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R-Type Ca^{2+}-channel Activity Is Associated with Chromogranin A Secretion in Human Neuroendocrine Tumor BON Cells

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Abstract. This electrophysiological study was undertaken to investigate the role of voltage-operated Ca^{2+} channels (VOCCs) in cultivated human neuroendocrine tumor (NET) cells. Patch-clamp techniques, measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$), and secretion analysis were performed using cultured human NET BON cells. Ba^{2+} inward currents through R-type channels ($Ca_V2.3$) were measured and identified by SNX-482 (10 nM), a novel voltage-sensitive R-type Ca^{2+} channel antagonist. In the presence of nifedipine (5 μM), ω-Conotoxin GVIA (100 nM) and ω-Agatoxin IVA (20 nM), R-type channel currents were also detectable. Release of Ca^{2+} from intracellular Ca^{2+} stores by intracellular application of inositol-1,4,5-trisphosphate ($InsP_3$; 10 μM) via the patch pipette during whole-cell configuration as well as induction of capacitative Ca^{2+} entry (CCE), a passive maneuver to release Ca^{2+} from intracellular Ca^{2+} stores, led to an increase in $[Ca^{2+}]_i$. This effect could be reduced by SNX-482 (20 nM). In addition, SNX-482 (25 nM) also decreased chromogranin A (CgA) secretion, whereas ω-Conotoxin GVIA (500 nM) and nifedipine (5 μM) failed to reduce CgA secretion. We conclude that these data reveal neuronal R-type channel activity ($Ca_V2.3$), for the first time associated with CgA secretion in BON cells. Influx of Ca^{2+} by activation of R-type channels may lead to an increase of intracellular Ca^{2+}, which stimulates CgA secretion. Thus, R-type channels could play an important role in certain clinical characteristics of NETs, such as the hypersecretion syndrome.

Key words: Neuroendocrine tumor — BON — R-type Ca^{2+} channels — Chromogranin A — Intracellular Ca^{2+} — Hypersecretion syndrome

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Introduction

Depending on the primary location, neuroendocrine tumor (NET[1]) cells release hormones and biogenic amines in distinct patterns leading to typical hypersecretion syndromes. For example, the release of serotonine is almost exclusively observed in midgut location, whereas the release of gastrointestinal hormones (e.g., gastrin, insulin) is primarily found in foregut location (Wiedenmann et al., 1998). The release of these peptide hormones, biogenic amines or growth factors is caused by Ca^{2+} entry. Therefore, Ca^{2+} channels could play an important role for secretion of these substances in NET cells. Since electrical properties of cell membranes are involved in these functions of NET, considerable attention has been directed to patch-clamp studies of NET cells. Therefore, voltage-operated Ca^{2+} channels (VOCCs) of the L-, N-, and P/Q-type have been investigated (Scherubl & Hescheler, 1991; Mangel et al., 1993; Snow et al., 1993; Glassmeier et al., 1997). These electrophysiological characteristics apply to both permanent as well as human primary NET cell cultures (Glassmeier et al., 1997). However, the role of each Ca^{2+} subtype channel remained unclear. Investigation of secretion in β-cells of the pancreas revealed that VOCCs of the R-type have an important trigger function for insulin release (Maechler & Wollheim, 1999; Vajna et al., 2001). Thus, the R-type Ca^{2+} channel has functional relevance in the neuroendocrine system (Pereverzev et al., 2002). So far, it is not clear whether R-type Ca^{2+} channels are also

[1]*Abbreviations:* Neuroendocrine tumor (NET); voltage operated calcium channels (VOCCs); capacitative Ca^{2+} entry (CCE), chromogranin A (CgA), ethylene glycol-bis-(β-aminoethyl ether) N, N, N′, N′,-tetraacetic acid (EGTA); N- [hydroxyethyl] piperazine-N′-[2 ethansulfonic acid] (HEPES); tetraethylammonium chloride (TEACl), tris(hydroxymethyl)-amino methane (Tris).

expressed in cultivated NET BON cells and whether these channels are participating in the secretion of certain tumor markers, such as CgA. The aim of this study was to investigate the relationship between distinct Ca^{2+}-channel activities and CgA secretion in human NET BON cells. We therefore analyzed the electrophysiological properties by using patch-clamp techniques (Hamill et al., 1981; Horn & Marty, 1988), fluorometric measurements (Grynkiewicz, Poenie & Tsien, 1985) and secretion analysis.

Materials and Methods

Cell Culture

NET BON cells were used as a representative model of NET disease. This cell line was derived from a human pancreatic NET and was systematically cultured using routine culture conditions and media as previously described (Evers et al., 1994; Ahnert-Hilger et al., 1996). Cultures aged 3–7 days were used for patch-clamp recordings and secretion analysis.

Patch-Clamp Recordings

Coverslips with human NET BON cells were mounted on the stage of a microscope (Olympus, BX50WI) and superfused with an extracellular sodium- and potassium-free bath solution containing (in mmol/L): N-methyl-D-glucamine, 120; CsCl, 5.4; $MgCl_2$, 1.0; glucose, 10; and HEPES acid, 10 (pH adjusted to 7.3). To isolate inward currents through voltage-gated Ca^{2+} channels, Ba^{2+} (10 mM) was used as the charge carrier. Pipettes of soft glass with a resistance of 2–5 MΩ were pulled using a Universal-Puller (Sutter, USA). Pipettes for whole-cell recordings were filled with a pipette solution containing (in mmol/L): CsCl, 130; $MgCl_2$, 4.0; EGTA 10; and HEPES salt, 10 (pH adjusted to 7.3). For perforated patch recordings, the pipette solution contained additionally 20–60 μg/ml nystatin (back-filled). Membrane currents were recorded using an EPC 8 (HEKA, Lamprecht, Germany). Electrical stimulation, data storage, and data processing were performed using TIDA 5.0 software (HEKA, Lamprecht, Germany) in conjunction with a conventional computer (PC). All electrophysiological experiments were performed at room temperature. Membrane capacitance and access resistance were calculated from the capacitance current transient induced by hyperpolarization voltage steps of 50 ms duration and 90 mV amplitude from the holding potential (−70 mV). In the whole-cell configuration, BON cells showed an access resistance of 45 ± 3 MΩ (n = 12) and a mean membrane capacitance of 64 ± 13 pF (n = 12). Pipette capacitance, membrane capacitance and access resistance were compensated by the patch-clamp amplifier. Voltage-dependent Ba^{2+} currents were induced by nine depolarizing voltage steps of 50 ms and 10 mV increasing amplitude at a frequency of 1 Hz from a holding potential of −70 mV. Data are regularly presented as mean ± SEM. Statistical analysis was performed using Student's t-test; p values lower than 0.05 were considered significant.

Measurements of Intracellular Free Ca^{2+}

Human NET BON cells were preincubated with culture medium containing the fluorescent dye fura-2 AM (1 μM) for 45 min. Subsequently, the cells were washed with the extracellular bath solution described above. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were measured by fura-2 fluorescence at room temperature with a digital imaging system (T.I.L.L. Photonics, Munich, Germany). Fura-2 fluorescence was excited alternatively at 340 nm and 380 nm wavelength and changes in $[Ca^{2+}]_i$ were observed based on the ratio of the fluorescence obtained. The concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (nM) was calculated according to Grynkiewicz et al. (1985). For calibration of $[Ca^{2+}]_i$, fluorescence in the presence of EGTA (1 mM) or ionomycin (1 μM) was obtained.

Chromogranin A (CgA) Secretion Analysis

For secretion experiments, human NET BON cells were cultured at a density of 5×10^5 cells/well for 2 days. Cells were preloaded for 2 h at 37°C in Krebs-Ringer-HEPES (KRH) buffer, washed three times with KRH buffer and then treated with the secretagogues or vehicle for 25 min at 37°C. CgA concentrations were then determined using a CgA-specific enzyme-linked immunosorbent assay (Dako, Hamburg, Germany) in supernatants and cell lysates in L-buffer (containing (in mmol/L) Tris-HCl, 130; $CaCl_2$, 10; and NaCl, 75 (pH adjusted to 8.0), and 0.4% Triton X-100). CgA secretion values were determined as percent of secretion related to the total amount of detected CgA. Experiments were routinely conducted at least in triplicates.

Chemicals

Media and supplements for cell culture were purchased from GIBCO-Life Technologies (Eggenstein, Germany) and/or BIOCHROM (Berlin, Germany). ω-Conotoxin GVIA, ω-Agatoxin VIA and SNX-482 were obtained from Alomone labs (Jerusalem, Israel). All other chemicals were obtained from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany), and from RBI Research Biochemicals (Köln, Germany).

Results

Identification of Ca^{2+} Subtype Channels in Human NET BON Cells

Under potassium- and sodium-free bath and pipette solutions, voltage-dependent Ca^{2+} channel inward currents were activated when 10 mM Ba^{2+} was used as charge carrier. These currents activated at potentials more positive than −45 mV (Fig. 1) and showed an inactivation time usually slower than 100 ms (estimated by single-exponential fit). The normalized maximal current amplitude was 3.54 ± 0.83 $pApF^{-1}$ (n = 4) and was reduced by the R-type channel antagonist SNX-482 (10 nM) to 80 ± 8% of control (set to 100%), indicative of R-type channel activity (Fig. 1*C*). Recovery occurred to 103 ± 6% of control (all n = 4). In addition, extracellular application of SNX-482 led to a reduction of intracellular free Ca^{2+} under resting membrane potential conditions from 116 ± 8 nM to 70 ± 4 nM (Fig. 2). Recovery occurred to 103 ± 9 nM (n = 4). Incidentally, L-, N-, and P/Q-type channel activity could also be demonstrated. Initially, L-type channels were measured and identified by the L-type channel opener Bay K 8644 (5 μM). Whereas Bay K 8644 increased the currents to 136 ±

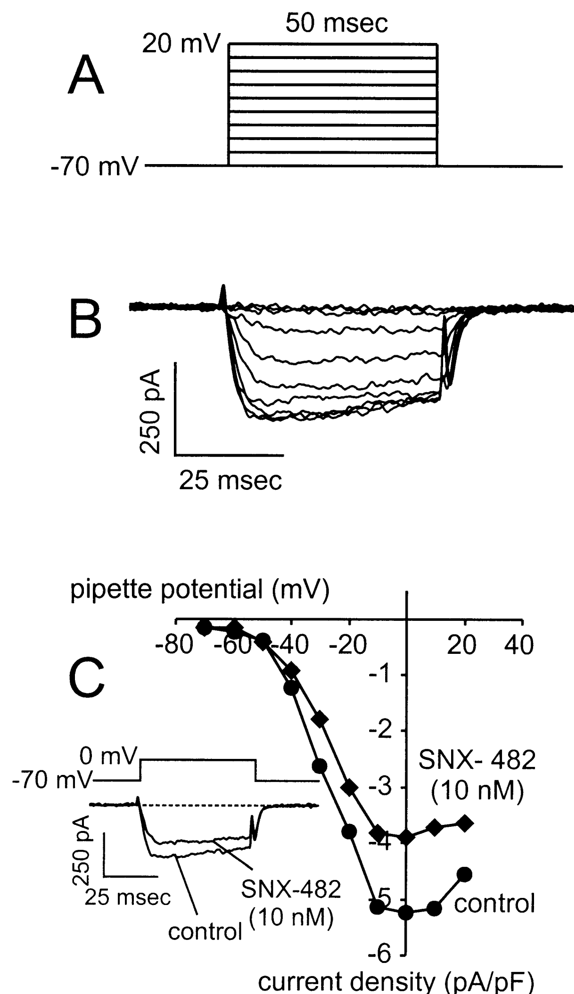


Fig. 1. Identification of Ba^{2+} currents through R-type Ca^{2+} channels ($Ca_V2.3$) in human NET BON cells. (*A*) Pattern of electrical stimulation to induce voltage-dependent inward currents. The membrane potential was clamped to −70 mV. The cell was stimulated by nine voltage steps of 50 ms duration and 10 mV increasing amplitudes. (*B*) Inward currents induced by the electrical stimulation with Ba^{2+} (10 mM) as the charge carrier under extra- and intracellular K^+-, and Na^+-free conditions. (*C*) Effect of extracellular application of SNX-482 (10 nM) summarized in a current/voltage plot and as current traces (*insert*). For the current/voltage relation, maximal peak current amplitudes (pA) were normalized to each cell membrane capacity (pF) (current density; pA/pF) and plotted against the pipette potential (mV) of the electrical stimulation.

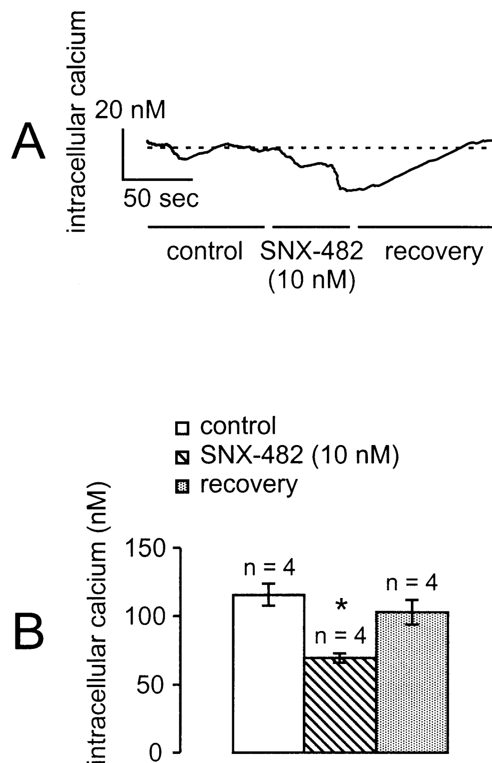


Fig. 2. R-type channel activity in non-stimulated human NET BON cells. (*A*) Effect of extracellular application of SNX-482 (10 nM) on cytosolic free Ca^{2+}. Changes in cytosolic free Ca^{2+} are depicted as the ratio of the fluorescence values induced by excitation wavelengths 340 and 380 nm. Intracellular Ca^{2+} concentration was calculated by the equation from Grynkiewicz et al. (1985). (*B*) Summary of the experiments with SNX-482.

9% (n = 3) of control (Fig. 3), the N-type blocker ω-Conotoxin GVIA (100 nM) (Olivera et al., 1985) decreased the currents only to 78 ± 4% (n = 3) of control (Fig. 4*A*, 4*B*), indicating L-type channel activity at higher levels than N-type channel activity. This also applied to R-type channels. R-type channel activity was at lower levels than L-type channel activity (Fig. 3). In addition, P/Q-type channels could be barely detected by the P/Q-type channel blocker ω-Agatoxin IVA (20 nM) (Fig. 4*C*). After blockage of L-, N-, and P/Q-type channels, residual Ca^{2+} channel currents were detectable (58 ± 7% of control; recovery occurred to 86 ± 11% of control; all n = 4) (Fig. 4*C*). Thus, R-type channel currents could be detected independently from SNX-482. In conclusion, human NET BON cells express R-type channels as well as L-, N-, and P/Q-type channels. These experiments revealed that L-type channel activity is at higher levels than any other subtype channel activity.

Active Depletion of Intracellular Ca^{2+} Stores

To obtain more information about the mechanism of R-type channel activation, release of Ca^{2+} from intracellular Ca^{2+} stores by intracellular application of inositol-1,4,5-trisphosphate ($InsP_3$; 10 μM) via the patch pipette during whole-cell configuration was performed. This led to an activation of Ca^{2+} channels as shown in Fig. 5. One min after establishing the whole-cell configuration, the relative current (measured from a voltage step to 0 mV from a holding potential of −70 mV) was 130 ± 15% (n = 3) of the control, measured after establishing the whole-cell configuration with $InsP_3$ in the patch pipette. Later, at the maximum $InsP_3$-induced increase of Ca^{2+}

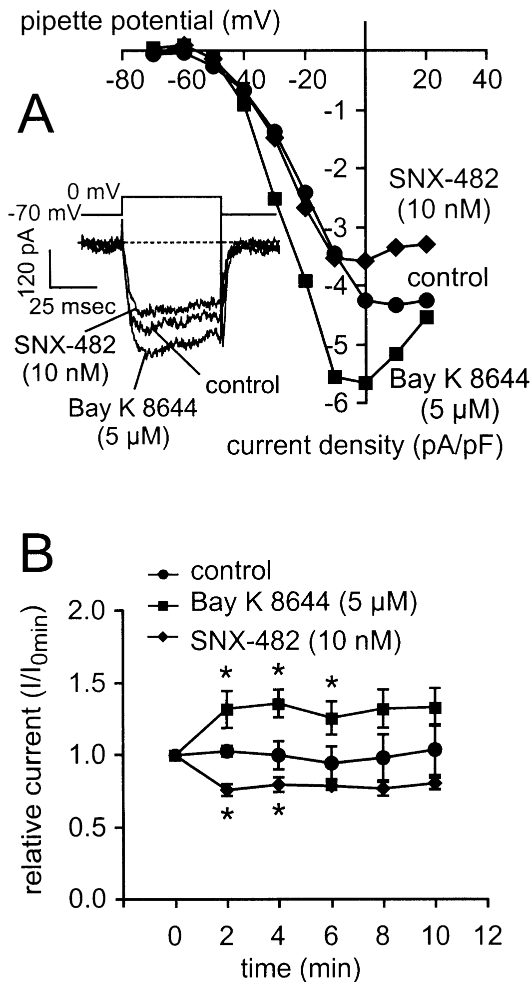
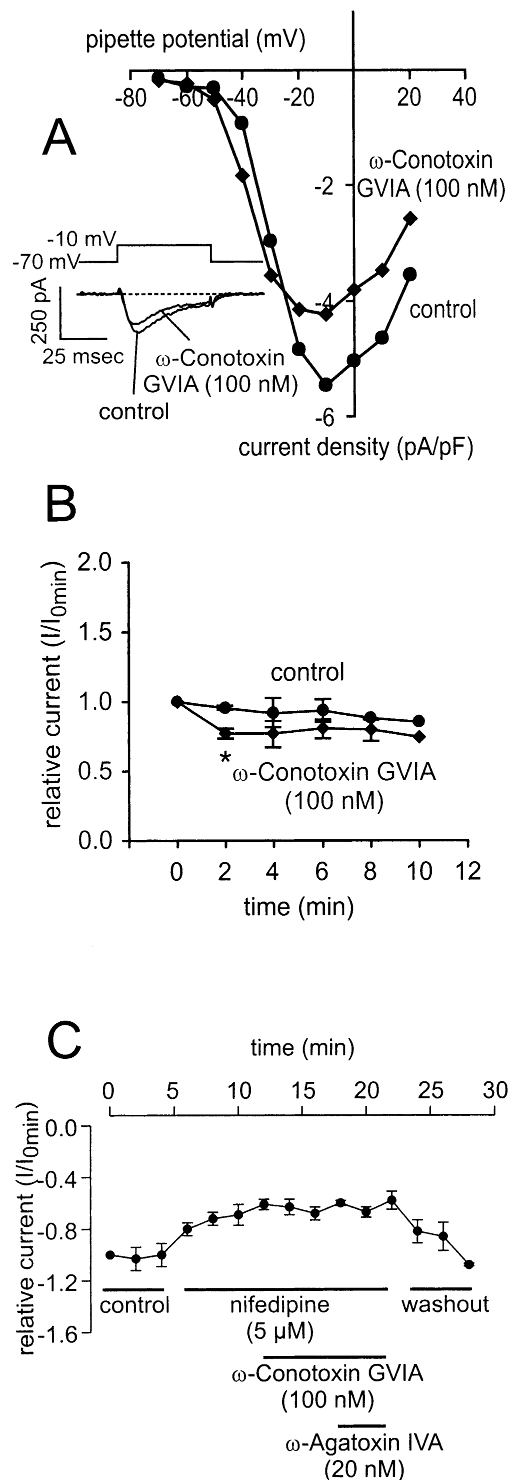


Fig. 3. Proportion of L- and R-type channel activity ($Ca_v1.3$ and $Ca_V2.3$) in human NET BON cells. (*A*) Effect of extracellular application of Bay K 8644 (5 μM) and SNX-482 (10 nM) summarized in a current/voltage plot and as current traces (insert panel). The cells were depolarized from −70 mV to +20 mV in 10-mV steps for the duration of 50 ms. For the current/voltage relation, maximal peak current amplitudes (pA) were normalized to each cell membrane capacity (pF) (current density; pA/pF) and plotted against the pipette potential (mV) of the electrical stimulation. (*B*) Time course (min) of changes in the relative current amplitudes ($I/I_{0\ min}$) of Ca^{2+}-channel currents during perforated-patch configuration. Maximal peak currents induced by depolarization from −70 mV to 0 mV were measured every 2 min and plotted against time. Currents were normalized to the control amplitude (I/I_{0min}) measured directly before application of the Ca^{2+}-channel modulator. The upper trace (quadrangles) was determined in the presence of the L-type channel opener Bay K 8644 (5 μM) ($n = 3 - 5$) and the middle trace (*circles*), in the absence of Ca^{2+}-channel modulators (*control*) ($n = 3 - 6$). The lower trace (*rhombs*) was determined in the presence of the R-type channel blocker SNX-482 (10 nM) ($n = 3 - 4$). The asterisks (*) indicate statistically significant differences ($p < 0.05$) compared with controls, using Student's *t*-test.

currents (at 4 min), it was 140 ± 10% ($n = 3$) (Fig. 5*C*). Interestingly, the currents were subsequently diminished. Establishing the whole-cell configuration without $InsP_3$ in the patch-pipette solution did not



change Ca^{2+}-channel current amplitude (after 6 min 94 ± 11% and after 10 min 89 ± 4% of the current amplitude, measured directly after break into whole-cell configuration; $n = 3–5$) (Fig. 5*C*). Measurements of intracellular free Ca^{2+} supported these findings. When the membrane potential was clamped to the mean resting potential of BON cells (about −50 mV), intracellular application of $InsP_3$ (10 μM) increased

Fig. 4. Identification of Ba^{2+} currents through different Ca^{2+} subtype channels in human NET BON cells. (*A*) Effect of extracellular application of ω-Conotoxin GVIA (100 nM) summarized in a current/voltage plot and as current traces (*insert panel*), indicating N-type channel activity. The cells were depolarized from −70 mV to +20 mV in 10-mV steps for the duration of 50 ms. For the current/voltage relation, maximal peak current amplitudes (pA) were normalized to each cell membrane capacity (pF) (current density; pA/pF) and plotted against the pipette potential (mV) of the electrical stimulation. (*B*) Time course (min) of changes in the relative current amplitudes ($I/I_{0\ min}$) of Ca^{2+}-channel currents during perforated-patch configuration. Maximal peak currents induced by depolarization from −70 mV to −10 mV were measured every 2 min and plotted against time. Currents were normalized to the control amplitude (I/I_{0min}) measured directly before application of the Ca^{2+}-channel modulator. The upper trace (*circles*) was determined in the absence of the N-type channel blocker (*control*) ($n = 3$). The lower trace (*rhombs*) was determined in the presence of the N-type channel blocker ω-Conotoxin GVIA (100 nM) ($n = 3$). The asterisk (*) indicates a statistically significant difference ($p < 0.05$) compared with controls, using Student's *t*-test. (*C*) Identification of residual (R-type) C^{2+} channel currents ($Ca_V2.3$) in human NET BON cells in the presence of the L-type channel blocker nifedipine (5 μM), the N-type channel blocker ω-Conotoxin GVIA (100 nM), and the P/Q-type channel blocker ω-Agatoxin IVA (20 NM) ($n = 3 - 8$). Voltage-dependent Ba^{2+} inward currents were measured every two minutes in the perforated patch configuration by depolarization the cells from −70 to 0 mV for 50 msec. Currents were normalized to −1 (control amplitude).

$[Ca^{2+}]_i$, beginning 2 min after establishing the whole-cell configuration (203 ± 21% of the control value directly after establishing the whole-cell configuration; $n = 3$) (Fig. 6*A*). This response was influenced by the R-type channel antagonist SNX-482 (10 nM). Several minutes after breaking into whole-cell configuration, SNX-482 decreased the slope of the $InsP_3$-induced rise in $[Ca^{2+}]_i$, indicating partial involvement of R-type channels. (Fig. 6*B*). Unfortunately, this effect was barely detectable because the whole-cell configuration was not stable after a certain time.

Passive Depletion of Intracellular Ca^{2+} Stores

To definitely demonstrate that R-type channels could be activated by release of Ca^{2+} from intracellular Ca^{2+} stores, we used an additional method for depletion of cytosolic Ca^{2+} stores. In this set of experiments, the so-called capacitative Ca^{2+} entry (CCE) was induced in human NET BON cells. Changes in $[Ca^{2+}]_i$ were monitored by fura-2 measurements. Fig. 7 summarizes the elevation of $[Ca^{2+}]_i$ and R-type channel activity achieved by passive depletion of intracellular Ca^{2+} stores by induction of CCE. CCE was stimulated at a holding potential of −50 mV as follows: cytosolic Ca^{2+} stores were depleted by cellular incubation in a Ca^{2+}-free bath solution containing the Ca^{2+} ionophore ionomycin (1 μM). This led to a significant increase in $[Ca^{2+}]_i$. After washout of ionomycin, cytosolic free Ca^{2+} decreased. Restoration of extracellular Ca^{2+} led to an influx of Ca^{2+}, resulting in an increase of $[Ca^{2+}]_i$ over the base value from 100 ± 4 nM; $n = 3$ to 285 ± 18 nM ($n = 3$) (Fig. 7*A*). This influx was significantly reduced by the R-type channel blocker SNX-482 (20 nM) to 192 ± 6 nM ($n = 3$) (Fig. 7*B*). Thus, passive depletion of cytosolic Ca^{2+} stores by inducing CCE led to an increase in $[Ca^{2+}]_i$ and R-type channel activity.

Association with Regulated CgA Secretion

To identify the participation of Ca^{2+} channel subtypes in the regulation of CgA secretion of human NET BON cells, Ca^{2+} channels were separately inhibited by the R-type channel blocker SNX-482 (25 nM), the L-type channel blocker nifedipine (5 μM), the N-type blocker ω-Conotoxin GVIA (500 nM) and the T/R-type channel inhibitor nickel chloride ($NiCl_2$; 100 μM) (Lee et al., 1999). Whereas ω-Conotoxin GVIA failed to modify CgA secretion, SNX-482 and $NiCl_2$ were able to significantly reduce CgA secretion from 46 ± 1% (with 30 mM KCl) to 34 ± 3% and from 26 ± 1% (even in the absence of 30 mM KCl) to 18 ± 1%, respectively, in the assay conditions used (all $n = 3$) (Fig. 8). In addition, nifedipine (5 μM) was not able to reduce CgA secretion (*not shown*). Taken together, regulated CgA secretion is more likely associated with R-type channel activity than with N- or L-type channel activity in human NET BON cells.

Discussion

In this study, we were able to demonstrate R-type channel activity corresponding to the class E Ca^{2+} channel ($Ca_V2.3$) in cultivated human NET BON cells for the first time. While the presence of L-, N-, and P/Q-type Ca^{2+} channels in human NE cells and NET cells of the gut has already been demonstrated (Glassmeier et al., 1997; Ahnert-Hilger et al., 1996; Magnelli et al., 1995, 1996; Bufler et al., 1998; Himmel et al., 1998; Basavappa et al., 1999), the expression of R-type channels in human NET BON cells has not been described to date. Recently, Vajna and coworkers characterized in the rat insulinoma cell line, INS-1, a tissue-specific splice variant of alpha1E (α_{1E}) at the mRNA and protein levels, indicating $Ca_V2.3$ Ca^{2+} channel proteins in the endocrine system (Vajna et al., 2001, Pereverzev et al., 2002).

R-Type Channel Activity

In our study, we found that Ca^{2+} channel currents in human NET BON cells were sensitive to the novel synthetic toxin peptide SNX-482, originated from *Hysterocrates gigas*, which has been described as a Ca^{2+} channel blocker with high specificity for

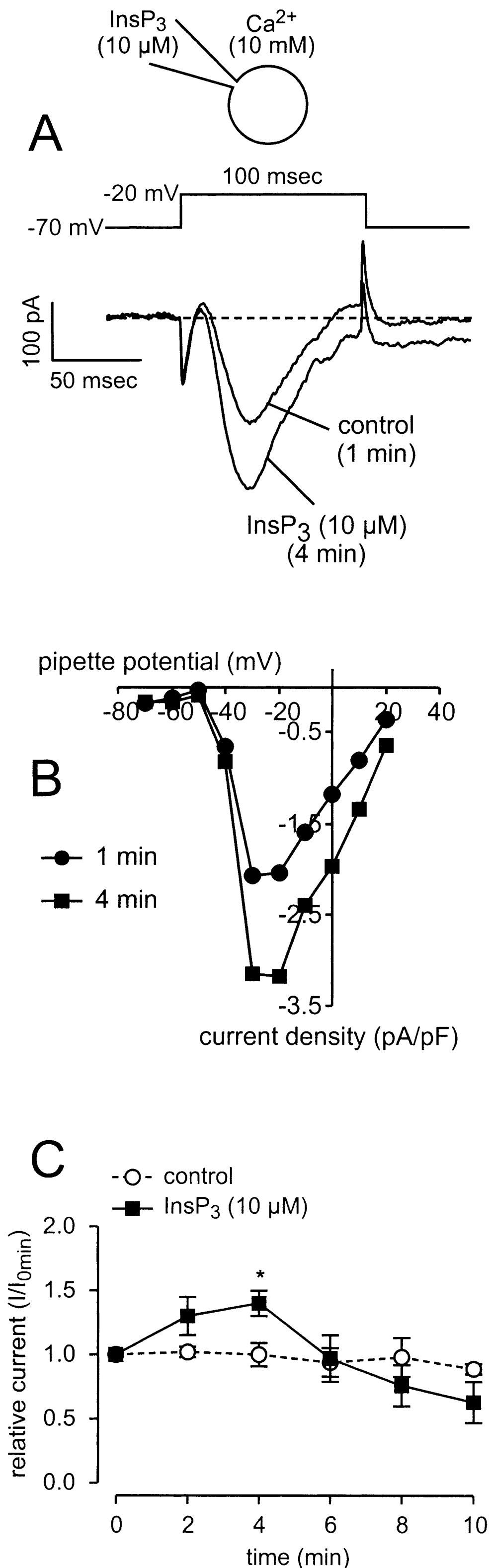


Fig. 5. Effect of intracellular application of $InsP_3$ on maximal Ca^{2+} channel amplitude in human NET BON cells. (*A*) Ca^{2+} currents through voltage-dependent Ca^{2+} channels during intracellular application of $InsP_3$ (10 μM). Currents were stimulated by a test potential from −70 mV to −20 mV of 100 msec duration. The upper Ca^{2+}-current trace was detected 1 min after breaking into whole-cell configuration. The lower Ca^{2+}-current trace was detected at the maximum of the $InsP_3$-induced rise in $[Ca^{2+}]_i$ (4 min after breaking into the whole-cell configuration). (*B*) Current/voltage relation of the currents in the same experiment shown in (*A*). Currents activated at potentials more positive than −50 mV. *Circles* signify currents measured 1 min after breaking with InsP3 (10 μM) into whole-cell configuration. *Squares* signify currents corresponding to the maximum effect of $InsP_3$-induced rise in $[Ca^{2+}]_i$ (4 min after breaking into the whole-cell configuration). (*C*) Time course (min) of changes in the relative current amplitudes ($I/I_{0\ min}$) of Ca^{2+}-channel currents after establishing the whole-cell configuration. Maximal peak currents induced by depolarization from −70 mV to −20 mV were measured every 2 min and plotted against time. Currents were normalized to the control amplitude (I/I_{0min}) measured directly after breaking into the whole-cell configuration. The line marked with squares was determined in the presence of $InsP_3$ (10 μM) in the pipette solution ($n = 3$). The line marked with circles was determined in the absence of $InsP_3$ in the pipette solution (*control*) ($n = 3 - 5$). The asterisk (*) indicates a statistically significant difference ($p < 0.05$) compared with controls, using Student's *t*-test.

R-type channel subtypes (Newcomb et al., 1998; Wang et al., 1999). In BON cells, however, the currents activate at potentials more positive than −45 mV, which is not typical for R-type channels usually classified as high-voltage activated (HVA) channels (Catterall, 2000). Interestingly, Kwiecien et al. (1998) observed nickel-sensitive Ca^{2+} channels in cells derived from a rat pituitary tumor (GC cell line) with similar activation threshold potentials. They described these channels as T/R-type channels because parallel experiments revealed the expression of the class E Ca^{2+} channel alpha1-subunit mRNA (for comparison of T-, and R-type channels, *see* Randall & Tsien, 1997). In our investigation, the currents were also nickel-sensitive (*data not shown*). However, nickel does not exhibit unequivocal specificity for R-type channels. Therefore, we used the voltage-sensitive R-type Ca^{2+} channel antagonist SNX-482. It is true that this compound does not exhibit unequivocal specificity for R-type channels, but SNX-482 very effectively antagonizes their activation (Bourinet et al., 2001). In addition, Newcomb et al. (1998) described this compound as a potent and selective blocker of the class E Ca^{2+} channel, corresponding electrophysiologically to R-type channels (Perez-Reyes & Schneider, 1995; Catterall, 2000). On the other hand, we were able to detect residual Ca^{2+} channel activity independently of SNX-482 by sequential blockage of L-, N-, and P/Q-type channel activity with specific Ca^{2+} channel blockers. This sequential blockage not only demonstrated L-, N-, and P/Q-type channel activity, but also R-type channel activity. Thus, R-type channels are expressed in human NET BON cells, and were registered electrophysiologically in two manners.

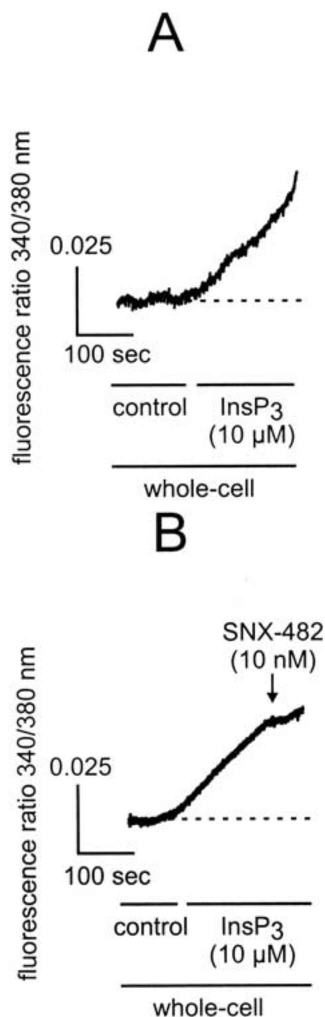


Fig. 6. Effect of intracellular application of $InsP_3$ on cytosolic free Ca^{2+} concentration in human NET BON cells. (*A*) Change in $[Ca^{2+}]_i$ (shown as fluorescence ratio) during intracellular application of $InsP_3$ (10 µM) via the patch pipette during whole-cell configuration. About 100 s after establishing the whole-cell configuration, an $InsP_3$-induced rise in $[Ca^{2+}]_i$ was detectable. (*B*) Several minutes after breaking into whole-cell configuration, extracellular application of the R-type channel antagonist SNX-482 (10 nM) slightly decreased the slope of the $InsP_3$-induced rise in $[Ca^{2+}]_i$, indicating putative involvement of voltage-dependent R-type channels.

Mechanism of Ca^{2+} Channel Activation

In different cell types, intracellular application of $InsP_3$ via the patch pipette during whole-cell configuration led to depletion of cytosolic Ca^{2+} stores (Jayaraman et al., 1996), an increase in $[Ca^{2+}]_i$ (Sneyd et al., 1995; Strauss, Wiederholt & Wienrich, 1996; Strauss et al., 1999), and activation of voltage-dependent Ca^{2+} channels (Mergler & Strauss, 2002). We could also demonstrate this in NET cells such as the BON cell line. In contrast to non-NET cells, however, the Ca^{2+}-channel activation during whole-cell configuration was only temporary, which could

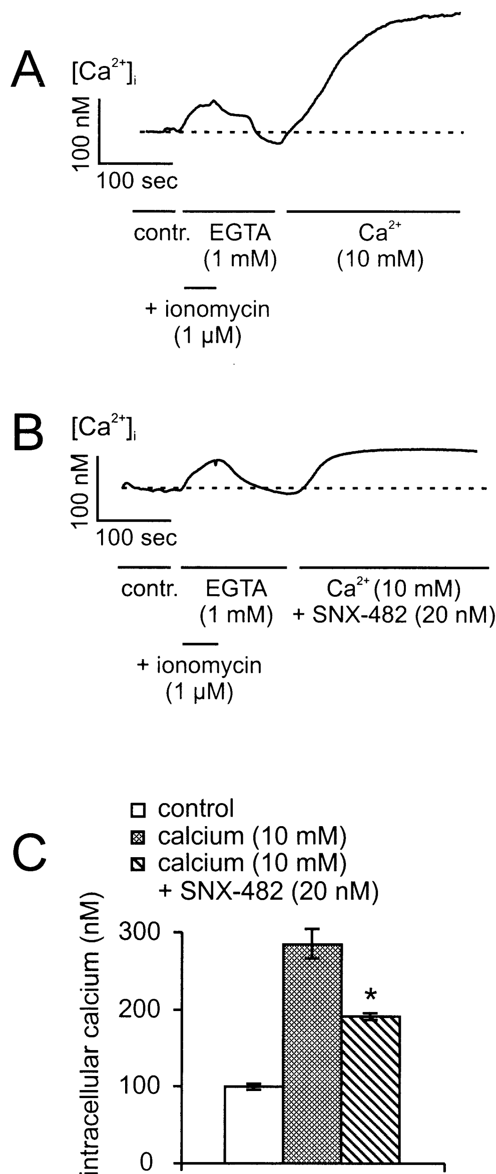


Fig. 7. Voltage-dependent R-type channels activated by depletion of cytosolic Ca^{2+} stores in human NET BON cells, demonstrated by induction of capacitative Ca^{2+} entry (CCE). (*A*) The control solution contains 10 mM Ca^{2+}. Subsequently, the cells were superfused with a Ca^{2+}-free bath solution (bath solution without Ca^{2+}, supplemented with 1 mM EGTA) and ionomycin (1 µM) to deplete intracellular Ca^{2+} stores. Following the washout of ionomycin, extracellular Ca^{2+} (10 mM) was added back (standard bath solution), inducing CCE. Changes in $[Ca^{2+}]_i$ were monitored on the basis of fura-2 fluorescence. Changes in $[Ca^{2+}]_i$ levels following the restitution of extracellular Ca^{2+} are indicative of the activity of membrane Ca^{2+} channels. (*B*) Reduction of the magnitude of CCE by the R-type channel antagonist SNX-482 (20 nM), indicating involvement of voltage-dependent R-type channels. (*C*) Comparison of $[Ca^{2+}]_i$ levels under control conditions, after induction of CCE (10 mM Ca^{2+}), and after induction of CCE in the presence of SNX-482 (10 nM) (all $n = 3$). The asterisk (*) indicates a statistically significant difference ($p < 0.05$) compared with controls, using Student's *t*-test.

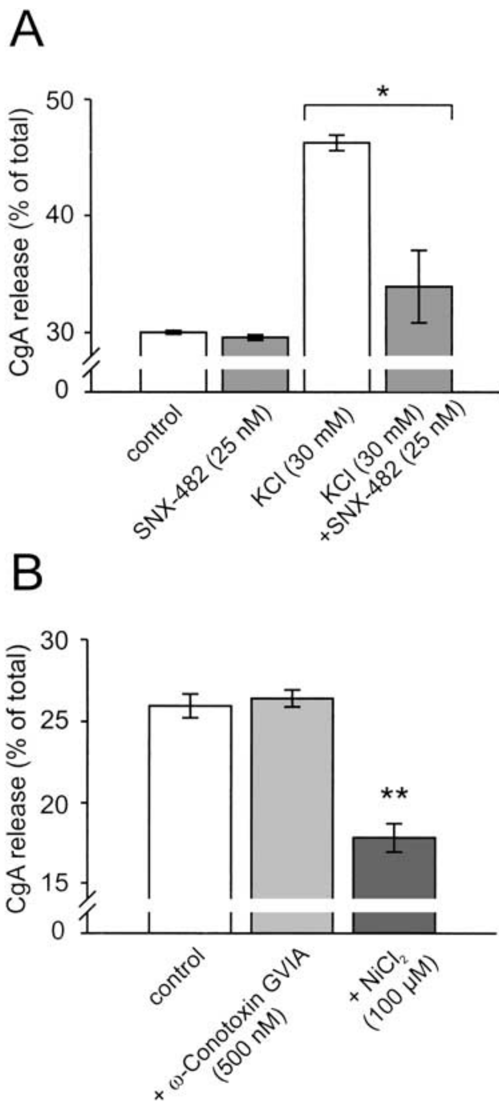


Fig. 8. Regulated CgA secretion in human NET BON cells. (*A*) Effect of the R-type channel antagonist SNX-482 (25 nM) in the presence of KCl (30 mM). (*B*) Effect of the N-type channel blocker ω-Conotoxin GVIA (500 nM) and the T- and R-type channel blocker nickel ($NiCl_2$) (100 μM). The asterisks (* and **) indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) compared with controls, using Student's *t*-test.

be a characteristic property of NET BON cells. The mechanism of Ca^{2+} channel activation by the Ca^{2+}/inositolphosphate second messenger system in non-NET cells is still under discussion. Some studies revealed that $InsP_3$ could directly activate Ca^{2+} channels (Kuno & Gardner, 1987). On the other hand, the release of Ca^{2+} from intracellular Ca^{2+} stores could induce a signal to the cell membrane leading to stimulation of L-type channels as discussed by Strauss and Mergler (Strauss et al., 1999; Mergler & Strauss, 2002). In our study, we presume an involvement of R-type channels activated by cytosolic Ca^{2+}-store depletion because the effect of intracellular application of $InsP_3$ via the patch pipette could be influenced by the R-type channel antagonist SNX-482. However, this effect was barely detectable. We presume that the moment of R-type channel activation is only temporary and difficult to detect. On the other hand, we are able to demonstrate R-type channel activation by cytosolic Ca^{2+}-store depletion independently from $InsP_3$. This supports our supposition of Ca^{2+} channel activation by an unknown Ca^{2+} influx signal from the cytosolic Ca^{2+} stores to the cell membrane, as discussed in non-NET cells (Putney, Jr. & Bird, 1993; Randriamampita & Tsien, 1995; Mergler & Strauss, 2002). In our study, we investigated Ca^{2+}-channel activation in NET BON cells by Ca^{2+}-store depletion via induction of the so-called capacitative Ca^{2+} entry (CCE) (Putney, Jr. & Bird, 1993; Putney, Jr., 1997; Putney, Jr. & McKay, 1999; Putney, Jr. et al., 2001) and checked the involvement of definite voltage-dependent Ca^{2+} channels by specific Ca^{2+}-subtype channel modulators. Indeed, Ca^{2+}-subtype channel blockers for L- or N-type channels decreased the magnitude of CCE (*data not shown*). We additionally tested SNX-482, which significantly decreased the magnitude of CCE as well as confirmed that voltage-dependent R-type channels are also activated by depletion of cytosolic Ca^{2+} stores.

Putative Role of R-type Ca^{2+} Channels

The function of voltage-dependent Ca^{2+} channels in human NET cells is not completely clear. We suggest that definite Ca^{2+}-channel subtypes could be linked to secretion of definite peptides or hormones, as described in the literature. For example, Pereverzew et al. found that alpha1E (electrophysiologically linked to R-type channels [Catterall, 2000]) is involved in glucose-induced insulin secretion probably by influencing the excitability of INS-1 cells (Vajna et al., 2001; Pereverzev et al., 2002). Furthermore, Wang et al. (1999) found that R-type Ca^{2+} currents in neurohypophysial terminals preferentially regulate oxytocin secretion (Wang et al., 1999). In our study, we therefore postulate that R-type channel activity is associated with CgA secretion in human NET BON cells because inhibitors/blockers for other Ca^{2+}-channel subtype activity, such as those for N- or L-type channel activity, did not induce reduction of CgA secretion. Incidentally, we could not definitely exclude that CgA secretion is associated with P/Q-type channel activity. However, we suppose that this doesn't have to be taken into consideration because P/Q-type channel conductance was only barely detectable. Predominantly, influx of Ca^{2+}, by activation of R-type channels may lead to an increase in intracellular Ca^{2+}, which likely stimulates CgA secretion. We conclude that in contrast to other

Ca^{2+}-channel subtypes, specific inhibition of R-type channel activity could be of clinical relevance for the control of hypersecretion in functional NET diseases.

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References

Ahnert-Hilger, G., Stadtbaumer, A., Strubing, C., Scherubl, H., Schultz, G., Riecken, E.O., Wiedenmann, B. 1996. gamma-Aminobutyric acid secretion from pancreatic neuroendocrine cells. *Gastroenterology* **110:**1595–1604

Basavappa, S., Mangel, A.W., Scott, L Liddle, R.A. 1999. Activation of calcium channels by cAMP in STC-1 cells is dependent upon Ca^{2+} calmodulin-dependent protein kinase II. *Biochem. Biophys. Res. Commun.* **254:**699–702

Bourinet, E., Stotz, S.C., Spaetgens, R.L., Dayanithi, G., Lemos, J., Nargeot, J,, Zamponi, G.W. 2001. Interaction of SNX482 with domains III and IV inhibits activation gating of alpha(1E) (Ca(V)2.3) calcium channels. *Biophys. J.* **81:**79–88

Bufler, J., Choi, G.C., Franke, C., Schepp, W., Prinz, C. 1998. Voltage-gated Ca^{2+} currents in rat gastric enterochromaffin-like cells. *Am. J. Physio.* **274:**C424–C429

Catterall, W.A., 2000. Structure and regulation of voltage-gated Ca^{2+} channels. *Annu. Rev. Cell Dev. Biol.* **16:**521–555

Evers, B.M., Ishizuka, J., Townsend, C.M., Jr., Thompson, J.C. 1994. The human carcinoid cell line, BON. A model system for the study of carcinoid tumors. *Ann. N. Y. Acad. Sci.* **733:**393–406

Glassmeier, G., Strubing, C., Riecken, E.O., Buhr, H., Neuhaus, P., Ahnert-Hilger, G., Wiedenmann, B., Scherubl, H. 1997. Electrophysiological properties of human carcinoid cells of the gut. *Gastroenterology* **113:**90–100

Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Himmel, H.M., Meyer zu, H.D., Windorfer, B., van Koppen, C.J., Ravens, U., Jakobs, K.H. 1998. Guanine nucleotide-sensitive inhibition of L-type Ca^{2+} current by lysosphingolipids in RINm5F insulinoma cells. *Mol. Pharmacol.* **53:**862–869

Horn, R., Marty, A. 1988. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* **92:**145–159

Jayaraman, T., Ondrias, K., Ondriasova, E., Marks, A.R. 1996. Regulation of the inositol 1,4,5-trisphosphate receptor by tyrosine phosphorylation. *Science* **272:**1492–1494

Kuno, M., Gardner, P. 1987. Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* **326:**301–304

Kwiecien, R., Robert, C., Cannon, R., Vigues, S., Arnoux, A., Kordon, C., Hammond, C. 1998. Endogenous pacemaker activity of rat tumour somatotrophs. *J. Physiol.* **508:**883–905.

Lee, J.H., Gomora, J.C., Cribbs, L.L., Perez-Reyes, E. 1999. Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. *Biophys. J.* **77:**3034–3042

Maechler, P., Wollheim, C.B. 1999. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* **402:**685–689

Magnelli, V., Grassi, C., Parlatore, E., Sher, E., Carbone, E. 1996. Down-regulation of non-L-, non-N-type (Q-like) Ca^{2+} channels by Lambert-Eaton myasthenic syndrome (LEMS) antibodies in rat insulinoma RINm5F cells. *FEBS Lett.* **387:**47–52

Magnelli, V., Pollo, A., Sher, E., Carbone, E. 1995. Block of non-L-, non-N-type Ca^{2+} channels in rat insulinoma RINm5F cells by omega-agatoxin IVA and omega-conotoxin MVIIC. *Pfluegers Arch.* **429:**762–771

Mangel, A.W., Snow, N.D., Misukonis, M.A., Basavappa, S., Middleton, J.P., Fitz, J.G., Liddle, R.A. 1993. Calcium-dependent regulation of cholecystokinin secretion and potassium currents in STC-1 cells. *Am. J. Physiol.* **264:**G103l–G1036

Mergler, S., Strauss, O. 2002. Stimulation of L-type $Ca^{(2+)}$ channels by increase of intracellular InsP3 in rat retinal pigment epithelial cells. *Exp. Eye Res.* **74:**29–40

Newcomb, R., Szoke, B., Palma, A., Wang, G., Chen, X., Hopkins, W., Cong, R., Miller, J., Urge, L., Tarczy-Hornoch, K., Loo, J.A., Dooley, D.J., Nadasdi, L., Tsien, R.W., Lemos, J., Miljanich, G. 1998. Selective peptide antagonist of the class E calcium channel from the venom of the tarantula *Hysterocrates gigas*. *Biochemistry* **37:**15353–15362

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

Pereverzev, A., Vajna, R., Pfitzer, G., Hescheler, J., Klockner, U., Schneider, T. 2002. Reduction of insulin secretion in the insulinoma cell line INS-l by overexpression of a Ca(v)2.3 (alpha1E) calcium channel antisense cassette. *Eur. J. Endocrinol.* **146:**881–889

Perez-Reyes, E., Schneider, T. 1995. Molecular biology of calcium channels. *Kidney Int.* **48:**1111–1124

Putney, J.W., Jr., 1997. Type 3 inositol 1,4,5-trisphosphate receptor and capacitative calcium entry. *Cell Calcium* **21:**257–261

Putney, J.W., Jr., Bird, G.S. 1993. The signal for capacitative calcium entry. *Cell* **75:**199–201

Putney, J.W., Jr., Broad, L.M., Braun, F.J., Lievremont, J.P., Bird, G.S. 2001. Mechanisms of capacitative calcium entry. *J. Cell Sci.* **114:**2223–2229

Putney, J.W., Jr., McKay, R.R. 1999. Capacitative calcium entry channels. *Bioessays* **21:**38–46

Randall, A.D., Tsien, R.W. 1997. Contrasting biophysical and pharmacological properties of T-type and R-type calcium channels. *Neuropharmacology* **36:**879–893

Randriamampita, C., Tsien, R.Y. 1995. Degradation of a calcium influx factor (CIF) can be blocked by phosphatase inhibitors or chelation of Ca^{2+}. *J. Biol. Chem.* **270:**29–32

Scherubl, H., Hescheler, J. 1991. Steady-state currents through voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels in GH3 pituitary cells. *Proc. R. Soc. Lond. B Biol. Sci.* **245:**127–131

Sneyd, J., Wetton, B.T., Charles, A.C., Sanderson, M.J. 1995. Intercellular calcium waves mediated by diffusion of inositol trisphosphate: a two-dimensional model. *Am. J. Physiol.* **268:**C1537–C1545

Snow, N.D., Mangel, A.W., Sharara, A.I., Liddle, R.A. 1993. Potassium channels regulate cholecystokinin secretion in STC-1 cells. *Biochem. Biophys. Res. Commun.* **195:**1379–1385

Strauss, O., Steinhausen, K., Mergler, S., Stumpff, F., Wiederholt, M. 1999. Involvement of protein tyrosine kinase in the InsP3-

induced activation of Ca^{2+}-dependent Cl^{-} currents in cultured cells of the rat retinal pigment epithelium. *J. Membrane Biol.* **169:**141–153

Strauss, O., Wiederholt, M., Wienrich, M. 1996. Activation of Cl^{-} currents in cultured rat retinal pigment epithelial cells by intracellular applications of inositol-1,4,5-triphosphate: differences between rats with retinal dystrophy (RCS) and normal rats. *J. Membrane Biol.* **151:**189–200

Vajna, R., Klockner, U., Pereverzev, A., Weiergraber, M., Chen, X., Miljanich, G., Klugbauer, N., Hescheler, J., Perez-Reyes, E., Schneider, T. 2001. Functional coupling between 'R-type' Ca^{2+} channels and insulin secretion in the insulinoma cell line INS-1. *Eur. J. Biochem.* **268:**1066–1075

Wang, G., Dayanithi, G., Newcomb, R., Lemos, J.R. 1999. An R-type Ca(2+) current in neurohypophysial terminals preferentially regulates oxytocin secretion. *J. Neurosci.* **19:**9235–9241

Wiedenmann, B., John, M., Ahnert-Hilger, G., Riecken, E.O. 1998. Molecular and cell biological aspects of neuroendocrine tumors of the gastroenteropancreatic system. *J. Mol. Med.* **76:**637–647.