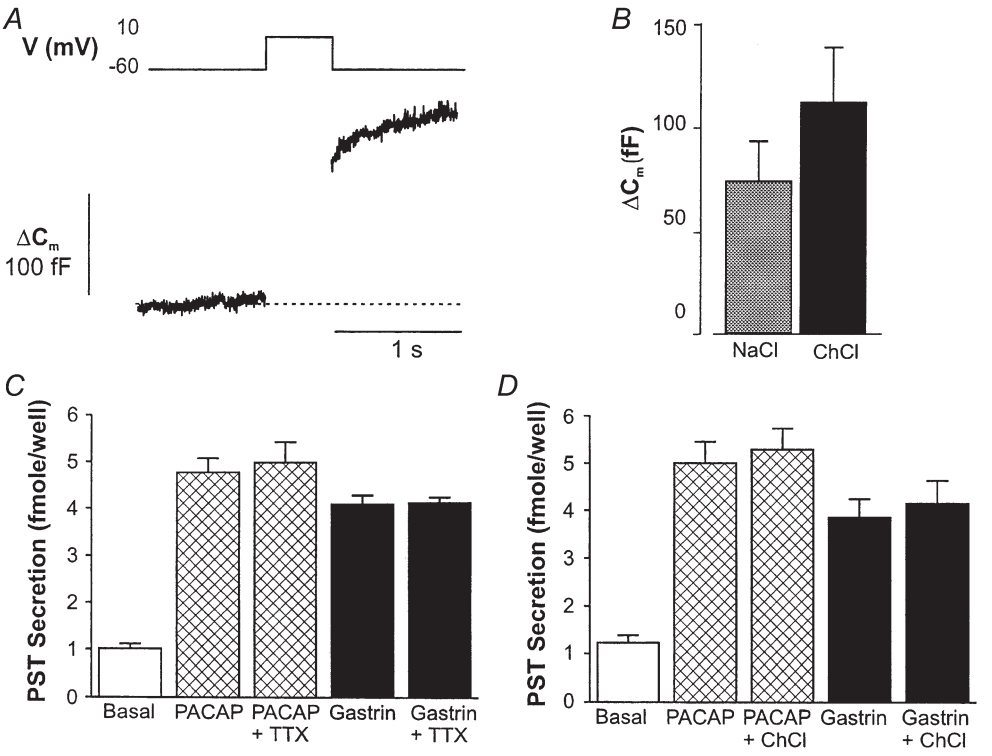


Figure 10. Effects of extracellular Na^+ on secretion from ECL cells

A, increase in membrane capacitance upon a 500 ms depolarisation from −60 to 0 mV measured on a single ECL cell in the presence of choline chloride (ChCl). *B*, the mean increase in membrane capacitance in the presence of NaCl or ChCl, respectively. *C*, histogram showing that 30 min pretreatment with 0.1 μg ml^{-1} TTX did not affect PACAP- or gastrin-evoked pancreastatin secretion. *D*, replacing extracellular NaCl with ChCl had no effect on PACAP- or gastrin-evoked pancreastatin secretion. Means ± S.E.M. of 6–8 experiments.

maximally effective concentrations of gastrin and PACAP (Fig. 1). It is therefore likely that gastrin- and PACAP-evoked secretion is dependent upon increases in $[Ca^{2+}]_i$. This conclusion reinforces earlier observations using permeabilised ECL cells, where elevations of $[Ca^{2+}]_i$ led to stimulated histamine secretion (Höhne-Zell *et al.* 1997).

Is Ca^{2+} entry or mobilisation of intracellular Ca^{2+} responsible for secretion in ECL cells?

PACAP- and gastrin-induced secretion did not occur in the absence of extracellular Ca^{2+} despite a transient increase in $[Ca^{2+}]_i$ (Figs 1 and 3). Stimulated secretion was also abolished in the presence of the non-selective Ca^{2+}-channel blocker $LaCl_3$ (Fig. 5*A*). These results illustrate the importance of Ca^{2+} influx. Interestingly, basal pancreastatin release in Ca^{2+}-free medium was the same as in 2 mM Ca^{2+}, suggesting that whereas extracellular Ca^{2+} is important for stimulated secretion, it is not required for basal, unstimulated (constitutive?) release.

The importance of intracellular stores of Ca^{2+} for secretion was examined by pretreatment with thapsigargin, which is known to deplete intracellular Ca^{2+} stores by irreversibly inhibiting the Ca^{2+} pumps of the sarco- and endoplasmic reticulae (for a review see Treiman *et al.* 1998). Pretreatment with 100 nM thapsigargin for 4 h failed to impair either gastrin- or PACAP-evoked secretion. The thapsigargin concentrations used (1–100 nM) are known to deplete Ca^{2+} from other cells (Mason *et al.* 1991; Cavallini *et al.* 1995). Hence, although both gastrin and PACAP are capable of mobilising Ca^{2+} from intracellular stores, this does not seen to be critical for secretion, and we therefore suggest that Ca^{2+} entry rather than IP_3-triggered mobilisation of intracellular Ca^{2+} is responsible for exocytosis in ECL cells.

Thapsigargin is sometimes used to open store-operated (capacitative) Ca^{2+} channels, an effect which is thought to be secondary to the thapsigargin-evoked depletion of intracellular Ca^{2+} stores (Jan *et al.* 1999). An acute thapsigargin challenge (30 min) failed to induce pancreastatin secretion, which suggests that Ca^{2+} entry through store-operated channels either does not occur, is sub-threshold or does not occur in the vicinity of those secretory vesicles that are docked to the plasma membrane in preparation for exocytosis (Zhao *et al.* 1999).

Gastrin- and PACAP-evoked secretion involve different Ca^{2+} entry pathways

The results described above strongly suggest that Ca^{2+} entry, rather than mobilisation of intracellular Ca^{2+}, is critical for secretion from the ECL cells. However, the level of extracellular Ca^{2+} seemed to be more critical for gastrin-evoked than for PACAP-evoked secretion. At low extracellular Ca^{2+} concentrations (< 1 mM), gastrin failed to stimulate secretion, while PACAP maintained its ability to stimulate secretion at Ca^{2+} levels as low as 0.1 mM. The PACAP-induced response at this Ca^{2+} concentration was 45% of that observed at 2 mM Ca^{2+}. Conceivably, PACAP-stimulated secretion also at low extracellular Ca^{2+} is due to activation of an additional second messenger pathway besides Ca^{2+} influx. Alternatively, PACAP and gastrin stimulate different (or differently located) Ca^{2+} entry pathways.

The importance of the various Ca^{2+} channels was studied by using selective blockers of the different channel types (see Table 2). No significant reduction in gastrin- or PACAP-evoked secretion was seen when blockers of T-, P- and Q-type Ca^{2+} channels were used. Hence, such channels do not appear to be involved in ECL cell exocytosis. On the other hand, various L-type channel blockers and the N-type blocker ω-conotoxin GVIA inhibited gastrin-evoked secretion (Fig. 5), L-type channel blockers being more effective ($\sim$75 *vs.* 40% for N-type). The presence of L-type and N-type Ca^{2+} channels in ECL cells was further confirmed by measurements of depolarisation-evoked whole-cell currents in single cells. The inward Ca^{2+} current was reduced to the same degree as secretion by nifedipine and ω-conotoxin GVIA. Indeed, in earlier studies the ECL cells have been shown to be equipped with L-type channels and the existence of N-type channels was also suggested (Bufler *et al.* 1998). The L-type channel blocker nifedipine also reduced the increase in $[Ca^{2+}]_i$ in response to gastrin (Table 1). These results are in contrast to the recent results of Zeng *et al.* (1999*b*) which indicated that L-type channels are not involved in gastrin-evoked increases in $[Ca^{2+}]_i$ or histamine secretion. The L-type Ca^{2+} channel activator Bay K 8644 raised cytosolic Ca^{2+} and induced pancreastatin secretion, thus providing further evidence for the involvement of L-type channels in exocytosis. Indeed, Bay K 8644 has been shown to stimulate secretion from other cell types as well (Yamada *et al.* 1996; Jordinson *et al.* 1998; Negishi *et al.* 1999). Surprisingly, Bay K 8644 did not enhance gastrin- or PACAP-evoked secretion, possibly because gastrin and PACAP at the concentrations used already induce sufficient activation of the L-type channels. Influx of Ca^{2+} through Ca^{2+} channels have been shown to be of importance in mediating secretion from several types of endocrine cells (Henkel & Almers, 1996) including cholecystokinin-secreting I cells (Chang *et al.* 2000) and insulin secreting β cells (Rorsman *et al.* 2000).

Although the results of the present study indicate that gastrin-induced secretion involves the opening of both L-type and N-type channels, the combination did not produce an additive inhibitory effect (Fig. 6*A*). SKF 96365, which is an inhibitor of both receptor-operated and L-type Ca^{2+} channels (Merritt *et al.* 1990), blocked gastrin-induced secretion to the same extent as nifedipine (Table 1). Combining nifedipine and SKF 96365 did not result in further inhibition of gastrin-evoked secretion, suggesting that gastrin opens L-type and N-type channels but not receptor-operated channels.

However, 25% of the secretory response to gastrin remained after treatment with L-type and N-type Ca^{2+} channel blockers. This might suggest that additional Ca^{2+} entry pathways exist or that L-type and N-type channels are not completely blocked by the inhibitors at the concentrations used.

At variance with gastrin-evoked secretion, PACAP-evoked secretion was reduced only by ~40% by nifedipine and not at all by ω-conotoxin GVIA, which agrees with the report by Zeng *et al.* (1999*b*). This suggests that PACAP-stimulated secretion is not as dependent on influx of Ca^{2+} through voltage-sensitive channels as gastrin-induced secretion. Instead, it is likely that a voltage-independent Ca^{2+} entry pathway is involved. SKF 96365 was by far the most potent blocker of PACAP-evoked secretion (~85% inhibition). This inhibitory effect is probably not due to L-type channel blockade, since the effect of SKF 96365 was more pronounced compared to the more selective L-type channel blocker nifedipine. Also, combining SKF 96365 with nifedipine resulted in total blockade of PACAP-evoked secretion suggesting that PACAP induces Ca^{2+} entry through both L-type and receptor-operated Ca^{2+} channels. PACAP is known to stimulate intracellular levels of cAMP and indeed this appears to be the case in ECL cells (Zeng *et al.* 1999*a*). The opening of receptor-operated channels has been suggested to be regulated by second messengers such as cAMP (Lenz & Kleineke, 1997; Barritt, 1999). Hence, this might explain why PACAP-induced secretion and not gastrin-stimulated secretion involves receptor-operated channels. The involvement of different Ca^{2+} channels might generate different types (and different locations) of intracellular Ca^{2+} signals.

Dynamics of exocytosis: quantitative, morphological considerations

When the membrane potential was depolarised to generate a maximal inward Ca^{2+} current the cells responded with an increase in membrane capacitance, reflecting exocytosis, of 100 fF. This gives further evidence for voltage-dependent exocytosis in ECL cells. Depolarisation was found to open TTX-sensitive Na^+ channels in addition to Ca^{2+} channels (Fig. 9). This is in contrast with Bufler *et al.* (1998) who failed to identify Na^+ channels in ECL cells. However, Na^+ influx through these channels does not seem to be necessary for exocytosis since addition of TTX and replacement of extracellular Na^+ with choline did not significantly affect depolarisation-induced exocytosis or gastrin- and PACAP-evoked pancreastatin secretion (Fig. 9).

Ultrastructural studies have indicated that the mean profile diameter of secretory vesicles close to the plasma membrane in ECL cells is around 200 nm (Zhao *et al.* 1999). This means that the surface area of the average secretory vesicle is approximately 0.125 μm^2. The specific capacitance for biological membranes is 10 fF μm^{-2} (Hille, 1991), hence upon fusion with the plasma membrane each vesicle adds about 1.25 fF of capacitance. Each cell contains a total of about 10 fmol histamine (Prinz *et al.* 1993; Sakai *et al.* 1995; E. Lindström *et al.* unpublished observations) and 5 amol pancreastatin (E. Lindström *et al.* unpublished observations) in approximately 10 000 secretory vesicles (calculated from Zhao *et al.* 1999). Depolarisation of the ECL cells resulted in an 100 fF increase in cell capacitance, and we can thus estimate the number of vesicles released from a single cell during 500 ms to be about 80. This readily releasable pool, which probably represents primed vesicles docked to the plasma membrane (Zhao *et al.* 1999), thus constitutes close to 1% of the secretory vesicle compartment (100 amol histamine per cell). Similar estimations have been made in other endocrine cells (see for example Eliasson *et al.* 1997).

Intracellular infusion of Ca^{2+} resulted in a sustained capacitance increase. Infusion of 1.5 μM Ca^{2+} resulted in a rate of capacitance increase of 9.4 fF s^{-1} which corresponds to an increase of 1.7 pF over a 3 min period. Using the above conversion factor (1.25 fF) we can thus estimate that approximately 1000 vesicles are being released during the 3 min time frame. This vesicle population possibly represents a less readily releasable pool, which needs to be translocated and chemically modified (primed) prior to release. This pool represents about 10% of the secretory vesicle compartment (1 fmol histamine per cell).

Concluding remarks

Gastrin controls the ECL cells via the circulation while the neuropeptide PACAP acts locally, being released from nerve fibres in the vicinity of the oxyntic glands. Thus, ECL cells are under hormonal as well as nervous control in turn controlling HCl secretion from parietal cells as part of the gastrin–ECL cell–parietal cell pathway. The results of the present study indicate that gastrin- and PACAP-induced secretion from ECL cells depends on the influx of extracellular Ca^{2+} rather than on the mobilisation of intracellular Ca^{2+}. The Ca^{2+} influx responsible for exocytosis upon gastrin stimulation appears to involve voltage-gated L-type and N-type Ca^{2+} channels. PACAP-induced secretion, on the other hand, involves L-type and receptor-operated Ca^{2+} channels. However, the mechanisms behind the opening of these Ca^{2+} channels in the ECL cells are unknown and will be the subject of further investigation.

Barritt, G. J. (1999). Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochemical Journal* **337**, 153–169.

Berridge, M. J. (1997). Elementary and global aspects of calcium signalling. *Journal of Physiology* **499**, 291–306.

Berridge, M. J. & Irvine, R. F. (1984). Inositol trisphosphate: a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321.

Bufler, J., Choi, G. C., Francke, C., Schepp, W. & Prinz, C. (1998). Voltage-gated Ca^{2+} currents in rat gastric enterochromaffin-like cells. *American Journal of Physiology* **274**, C424–429.

Burgoyne, R. D. (1991). Control of exocytosis in adrenal chromaffin cells. *Biochimica et Biophysica Acta* **1071**, 174–202.

Cavallini, L., Coassin, M. & Alexandre, A. (1995). Two classes of agonist-sensitive Ca^{2+} stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. *Biochemical Journal* **310**, 449–452.

Chang, C. H., Chey, W. Y. & Chang, T.-M. (2000). Cellular mechanism of sodium oleate-stimulated secretion of cholecystokinin and secretin. *American Journal of Physiology – Gastrointestinal and Liver Physiology* **279**, G295–303.

Chen, D., Mårvik, R., Ronning, K., Andersson, K., Waldum, H. L. & Håkanson, R. (1996). Gastrin-evoked secretion of histamine, pancreastatin and acid from the isolated, vascularly perfused rat stomach. Effects of isobutylmethylxanthin and α-fluoromethylhistidine. *Regulatory Peptides* **65**, 133–138.

Chen, D., Monstein, H.-J., Nylander, A.-G., Zhao, C.-M., Sundler, F. & Håkanson, R. (1994). Acute responses of rat stomach enterochromaffin-like cells to gastrin: Secretory activation and adaptation. *Gastroenterology* **107**, 18–27.

Davila, H. M. (1999). Molecular and functional diversity of voltage-gated calcium channels. *Annals of the New York Academy of Sciences* **868**, 102–117.

Eliasson, L., Proks, P., Ämmälä, C., Ashcroft, F. M., Bokvist, K., Renström, E., Rorsman, P. & Smith, P. A. (1996). Endocytosis of secretory granules in mouse pancreatic β-cells evoked by transient elevation of cytosolic calcium. *Journal of Physiology* **493**, 755–767.

Eliasson, L., Renström, E., Ding, W.-Q., Proks, P. & Rorsman, P. (1997). Rapid ATP-dependent priming of secretory granules precedes Ca^{2+}-induced exocytosis in mouse pancreatic B-cells. *Journal of Physiology* **503**, 399–412.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

Håkanson, R., Böttcher, G., Ekblad, E., Panula, P., Simonsson, M., Dohlsten, M., Hallberg, T. & Sundler, F. (1986). Histamine in endocrine cells in the stomach. A survey of several species using a panel of histamine anibodies. *Histochemistry* **86**, 5–17.

Håkanson, R., Chen, D., Lindström, E., Norlén, P., Björkqvist, M. & Lehto-Axtelius, D. (1998). Physiology of the ECL cell. *Yale Journal of Biology and Medicine* **71**, 163–171.

Håkanson, R., Chen, D. & Sundler, F. (1994). The ECL cells. In *Physiology of the Gastrointestinal Tract*. ed. Johnson, L. R., 3rd edn, vol. 2, pp. 1171–1184. Raven Press, New York.

Håkanson, R., Ding, X.-Q., Norlén, P. & Chen, D. (1995). Circulating pancreastatin is a marker for the enterochromaffin-like cells of the rat stomach. *Gastroenterology* **108**, 1445–1452.

Henkel, A. W. & Almers, W. (1996). Fast steps of exocytosis and endocytosis by capacitance measurements in endocrine cells. *Current Opinion in Neurobiology* **6**, 350–357.

Hille, B. (1991). *Ionic Channels of Excitable Membranes*. Sinauer, Sunderland, MA, USA.

Höhne-Zell, B., Galler, A., Schepp, W., Gratzl, M. & Prinz, C. (1997). Functional importance of synaptobrevin and SNAP-25 during exocytosis of histamine by rat gastric enterochromaffin-like cells. *Endocrinology* **138**, 5518–5526.

Jan, C. R., Ho, C. M., Wu, S. N. & Tseng, C. J. (1999). Mechanism of rise and decay of thapsigargin-evoked calcium signals in MDCK cells. *Life Sciences* **64**, 259–267.

Jones, S. W. (1998). Overview of voltage-dependent calcium channels. *Journal of Bioenergetics and Biomembranes* **30**, 299–312.

Jordinson, M., Beales, I. L. & Calam, J. (1998). Soybean agglutinin stimulated cholecystokinin release from cultured rabbit jejunal cells requires calcium influx via L-type calcium channels. *Peptides* **19**, 1541–1547.

Kinoshita, Y., Nakata, H., Kishi, K., Kawanami, C., Sawada, M. & Chiba, T. (1998). Comparison of the signal transduction pathways activated by gastrin in enterochromaffinlike cells and parietal cells. *Gastroenterology* **115**, 93–100.

Läuffer, J. M., Modlin, I. M., Hinoue, T., Kidd, M., Zhang, T., Schmid, S. W. & Tang L. H. (1999). Pituitary adenylate cyclase activating polypeptide modulates gastric enterochromaffin-like cell proliferation in rats. *Gastroenterology* **116**, 623–635.

Lenz, T. & Kleineke, J. W. (1997). Hormone-induced rise in cytosolic Ca^{2+} in axolotl hepatocytes: properties of the Ca^{2+} influx channel. *American Journal of Physiology* **273**, C1526–1532.

Lindström, E., Björkqvist, M., Boketoft, Å., Chen, D., Zhao, C.-M., Kimura, K. & Håkanson, R. (1997). Neurohormonal regulation of histamine and pancreastatin secretion from isolated rat stomach ECL cells. *Regulatory Peptides* **71**, 73–86.

Lindström, E., Björkqvist, M. & Håkanson, R. (1999). Pharmacological analysis of CCK_2 receptor antagonists using isolated rat stomach ECL cells. *British Journal of Pharmacology* **127**, 530–536.

Lindström, E. & Håkanson, R. (2001). Neurohormonal regulation of secretion from isolated rat stomach ECL cells: a critical reappraisal. *Regulatory Peptides* **97**, 169–180.

Loo, D. D. F., Sachs, G. & Prinz, C. (1995). Potassium and chloride currents in rat gastric enterochromaffin-like cells. *American Journal of Physiology* **270**, G739–745.

Martell, A. E. & Smith, R. M. (1971). Critical stability constants. *Amino Acids*, vol.1 and *Amines*, vol. 2. Plenum Press, New York.

Mason, M. J., Garcia-Rodriguez, C. & Grinstein, S. (1991). Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *Journal of Biological Chemistry* **266**, 8801–8806.

Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. & Rink, T. J. (1990). SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochemical Journal* **271**, 515–522.

Negishi, T., Chik, C. L. & Ho, A. K. (1999). Ceramide enhances growth hormone (GH)-releasing hormone-stomulated cyclic adenosine 3′,5′-monophosphate accumulation but inhibits GH release in rat anterior pituitary cells. *Endocrinology* **140**, 5691–5697.

Neher, E. & Marty, A. (1982). Discrete changes in cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **79**, 6712–6716.

Norlén, P., Kitano, M., Lindström, E. & Håkanson, R. (2000). Anaesthetic agents inhibit gastrin-stimulated but not basal histamine release from rat stomach ECL cells. *British Journal of Pharmacology* **130**, 725–730.

Olivera, B. M., Cruz, L. J., de Santos, V., LeCheminant, G. W., Griffin, D., Zeikus, R., McIntosh, J.M., Galyean, R., Varga, J., Gray, W. R. & Rivier, J. (1987). Neuronal calcium channel antagonists. Discrimination between calcium channel subtypes using omega-conotoxin from conus magus venom. *Biochemistry* **26**, 2086–2090.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V. & Cruz, L. J. (1985). Peptide neurotoxins from fish-hunting cone snails. *Science* **230**, 1338–1343.

Panula, P., Häppölä, O., Airaksinen M. S., Auvinen, S. & Virkamäki, A. (1988). Carbodiimide as a fixative in histamine immunohistochemistry and its application in developmental neurobiology. *Journal of Histochemistry and Cytochemistry* **36**, 259–269.

Prinz, C., Kajimura, M., Scott, D. R., Mercier, F., Helander, H. & Sachs, G. (1993). Histamine secretion from rat enterochromaffin-like cells. *Gastroenterology* **105**, 449–461.

Prinz, C., Scott, D. R., Hurwitz, D., Helander, H. F. & Sachs, G. (1994). Gastrin effects on isolated enterochromaffin-like cells in primary culture. *American Journal of Physiology* **267**, G663–675.

Prinz, C., Zanner, R., Gerhard, M., Mahr, S., Neumayer, N., Höhne-Zell, B. & Gratzl, M. (1999). The mechansim of histamine secretion from gastric enterochromaffin-like cells. *American Journal of Physiology* **277**, G945–955.

Rorsman, P., Eliasson, L., Renström, E., Gromada, J., Barg, S. & Göpel, S. (2000). The cell physiology of biphasic insulin secretion. *News in Physiological Sciences* **15**, 72–77.

Sakai, H., Tabuchi, Y., Kakinoki, B., Seike, H., Kumagai, S., Matsumoto, C. & Takeguchi, N. (1995). Ca^{2+}-activated outward-rectifier K^+ channels and histamine release by rat gastric enterochromaffin-like cells. *European Journal of Pharmacology* **291**, 153–158.

Sandvik, A. K. & Waldum, H. L. (1991). CCK-B (gastrin) receptor regulates histamine release and acid secretion. *American Journal of Physiology* **260**, G925–928.

Schweitz, H., Heurteaux, C., Bois, P., Moinier, D., Romey, G. & Lazdunski, M. (1994). Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, is a potent blocker of high-threshold Ca^{2+} channels with a high affinity for L-type channels in cerebellar granule neurons. *Proceedings of the National Academy of Sciences of the USA* **91**, 878–882.

Scott, R. H., Sweeney, M. I., Kobrinsky, E. M., Pearson, H. A., Timms, G. H., Pullar, I. A., Wedley, S. & Dolphin, A. C. (1992). Actions of arginine polyamine on voltage- and ligand-activated whole cell currents recorded from cultured neurons. *British Journal of Pharmacology* **106**, 199–207.

Seva, C., Scemama, J. L., Pradayrol, L., Sarfati, P. D. & Vaysse, N. (1994). Coupling of pancreatic gastrin/cholecystokinin-B (G/CCK_B) receptors to phospholipase C and protein kinase C in AR4–2J tumoral cells. *Regulatory Peptides* **52**, 31–38.

Taylor, C. W. & Broad, L. M. (1998). Pharmacological analysis of Ca^{2+} signalling: problems and pitfalls. *Trends in Pharmacological Sciences* **19**, 370–375.

Teramoto, T., Niidome, T., Miyagawa, T., Nishizawa, Y., Katayama, K. & Sawada, K. (1995). Two types of calcium channels sensitive to omega-agatoxin-TK in cultured rat hippocampal neurones. *NeuroReport* **6**, 1684–1688.

Treiman, M., Caspersen, C. & Christensen, S. B. (1998). A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases. *Trends in Pharmacological Sciences* **19**, 131–135.

Triggle, D. J. & Janis, R. A. (1987). Calcium channel ligands. *Annual Review in Pharmacology & Toxicology* **27**, 347–369.

Triggle, D. J. & Rampe, D. (1989). **1**,4-Dihydropyridine activators and antagonists: structural and fuctional distinctions. *Trends in Pharmacological Sciences* **10**, 507–511.

Yamada, H., Yamamoto, A., Takahashi, M., Michibata, H., Kumon, H. & Moriyama, Y. (1996). The L-type Ca^{2+} channel is involved in microvesicle glutamate exocytosis from rat pinealocytes. *Journal of Pineal Research* **21**, 165–174.

Zeng, N., Athmann, C., Kang, T., Lyu, R. M., Walsh, J. H., Ohning, G. V., Sachs, G. & Pisegna, J. R. (1999*a*). PACAP type 1 receptor activation regulates ECL cells and gastric acid secretion. *Journal of Clinical Investigation* **104**, 1383–1391.

Zeng, N., Athmann, C., Kang, T., Walsh, J. H. & Sachs, G. (1999*b*). Role of neuropeptide-sensitive L-type Ca^{2+} channels in histamine release in gastric enterochromaffin-like cells. *American Journal of Physiology* **277**, G1268–1280.

Zeng, N., Kang, T., Lyu, R.-M., Wong, H., Wen, Y., Walsh, J. H., Sachs, G. & Pisegna, J. R. (1998). The pituitary adenylate cyclase activating polypeptide type 1 receptor (PAC-R) is expressed on gastric ECL cells: evidence by immunocytochemistry and RT-PCR. *Annals of the New York Academy of Sciences* **865**, 147–156.

Zeng, N., Walsh, J. H., Kang, T., Helander, K. G., Helander, H. F. & Sachs, G. (1996). Selective ligand-induced intracellular calcium changes in a population of rat isolated gastric endocrine cells. *Gastroenterology* **110**, 1835–1846.

Zhao, C.-M., Chen, D., Lintunen, M., Panula, P. & Håkanson, R. (1999). Secretory organelles in rat stomach ECL cells. An immunohistochemical and electron-microscopic study. *Cell and Tissue Research* **298**, 457–470.

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