Role of neuropeptide-sensitive L-type Ca\textsuperscript{2+} channels in histamine release in gastric enterochromaffin-like cells

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Zeng, Ningxin, Christoph Athmann, Tao Kang, John H. Walsh, and George Sachs. Role of neuropeptide-sensitive L-type Ca\textsuperscript{2+} channels in histamine release in gastric enterochromaffin-like cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1268–G1280, 1999.—Peptides release histamine from enterochromaffin-like (ECL) cells because of elevation of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) by either receptor-operated or voltage-dependent Ca\textsuperscript{2+} channels (VDCC). To determine whether VDCCs contribute to histamine release stimulated by gastrin or pituitary adenylate cyclase-containing neuroendocrine cells in fun dic gastric mucosa that plays a central role in the peripheral regulation of mammalian gastric acid secretion (19, 20, 52).

Studies using a preparation of isolated, highly enriched ECL cells have shown that these cells display some of the characteristic features of neuroendocrine cells, but their stimulus-secretion coupling is often like that of nonexcitable cells such as the mast cell (3, 34, 47). Activation of histamine secretion requires specific signaling on a distinct receptor distribution on ECL cells (32, 47, 48, 52). ECL cells respond to the antral hormone gastrin with histamine secretion, activation of the histamine-synthesizing enzyme histidine decarboxylase (HDC), and cellular proliferation (49). Other stimulatory receptors such as pituitary adenylate cyclase-activating polypeptide (PACAP), adrenergic, and cytokine receptors have been found, as well as inhibitory receptors such as somatostatin, galanin, and peptide YY (PYY) receptors (59–62). Although several studies clearly indicate that an intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}-dependent pathway is required for histamine release, the specific cellular mechanisms that regulate histamine release have yet to be delineated (47, 61).

Electrophysiological studies have shown that the ECL cell has a resting potential of −58 mV, and pharmacological studies demonstrate that L-type Ca\textsuperscript{2+} channels are the main VDCCs present in rat ECL cells (3, 34). L-type Ca\textsuperscript{2+} channels are believed to

CALCIUM CHANNELS in neuroendocrine cells are important because Ca\textsuperscript{2+} entry through these channels controls a wide variety of their physiological functions, such as transmitter release, firing patterns, biochemical processes, bioenergetics, and gene expression (28). The three types of known Ca\textsuperscript{2+} entry pathways are voltage-dependent Ca\textsuperscript{2+} channels (VDCC), receptor-operated Ca\textsuperscript{2+} channels (ROCC), and reversal of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (21, 28, 38, 42). VDCCs are expressed in excitable neurons, muscle cells, and all neuroendocrine cells so far studied, such as hypothalamic, pituitary, glomus, and C, neuroendocrine pulmonary, chromaffin, enterochromaffin, CCK-secreting, and pancreatic \(\alpha\)- and \(\beta\)-cells (28).

Distinct types of VDCCs have been defined according to various pharmacological and electrophysiological criteria: L-type channels are dihydropyridine sensitive, whereas N-type channels are \(\omega\)-conotoxin GVIA (\(\omega\)-CTx GVIA) sensitive. P- and Q-type channels are distinguished on the basis of differential sensitivities to \(\omega\)-agatoxin IVA, and the R-type component of the high-voltage-activated current is resistant to dihydropyridines and toxins (28, 54). Cloning has shown an even greater diversity among VDCCs. At least six different genes have been identified, and even greater functional diversity might be produced by alternative splicing of the different gene products (28, 54). These channels differ considerably in their responsiveness to neuromodulators, their distribution among various cell types, and their localization in different regions within individual cells (9, 14, 35). There is now general agreement that, even in a single cell, different subtypes of VDCCs can be coexpressed (9, 14, 35).

The enterochromaffin-like (ECL) cell is a histamine-containing neuroendocrine cell in fundic gastric mucosa, and its stimulus-secretion coupling is often like that of nonexcitable cells such as the mast cell (3, 34, 47). Activation of histamine secretion requires specific signaling on a distinct receptor distribution on ECL cells (32, 47, 48, 52, 61). ECL cells respond to the antral hormone gastrin with histamine secretion, activation of the histamine-synthesizing enzyme histidine decarboxylase (HDC), and cellular proliferation (49). Other stimulatory receptors such as pituitary adenylate cyclase-activating polypeptide (PACAP), adrenergic, and cytokine receptors have been found, as well as inhibitory receptors such as somatostatin, galanin, and peptide YY (PYY) receptors (59–62). Although several studies clearly indicate that an intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i})-dependent pathway is required for histamine release, the specific cellular mechanisms that regulate histamine release have yet to be delineated (47, 61).

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contribute to exocytosis in many neuroendocrine cells, such as insulin secretion from pancreatic β-cells (34), catecholamines from chromaffin cells (14, 30, 33, 35, 50), 5-HT from enterochromaffin cells (59), and many other cell types (9–11, 27, 39). However, whether L-type Ca²⁺ channels contribute to activation of secretion of histamine from rat ECL cells is not known. In the present study, this issue was addressed by RT-PCR to analyze the expression of L-type Ca²⁺ channels and by video imaging to monitor single cell [Ca²⁺]i responses. Contributions of the distinct Ca²⁺ receptor antagonists to the release of histamine measured by RIA were evaluated by using L- and N-type antagonists after cell depolarization by high K⁺ or by tetraethylammonium (TEA⁺). In addition, we also investigated whether L-type Ca²⁺ channels are functionally altered by gastrin and neuropeptides such as PACAP, PYY, somatostatin, and galanin and whether histamine release from ECL cells induced by depolarization affects parietal cells in intact gastric gland preparations.

We found that high-K⁺ solution induced dose-related histamine release from rat ECL cells and H₂ histamine-receptor antagonist-sensitive Ca²⁺ signals in rabbit glands. L-type VDCCs were found to be the main contributors, accounting for 50% of K⁺ depolarization and 95% of TEA⁻-induced histamine release. L-type Ca²⁺ channels also contribute 40% of the PACAP-induced Ca²⁺ entry signal, as well as histamine release, but are probably not involved in gastrin-induced Ca²⁺ entry and histamine release (<10%). In ECL cells, L-type Ca²⁺ channels are completely inhibited by somatostatin, galanin, and PYY.

**METHODS**

Isolation and purification of ECL cells. The rat gastric ECL cells were isolated by a combination of elutriation and density gradient centrifugation as described previously (47, 61). Five rat stomachs provided 1 × 10⁶ small cells in the low-density layer. Cell viability was >95% as determined by trypan blue exclusion. Cell purification was quantified by immunostaining with anti-HDC antibody and antihistamine antibody and by using the fluorescent dye acridine orange, which accumulates with a red shift in fluorescence in the acidic, histamine-containing vacuoles. A majority (65–75%) of cells in the freshly isolated cell population were ECL cells. Freshly isolated ECL cells were rinsed by gentle centrifugation in growth medium containing DMEM/F-12, supplemented with 2 mg/ml BSA, 2.5% FCS, 100 µM hydrocortisone, 1% penicillin, streptomycin, and 5 mg/ml insulin, 5 mg/ml transferrin, and 5 µg/l sodium selenite. After 48 h in culture, the ECL cell population was enriched to >85%. All Ca²⁺ imaging experiments and histamine release studies were carried out on these cultured cells after 2–4 days in culture at 37°C.

RT-PCR analysis of dihydropyridine and α-CTx-GVIA-sensitive Ca²⁺ channels. Molecular cloning and heterologous expression systems have shown that the different α₁-subunits give rise to Ca²⁺ channels that reflect the electrophysiological and pharmacological classification. The α₁C- and α₁D-subunits produce L-type Ca²⁺ currents that are sensitive to dihydropyridines, and the α₁E-subunit produces an N-type Ca²⁺ channel that is sensitive to α-CTx-GVIA. The α₁A-subunit expresses a ω-agatoxin-IVA-sensitive current resembling both P- and Q-type Ca²⁺ channels, possibly differentiated by alternative splicing of the α₁A-subunit transcript or by association with different auxiliary subunits. The α₁E-subunit encodes for a Ca²⁺ channel characterized by voltage activation properties at relatively negative potentials, fast kinetics, and insensitivity to known toxins or drugs. Whether the α₁A-subunit represents the R-type or other types of Ca²⁺ currents remains to be determined.

RT-PCR was used to identify the presence of VDCCs in ECL cells and parietal cells. The same amount of total RNA (5 µg) isolated from enriched rat ECL cells or from parietal cells was used to make cDNA by RT with oligo(dT)₁₅ primers. The sample without RT was used as a control. PCR was performed in low-salt TaqDNA polymerase buffer and 5 units Taq DNA polymerase (Stratagene) in the presence of oligonucleotide primers under the following conditions: initial step (one cycle), 94°C for 2 min, 57°C for 1 min, and 72°C for 2 min; repeating steps (35 cycles), 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; and extension step (one cycle), 94°C for 1 min, 57°C for 1 min, and 72°C for 5 min. The primers were designed based on the cloned rat Dar1 sequence, and the primer sense and antisense sequences are summarized in Table 1. The RT-PCR products were amplified and cloned into pCR-II vector (Invitrogen, San Diego, CA). The cloned PCR products were sequenced on both strands by the chain termination method using T7 and SP6 primers to confirm the fragments were the portions of the Ca²⁺ channels.

Measurement of [Ca²⁺]i in isolated cells using video microscopy. After 48-h culture, cells were loaded with 2 µM fura-2 AM for 30 min at 37°C, and then the coverslip was washed with growth medium and placed in a heated chamber (Medical Systems, Greenvale, NY). The temperature was maintained constant at 37°C. The coverslip was perfused with Ringer buffer with different concentrations of KCl or TEA⁺ at a rate of 5 ml/min, and the effluent was constantly removed with a peristaltic pump. Fura 2 fluorescence was measured by using a Nikon Fluov ×40 objective with a Zeiss Axiovert 100TV

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HDC, histidine decarboxylase.
microscope (Zeiss, Thornwood, NY) connected to a PC-XT computer programmed to rapidly alternate between the excitation wavelengths of 340 and 380 nm. The emission wavelength (505 nm) was constant. Image pairs were captured under the control of Image-1/FL software (Universal Imaging, West Chester, PA) and expressed as the ratio of fluorescence level in the chosen field. Maximal and minimal fluorescence was achieved by adding 10 μM ionomycin followed by 20 mM EGTA. [Ca$^{2+}$]$_i$ was calculated using previously described formulas with slight modification (47, 61). All data presented in the Figs. 1–10 are representative of at least four experiments.

Confocal microscopy of rabbit gastric glands. The preparation of rabbit fundic gastric glands and measurement of [Ca$^{2+}$]$_i$ by confocal microscopy are the same as described elsewhere (N. Zeng, unpublished observations). Briefly, under anesthesia, high-pressure aortic perfusion with mammalian Ringer was used to loosen the epithelial layer from the submucosa. The stomach was removed, and the epithelium was stripped off, minced with scissors, and digested with collagenase. The glands were washed by allowing them to settle on the coverslips and then kept at room temperature until use. This is a preparation that responds to histamine, gastrin, and carbachol in terms of stimulation of acid secretion. Changes in Ca$^{2+}$ were monitored on a selected region during the perfusion with Ringer buffer as noted. The relative depolarization on ECL cells in situ and consequent effects on parietal cells. It was expected that the ECL cell of rabbit has a generally similar response to that of rat, and this expectation is borne out by our experiments.

The glands were loaded with fluo 4-AM at a concentration of 5 μM for 30 min on a coverslip coated with Cell-TAK before they were transferred to a superfusion chamber at 37°C. The dye fluorescence was measured at 525 nm, with excitation at 488 nm. The high-K$^+$ solutions (40 mM) were added during the perfusion with Ringer buffer as noted. The relative changes in [Ca$^{2+}$]$_i$ were monitored on a selected region during the experiment, visualizing individual gastric glands at ×63 magnification, scanning 512 × 512 pixels every other second. The region of interest was highlighted on the image, with numbers indicating the position of each image on the scan. Changes in Ca$^{2+}$ in ECL cells and parietal cells were measured. The images are representative of at least four experiments.

Histamine release. Histamine release was determined following 48-h culture by incubating ECL cells on Cell-TAK precoated coverslips in six-well plates. Growth medium was replaced 3 h before the experiment. The cultured ECL cells were rinsed with Ringer buffer and incubated in test medium for 60 min at 37°C. Approximately 20,000 cells per well were seeded for the experiment. The test medium contained, as necessary, different concentrations of K$^+$ or TEA$^-$ in Ringer medium. Na$^+$ was replaced by K$^+$ or TEA$^-$, retaining the same osmolarity in different test solutions. Histamine concentrations were determined as previously described by using a commercially available kit (AMAC, Westbrook, MA). The data are presented as percent over basal (control).

Statistical analysis. Results are means ± SE. Where appropriate, statistical analysis of the data was performed by Mann-Whitney U test if the Kruskal-Wallis indicated a significant difference between multiple groups. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni. Differences between paired groups were determined using a paired t-test. Median effective concentration and IC$_{50}$ were calculated by using linear regression analysis. Values were considered statistically significant when P < 0.05.

Materials. Rabbit anti-HDC antibody and monoclonal antibody against somatostatin (S6) were obtained from the Antibody Core of the CURE Digestive Diseases Research Center. All other reagents were analytical grade and were purchased from the indicated sources. Pronase E was from Boehringer Mannheim. Taq$^+$ DNA polymerase and pCR-Script Amp SK(+) plasmid were from Stratagene (La Jolla, CA). PCR primers for β-actin were from Clontech (Palo Alto, CA). Model 391 PCR-MATE ABI was from Perkin-Elmer (Foster City, CA). Gastrin-17 and galanin were from Peninsula. PACAP-27 was from American Peptide. BSA, acridine orange, DMEM/F-12, insulin-transferrin-sodium selenite media supplement, hydrocortisone, trypan blue, gastrin, somatostatin, goat anti-rabbit fluorescein-conjugated IgG, ionomycin, TEA, and EGTA were all from Sigma; Cell-TAK was from Collaborative Research; Nycodenz was from Accurate Chemical (Westbury, NY); and fura 2 was from Molecular Probes (Eugene, OR).

RESULTS

Selective expression of dihydropyridine- and ω-CTx-GVIA-sensitive Ca$^{2+}$ channels on ECL cells. For analysis of dihydropyridine- and ω-CTx-GVIA-sensitive Ca$^{2+}$ channel expression by RT-PCR, 5 μg total RNA extracted from purified rat ECL or parietal cells was used to make cDNAs as described previously using RT. The same amount of cDNA from purified ECL cells and parietal cells was used for PCR and amplification of HDC; H$^-$-K$^+$-ATPase and β-actin were used as internal controls.

By using dihydropyridine-sensitive Ca$^{2+}$ channel primers, as shown in Fig. 1, a band of 437-bp DNA fragment was obtained from ECL cDNA but not from the purified parietal cell cDNA.

Amplification of the same cDNA samples using primers for ω-CTx-GVIA-sensitive Ca$^{2+}$ channels provided a 671-bp product from ECL cDNA but not from parietal cell cDNA. These products were identical in size to that calculated from the cDNA sequences of rat dihydropyridine- and ω-CTx-GVIA-sensitive Ca$^{2+}$ channels, respectively (28, 45, 54). No products were obtained from negative controls, which contained all components except RT, ruling out the possibility of genomic DNA contamination (data not shown). The products of PCR were then sequenced and were 100% identical to dihydropyridine- and ω-CTx-GVIA-sensitive Ca$^{2+}$ channels.

These data demonstrated that the α1-subunits of dihydropyridine-sensitive and of ω-CTx-GVIA-sensitive Ca$^{2+}$ channels are expressed on rat ECL cells but not on parietal cells.

K$^+$ depolarization induced elevation of [Ca$^{2+}$]$_i$. Elevation of extracellular K$^+$ concentration depolarizes cells because of changes in the K$^+$ equilibrium potential. To determine whether VDCCs are involved in the Ca$^{2+}$ entry in ECL cells, fura 2-loaded ECL cells were perfused with HEPES buffer containing from 5 (basal) to 60 mM K$^+$ that substituted for Na$^+$. In the presence of Ringer buffer containing 5 mM extracellular K$^+$, the resting [Ca$^{2+}$]$_i$ in ECL cells was found to be from 70 to
146 nM and remained unchanged for up to 3–5 h at 37°C as described previously (61). Brief exposure (1 min) to elevated extracellular K⁺ from 5 (basal) to 10, 20, 40, and 60 mM resulted in a rapid (peak in 5–10 s) transient increase in [Ca²⁺]i that fell rapidly, as shown in Fig. 2A. With prolonged exposure to an increase in K⁺, for example 40 mM, a typical biphasic [Ca²⁺]i increase was found in 85% of the cells observed as an initial transient followed by a plateau phase (Fig. 2B), reminiscent of the gastrin or PACAP response of the ECL cells. Some cells even had a sustained [Ca²⁺]i increase when the K⁺ concentration was ,20 mM (data not shown).

The increase of [Ca²⁺]i, induced by K⁺ was dependent on extracellular Ca²⁺ because addition of an excess of EGTA (3 mM) during the plateau phase resulted in a rapid decline of [Ca²⁺] toward basal levels (Fig. 2C). Both initial and sustained Ca²⁺ signals were absent during brief or prolonged K⁺ stimulation when Ca²⁺-free solutions were used containing 100 µM EGTA (data not shown).

Fig. 1. Selective expression of dihydropyridine- and ω-conotoxin-GVIA (ω-CTx-GVIA)-sensitive Ca²⁺ channels on enterochromaffin-like (ECL) cells as determined by RT-PCR. RNA isolated from parietal cell and ECL cell preparations (top) was probed with selective primers for these channels. Bands shown by ethidium bromide staining (bottom) were confirmed by dideoxynucleotide sequencing.

TEA⁺, which blocks outward K⁺ currents and depolarizes the membrane potential, evoked Ca²⁺ oscillations, which were dependent on extracellular Ca²⁺ at concentrations up to 30 mM. At 40 mM, the effect mimicked that of K⁺ depolarization, inducing a transient and steady-state response (Fig. 3). Hence, membrane depolarization activates Ca²⁺-signaling pathways in ECL cells, whether typically biphasic or oscillatory.

Flufenamic acid has been shown to stimulate outward K⁺ currents and to hyperpolarize the resting membrane potential of a variety of cell types as a result of activation of K⁺ channels (51). The effects of flufenamic acid (200 µM) on [Ca²⁺]i are shown in Fig. 4. Perfusion with flufenamic acid significantly reduced basal [Ca²⁺] and K⁺ depolarization-induced increase of [Ca²⁺].

High-K⁺ solution- and TEA⁺-induced histamine release requires external Ca²⁺. To determine the functional effect of high-K⁺ and TEA⁺ depolarization on isolated gastric ECL cells, the release of histamine from ECL cells was examined after exposure to different concentrations of KCl and TEA⁺.

Under basal conditions, cultured ECL cells release 40 pmol histamine per well. In the presence of 1.8 mM Ca²⁺ in the incubation medium, K⁺ depolarization elevated histamine release from cultured ECL cells in a dose-related manner, with maximal release being 500% of basal at 40 mM (Fig. 5A). Addition of TEA⁺ to the incubation medium induced a dose-dependent increase in histamine release, with a maximal threefold increase at 30 mM (Fig. 5B). Increasing TEA⁺ to 40 mM did not increase the histamine release further. The minimum effective dose of TEA⁺ was 10 mM, and the
EC_{50} was 20 mM. However, in Ca^{2+}-deficient medium, high-K^{+} depolarization- and TEA^{1-}-induced histamine release were completely abolished (Fig. 5C). These results suggested that K^{+} depolarization- and TEA^{1-}-induced histamine release from ECL cells depended on Ca^{2+} entry from the extracellular space.

Effects of high-K^{+} depolarization on rabbit gastric glands. As shown in Fig. 6, ECL cells load with the fluo 4 dye to a greater extent than parietal cells, presumably because of accumulation of the dye in the secretory vacuoles. In the confocal microscope, only single-plane sections are shown here, visualizing only a single ECL cell but several parietal cells, reflecting the relative numbers of these two cell types. The scan of a defined region of the images in the whole sequence shows the Ca^{2+} signal in a specific cell type.

As shown in Fig. 6A, there was a biphasic increase of [Ca^{2+}] in the ECL cells (identified either by high-fluo 4 fluorescence or subsequent accumulation of acridine orange in their vacuoles) due to the addition of 40 mM KCl to the perfusate (green line). This is visualized in the displayed images selected from the recorded sequence and numbered on the recording of the signal. The increase of [Ca^{2+}] in the ECL cells was followed, after a lag phase, by a Ca^{2+} transient in parietal cells, as shown in the Ca^{2+} scans of selected cells illustrated (red and orange lines). The data presented are typical of several experiments in which both cell types were stimulated, but the parietal cell signal always followed the ECL cell signal with a significant delay (~100 s).

The transient in the parietal cells but not in the ECL cells was blocked by the H_{2}-receptor antagonist ranit-
Histamine itself produced a transient signal in parietal cells but not in ECL cells, as has been shown previously. Thus high-K\textsuperscript{+} depolarization in this more integrated model stimulates ECL cell Ca\textsuperscript{2+} signaling and then, via histamine release and activation of the H\textsubscript{2} receptor, parietal cell Ca\textsuperscript{2+} signals. Because the presence of H\textsubscript{2}-receptor antagonists ablated the effect of depolarization by high K\textsuperscript{+} on parietal cells but not on ECL cells, there can be no functional VDCCs on parietal cells, confirming the RT-PCR results.

Contribution of L-type Ca\textsuperscript{2+} channels to high K\textsuperscript{+} and TEA\textsuperscript{+}-induced Ca\textsuperscript{2+} entry. K\textsuperscript{+} depolarization induces Ca\textsuperscript{2+} entry via VDCCs in many types of neuroendocrine cells. VDCCs have been classified into at least six types (L, N, P, Q, T, and O) on the basis of electrophysiological and pharmacological criteria. Multiple brief applications of 40 mM K\textsuperscript{+} (1 min) produce [Ca\textsuperscript{2+}]\textsubscript{i} transients of identical amplitude, as described. Therefore, we pulsed KCl to examine the pharmacology of subtypes of VDCCs in ECL cells.

L-type channel blockade by 2 µM nifedipine inhibited 50% of [Ca\textsuperscript{2+}]\textsubscript{i} elevation (Fig. 7A) and histamine release (Fig. 7B). N-type channel blockade by 1 µM ω-CTx-GVIA inhibited 25% of [Ca\textsuperscript{2+}]\textsubscript{i} elevation and 14% of histamine release, which was not statistically significant (Fig. 7, A and B). The combination was apparently additive, resulting in 70% inhibition. The elevation of [Ca\textsuperscript{2+}]\textsubscript{i} due to TEA\textsuperscript{+} blockade and the histamine release were inhibited by 2 µM nifedipine and Ca\textsuperscript{2+} removal (Fig. 7, C and D). BAY K 8644, a compound that activates L-type Ca\textsuperscript{2+} channels, at 1 µM alone also induced a biphasic Ca\textsuperscript{2+} increase in 48% of the ECL cell population as well as a significant increase in basal and high K\textsuperscript{+} or TEA\textsuperscript{+}-induced histamine release (Fig. 7, E and F).

Modulation of L-type Ca\textsuperscript{2+} channels by peptide ligands. Gastrin and PACAP showed the same efficacy in stimulation of histamine release, namely a fourfold increase over basal from isolated rat ECL cells (49, 59). As shown in Fig. 8, both gastrin (10\textsuperscript{-9} M) and PACAP (10\textsuperscript{-9} M) stimulated a biphasic Ca\textsuperscript{2+} signal. Nifedipine gave <10% inhibition on gastrin-induced Ca\textsuperscript{2+} entry and histamine release (Fig. 8A). However, nifedipine partially inhibited the PACAP-induced Ca\textsuperscript{2+} transient and 40% of the PACAP-induced histamine release (Fig. 8B). Part of the action of PACAP is via modulation of other Ca\textsuperscript{2+} pathways.

Fig. 5. High K\textsuperscript{+} and TEA\textsuperscript{+}-induced histamine release requires external Ca\textsuperscript{2+}. A: in presence of 1.8 mM Ca\textsuperscript{2+} in incubation medium, K\textsuperscript{+} depolarization induced histamine release from cultured ECL cells in a dose-related manner, with maximal release (fivefold above basal) at 40 mM. B: addition of TEA at incubation medium from 5 to 30 mM induced a dose-dependent increase in histamine release, with maximal release at 30 mM (threefold of basal). C: in Ca\textsuperscript{2+}-deficient medium, high-K\textsuperscript{+} depolarization- and TEA\textsuperscript{+}-induced histamine release was completely abolished.

Fig. 4. Effect of flufenamic acid. Perfusion with flufenamic acid significantly reduced basal [Ca\textsuperscript{2+}]\textsubscript{i} (top) and K\textsuperscript{+} depolarization-induced increase of [Ca\textsuperscript{2+}]\textsubscript{i} (bottom). Top: typical set of basal Ca\textsuperscript{2+} recordings from different ECL cells. Bottom: effect on peak Ca\textsuperscript{2+} induced by K\textsuperscript{+} depolarization.
The effect of inhibitory peptides on L-type Ca\(^{2+}\) channels was also investigated in this isolated cell model. As shown in Fig. 9, somatostatin, PYY, and galanin completely inhibited TEA\(^{-}\)-induced Ca\(^{2+}\) oscillations as well as histamine release, with maximal inhibition of 90%. The BAY K 8644-induced histamine release was also totally inhibited by these neuropeptides (Fig. 9B). Somatostatin, PYY, and galanin also inhibited high-K\(^{+}\)-induced [Ca\(^{2+}\)]\(_i\) increases and histamine release, with an EC\(_{50}\) of 10\(^{-9}\), 10\(^{-9}\), and 10\(^{-8}\) M, respectively (data not shown). These are universal inhibitors of ECL cell function.

Modulation of G proteins in VDCCs. The inhibitory effects of somatostatin, galanin, and PYY on gastrin-induced Ca\(^{2+}\) entry and histamine release are sensitive to pertussis toxin, indicating that these neuropeptide receptors are coupled to G\(_i\) or G\(_o\) proteins (12, 23, 25, 26, 37). Pretreatment of the cultured ECL cells with pertussis toxin (200 ng/ml) for 16 h abolished the inhibitory action of somatostatin and PYY on both Ca\(^{2+}\) signaling...
and histamine release and partially reduced the inhibition by galanin, as shown in Fig. 10.

**DISCUSSION**

Some neuroendocrine cells share characteristics with neuronal cells, such as their secretory mechanisms, membrane trafficking proteins, and electrical excitability (11, 28, 52, 58). Some neuroendocrine cells can fire action potentials in response to membrane depolarization (11, 28, 52, 58). The depolarization-induced action potentials cause corresponding rises in \([Ca^{2+}]_i\) and thereby \(Ca^{2+}\)-dependent hormone or transmitter release (11). The \(Ca^{2+}\) influx due to membrane depolarization occurs mainly by voltage-dependent \(Ca^{2+}\) channels in neurons as well as in most types of neuroendocrine cells.

A rise in \([Ca^{2+}]_i\) is an obligatory step in stimulus-secretion coupling in ECL cells due to gastrin or PACAP (47, 52, 61). The ECL cells produce and store histamine (19, 20). This biogenic amine is stored in vesicles with a total content of 2.8–4.3 pg/cell of histamine, which is a relatively low amount compared with mast cells (12–20 pg/cell). The cytoplasm of ECL cells shows numerous hollow vesicles, which contain histamine, and a characteristic electron-dense core inside these electron-lucent vesicles, which is thought to represent the storage of a peptide hormone (19, 20). The exocytotic process of histamine release involves members of membrane-trafficking protein families such as vesicle-associated membrane protein, synaptosome-associated protein, or syntaxins (24). In isolated rat ECL cells, the removal of extracellular \(Ca^{2+}\) abolishes the secretion of histamine in response to gastrin, showing that influx of \(Ca^{2+}\) into the ECL cell is required for histamine release (47, 61). Patch-clamp analysis has shown the presence of voltage-gated \(Ca^{2+}\) currents in rat gastric ECL cells, inhibited by verapamil and augmented by the L-type \(Ca^{2+}\) channel activator BAY K 8644 (3), but their functional significance was not analyzed.

Excitation-secretion coupling in many endocrine cells is dependent on changes in membrane voltage, which is controlled by ion channels (57, 58). Modulation of ion channels causes changes in resting membrane potential that lead to alteration in \(Ca^{2+}\) influx and hormone secretion. In most endocrine cells, the resting membrane voltage is regulated by \(K^+\) channels (57, 58). ECL cells have a resting potential of \(-58\) mV. Electrophysiological and pharmacological studies have provided...
evidence for depolarization-activated K⁺ channels and Ca²⁺ channels in gastric ECL cells (3). It has been established that K⁺ channels are important modulators of hormone secretion in many types of endocrine cells (3, 34). Blocking K⁺ channels induces catecholamine release from adrenal medullary cells, corticoid secretion from adrenocortical cells, insulin secretion from pancreatic β-cells, and prolactin secretion from pituitary cells (4, 17, 57).

In this study, we confirmed that L- and N-type Ca²⁺ channels are selectively expressed in the gastric ECL cells by measurements of Ca²⁺ signals and RT-PCR. In addition, we found that Ca²⁺ influx through L-type VDCCs induced increases in [Ca²⁺]ᵢ and exocytosis in gastric ECL cells.

First, both high-K⁺ and TEA⁺ depolarization-induced histamine release depended on external Ca²⁺ and were partially or completely inhibited by nifedipine, respectively, at the concentrations that target L-type channels. Second, BAY K 8644 significantly enhanced the depolarization-induced histamine release. BAY K 8644 alone also induced a biphasic Ca²⁺

Fig. 8. Modulation of L-type Ca²⁺ channels by neuropeptides. Gastrin (A) and pituitary adenylate cyclase-activating peptide (PACAP; B) stimulated a biphasic Ca²⁺ signal, but nifedipine had <10% inhibition on gastrin-induced Ca²⁺ entry and histamine release (A). However, nifedipine partially inhibited PACAP-induced Ca²⁺ transient and 40% of histamine release (B). *P < 0.05.

Fig. 9. Inhibition of L-type Ca²⁺ channels by neuropeptides. Somatostatin (SS), peptide YY (PYY), and galanin (Gal) completely inhibited 20 mM TEA⁺-induced Ca²⁺ oscillations (A) as well as histamine release (B). BAY K 8644-induced histamine release was also totally inhibited by these neuropeptides (B). *P < 0.05.
signal and histamine release from ECL cells, which was totally inhibited by nifedipine. Therefore, an L-type Ca\(^{2+}\) channel, as in other neuroendocrine cells, is present in ECL cells and may play a role in histamine release. This depolarization-induced Ca\(^{2+}\) elevation in isolated ECL cells is also found in intact rabbit gastric glands, and the subsequent [Ca\(^{2+}\)]i response of the parietal cell in this integrated preparation shows that there is depolarization-dependent signaling between these two cell types, implying a physiological significance to the VDCCs found only in the ECL cell.

In the present study, we have also evaluated the effect of blocking K\(^{+}\) channels on histamine release from isolated gastric ECL cells by using the K\(^{+}\) channel blocker TEA\(^{+}\). We found that TEA\(^{+}\) (10–40 mM) evoked histamine release from cultured ECL cells. We have also shown that TEA\(^{+}\)-induced histamine release is absolutely dependent on the presence of external Ca\(^{2+}\) and that this histamine release is completely blocked by nifedipine and profoundly enhanced by the L-type Ca\(^{2+}\) channel agonist BAY K 8644.

Gastrin is the major endocrine ligand that serves to stimulate histamine secretion. This effect is mediated by the binding of gastrin to CCK-B receptors located on the surface of the ECL cell, and secretion can be detected within 5 min (52). The effect of binding the ligand is displayed as a typical G7 receptor-mediated change in [Ca\(^{2+}\)], namely a transient followed by a steady-state elevation. The elevation of Ca\(^{2+}\) during cellular activation by Ca\(^{2+}\)-mobilizing hormones may originate from both release from intracellular Ca\(^{2+}\) stores and entry via membrane Ca\(^{2+}\) channels (61). Patch-clamp experiments have shown that both L- and N-type VDCCs could be activated during exocytosis because of electrical changes after fusion of the histamine-containing vacuole with the plasma membrane of ECL cell (3). The histamine-containing vacuole has a membrane V-type ATPase (VMAT2) that is an electrogenic proton pump. Acidification by this pump depends on the presence of a Cl\(^{-}\) conductance that allows electrogenic proton pumping. Stimulation of histamine release by gastrin resulted in activation of a Cl\(^{-}\)-current, presumably because of fusion of the vacuole membrane with the plasma membrane (34). The depolarization because of this channel could activate VDCCs. However, our data show that the L-type channel is not important in the gastrin-induced histamine release in vitro because nifedipine had no effects on gastrin-induced Ca\(^{2+}\) entry and histamine release. Different results were obtained for stimulation of ECL cells by PACAP.

We previously found that PAC1 receptors are expressed on gastric ECL cells by using RT-PCR and by pharmacological studies measuring Ca\(^{2+}\) signals and histamine release in isolated ECL cells and gastric glands (59). Influx of Ca\(^{2+}\) through different VDCCs has been reported to mediate PACAP-induced catecholamine release from bovine chromaffin cells or insulin release from pancreatic islet \(\beta\)-cells (6, 15, 18, 40, 59). Here, activation of L-type VDCCs also contributed to PACAP-induced Ca\(^{2+}\) entry and histamine release because nifedipine significantly reduced histamine release as well as Ca\(^{2+}\) entry induced by PACAP. It is of interest that PACAP, in contrast to gastrin, is coupled to both adenylyl cyclase and Ca\(^{2+}\) elevation. Elsewhere, we have shown that PACAP addition to rabbit gastric glands elevates ECL cell Ca\(^{2+}\) and also parietal cell Ca\(^{2+}\), the latter effect being completely blocked by H\(_2\)-receptor antagonists. Furthermore, PACAP injection can result in gastric acid stimulation in intact rats, provided the response of the fundic \(D\) cell is blunted by the presence of neutralizing somatostatin antibody (60a). It appears that VDCCs may play a role in PACAP activation of acid secretion.

There are many neuropeptides involved in the inhibition of ECL cell function (48, 60, 62). Somatostatin is known to be a major peptide inhibitor of gastric acid secretion with distinct cellular targets, such as the G cell of the antrum and the ECL cell of the fundus. In the case of the ECL cell, somatostatin, by binding at a

![Fig. 10. Role of G proteins in modulation of L-type activity by neuropeptides. ECL cells pretreated with pertussis toxin (200 ng/ml) for 16 h abolished inhibitory action of somatostatin (SS) and peptide YY (PYY) on BAY K 8644-induced Ca\(^{2+}\) entry (A) and TEA\(^{+}\)-induced histamine release (B) as well as partially reduced inhibition by galanin (Gal; B). \(P < 0.05\).](attachment:image.png)
somatostatin 2 subtype receptor, inhibits both histamine release and Ca\textsuperscript{2+} signaling. PYY is a peptide that also inhibits gastric acid secretion by acting at a variety of locations. Pharmacological evidence in the rat suggests that its site of action on the ECL cell is at a Y\textsubscript{1} receptor of PYY. Interestingly, this peptide blocks the plateau phase of Ca\textsuperscript{2+} signaling but is much less active against the transient elevation of [Ca\textsuperscript{2+}]. (23). The PYY effect may account for much of the inhibitory effect of nutrient presentation to the intestine, classified as an “enterogastrone” effect. Recently, we found that galanin has an equipotent effect to somatostatin in inhibition of ECL function (60).

In the β-cell of the pancreas, somatostatin and galanin inhibit insulin release by blockade of the L-type Ca\textsuperscript{2+} channels (12, 22, 23, 25, 26, 29, 39, 41, 43, 44, 53, 56, 60). PYY is also effective in inhibition of L-type Ca\textsuperscript{2+} channels in PC-12 cells and other neuroendocrine cell lines (55, 62). In our study, we found that these peptides inhibit high-K\textsuperscript{+} and TEA\textsuperscript{1} depolarization-induced Ca\textsuperscript{2+} signals and histamine release as well as BAY K 8644-induced Ca\textsuperscript{2+} signals and histamine release, indicating that, in this cell type, these inhibitory peptides are able to inhibit L-type Ca\textsuperscript{2+} channels. Their effects on TEA\textsuperscript{1} or BAY K 8644-induced Ca\textsuperscript{2+} signals and histamine release were abolished by pretreatment with pertussis toxin, similar to their effects on gastrin stimulation of the ECL cell. Hence, inhibition of either VCDCs or ROCs of these inhibitory peptides is mediated by G\textsubscript{i} trimeric proteins (12, 22, 23, 26, 29, 39, 41, 53).

The importance of N-type Ca\textsuperscript{2+} channels in the stimulation of neuronal transmitter release has been well demonstrated, and numerous neurotransmitters have been found to inhibit transmitter release through inhibition of this channel subtype (9, 14, 30, 33). Our data indicate that N-type Ca\textsuperscript{2+} channels are expressed on ECL cells, but, functionally, they seem less important in stimulus secretion coupling than the L-type Ca\textsuperscript{2+} channels, similar to data obtained for catecholamine release from chromaffin cells (14, 30, 35).

In vivo studies have implied that VCDCs may be involved in the regulation of gastric acid secretion because nifedipine, an L-type Ca\textsuperscript{2+} channel antagonist, could significantly reduce gastric acid secretion in humans and rats (1, 2, 5, 8, 13, 16, 31, 36). This investigation therefore suggests that L-type Ca\textsuperscript{2+} channels, expressed on gastric ECL cells, are functionally coupled to histamine release induced by a neuropeptide, PACAP. The finding that activation of these L-type Ca\textsuperscript{2+} channels is blocked by somatostatin, galanin, and PYY is pertussis toxin sensitive is in agreement with our previous investigations into mechanisms of inhibition of ECL cell function by these neuropeptides. This study, in combination with our previous work, provides additional evidence that regulation of gastric acid secretion by neuropeptides from enteric neurons is exerted via regulation of ECL cell function, in part by activation of VCDCs.

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