γ-Aminobutyric Acid Secretion From Pancreatic Neuroendocrine Cells

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Background & Aims: **Neuroendocrine cells and tumors derived therefrom contain hormone-storing large dense core vesicles and neuron-like small synaptic vesicle analogues with unknown function. The aim of this study was to characterize the small synaptic vesicle pathway in detail.** ***Methods:*** **In human pancreatic neuroendocrine tumors and corresponding mammalian cell lines, the expression of key proteins of regulated secretion were detected by immunofluorescence microscopy. Using ^{3}H–γ-aminobutyric acid (GABA), uptake and release by small synaptic vesicle analogues were studied.** ***Results:*** **Tumor tissues obtained from 14 patients expressed key proteins of neurosecretion such as synaptobrevin, syntaxins, and SNAP 25. These proteins were also found in the cell lines AR42J, BON, RIN, and INR. The cell lines specifically transported GABA by a low-affinity plasma membrane transporter and showed an adenosine triphosphate–sensitive GABA uptake into an intracellular compartment. Stored GABA was released upon stimulation by regulated exocytosis. Electrophysiological analyses suggested that calcium-dependent secretion was mediated by activation of voltage-dependent calcium channels of mainly the L type, but also of the N and probably the T type.** ***Conclusions:*** **Small synaptic vesicle analogues in neuroendocrine cells and tumors can store and secrete GABA and probably other amino acid transmitters by regulated exocytosis comparable with neurons.**

Neuroendocrine tumors, irrespective of clinical symptoms, express proteins characteristic of secretory vesicles.[1–3] In the past, diagnostic and therapeutic efforts have focused on large dense core vesicles (LDCVs), the storage organelles for hormones and other polypeptides.[2] It turned out that secretion by the LDCV pathway is operative regardless of whether patients have clinical symptoms.[4–6] Besides LDCVs, neuroendocrine cells and tumors derived therefrom contain another secretory pathway that uses vesicles resembling the small synaptic vesicles (SSVs) of neurons and thus are termed synaptic-like microvesicles or SSV analogues.[1,2,7–13]

Recently, it has been suggested that a similar basic fusion machinery may be used by all secretory cells. Membrane fusion is based primarily on the interaction between soluble fusogens and their receptors. Soluble fusogens are the *N*-ethylmaleimide–sensitive factor (NSF) and α-, β-, and γ-soluble NSF attachment proteins (SNAPs).[14] NSF binds to membranes when one or more of the SNAPs link up to their SNAP receptors (SNAREs). An integral membrane protein of a transport vesicle (v-SNARE) pairs with one or more integral membrane proteins of the target membrane (t-SNARE). According to this hypothesis, different v-SNAREs and t-SNAREs confer specificity to intracellular fusion events, whereas NSF and SNAPs are common to all forms.[14–16] In neurons and chromaffin cells, the v-SNAREs for SSVs and LDCVs are synaptobrevin, whereas the t-SNAREs are syntaxins and SNAP 25 (synaptosomal-associated protein of 25 kilodaltons).[14,17–19]

So far, little information exists concerning the secretory pathway of SSV analogues in neuroendocrine cells. The idea that SSV analogues of pancreatic neuroendocrine cells may store amino acid transmitters has been supported by studies on subcellular fractionation of TCβ cells,[9] by uptake studies in synaptophysin-immunoisolated vesicles,[12] and, most importantly, by the regulated release of γ-aminobutyric acid (GABA) from the amphicrine cell line AR42J.[11] The present study was undertaken to characterize in detail the SSV pathway. For pancreatic neuroendocrine tumors and the corresponding mammalian cell lines, we show the expression of SNARE proteins found to regulate exocytosis from neurons[17,19]

Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle medium; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GABA, γ-aminobutyric acid; I_{Ca}, whole-cell calcium current; KR-HEPES, Krebs'-Ringer-HEPES; LDCV, large dense core vesicles; NSF, *N*-ethylmaleimide–sensitive factor; SNAP, synaptosomal-associated protein; SNARE, synaptosomal-associated protein receptor; SSV, small synaptic vessel.

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and nonneoplastic neuroendocrine cells.[18,20–22] Furthermore, we show that mammalian pancreatic neuroendocrine cell lines specifically take up GABA, which is stored in SSV analogues and released by regulated exocytosis.

Materials and Methods

Tissues and Cell Cultures

Human tumor tissues were obtained from 14 patients by tumor resection, immediately shock frozen, and used according to the standards set by the Ethical Committee of the Klinikum Benjamin Franklin, Freie Universität Berlin. PC12 cells[23] were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% horse and 5% fetal calf serum and AR42J[24] in DMEM supplemented with 20% fetal calf serum at 10% CO_2. RIN 38[25] and INR[26] were grown in RPMI supplemented with 5% fetal calf serum and 5% calf newborn serum, BON cells[27] in a 1:1 mixture of DMEM, and F12K medium containing 10% fetal calf serum and HeLa cells in DMEM containing 10% fetal calf serum in an atmosphere of 5% CO_2. Primary cultures of mouse hypothalamus were prepared and grown as previously described.[28]

Antibodies

Monoclonal antibody against synaptobrevin II and a polyclonal antiserum against synaptobrevin I[29] were provided by Dr. R. Jahn, Yale University, Howard Hughes Medical Institute, New Haven, CT. Polyclonal antisera against human synaptobrevin II and syntaxin were kindly provided by Dr. T. Rapoport, Harvard University, Boston, MA. Monoclonal antibody SY 38[7] and a polyclonal antiserum against synaptophysin[10] and monoclonal antibody against cytochrome b561[13] were used as previously described. Monoclonal antibodies against SNAP 25 and syntaxin (HPC-1 Barnstacle) were obtained from Sternberger Monoclonals (Baltimore, MD) or Sigma Chemical Co. (Deisenhofen, Germany), respectively. An anti-rabbit immunoglobulin (Ig) G antiserum from goat coupled to Texas Red and an anti-mouse IgG antiserum from donkey coupled to Texas Red were obtained from Dianova (Hamburg, Germany).

Other Chemicals

Streptolysin O, purified[30] and provided by Dr. U. Weller, Institut für Mikrobiologie, Johannes Gutenberg-Universität, Mainz, Germany, was used as described earlier.[31,32] (+)Isradipine was a gift from Sandoz AG (Basel, Switzerland). The calcium ionophore A23187, aminooxyacetic acid, gabaculin, betain, β-alanin, and diaminobutyric acid were obtained from Sigma Chemical Co. α-Latrotoxin and ω-conotoxin GVIA were purchased from Alamone Laboratories (Jerusalem, Israel). GABA (sp act, 60 Ci/mmol) and ^{125}J–protein A (sp act, 30 mCi/mg) were obtained from Amersham (Braunschweig, Germany).

Immunofluorescence Microscopy and Immunoreplica Analysis

Immunofluoresecence microscopy from cryosections of human tumor tissues was performed as described.[7] The various cell lines were grown on coverslips and processed for immunofluoresecence microscopy as previously described[33,34] using either Texas Red coupled to anti-mouse or anti-rabbit IgG. Immunoreplica analysis was performed as previously described[34] except that ^{125}J–protein A was used to detect immunoreactive signals.

GABA Uptake and Secretion

GABA uptake into intact cells was performed in the presence of 1 mmol/L aminooxyacetic acid and gabaculine in Krebs'-Ringer-HEPES buffer containing (in mmol/L): NaCl, 130; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; glucose, 11; and HEPES, 10, pH 7.4 (KR-HEPES buffer), for 30 minutes at 37°C in the presence of the various inhibitors. The incubation was stopped by diluting the sample with 500 μL of ice-cold KR-HEPES buffer followed by rapid centrifugation. The supernatant was discarded, and the pellet was washed again with KR-HEPES buffer and then dissolved in lysis buffer containing (in mmol/L): Tris-HCl, 130; $CaCl_2$, 10; NaCl, 75, pH 8, supplemented with 0.4% Triton X-100. One part of the cell lysate was used to count the amount of radioactivity; from the other part the protein content was determined using the bicinchoninic acid method. Values are calculated as picomoles per milligram of protein and expressed as percent of the uptake in the absence of inhibitors.

Uptake into permeabilized cells was performed using an intracellular buffer consisting of (in mmol/L): sucrose, 200; KCl, 50; piperazine-*N,N′*-bis(2-ethanesulfonic acid), 20; and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4, pH 7.0, with or without 2 mmol/L Mg/adenosine triphosphate (ATP) and ^{3}H-GABA. Incubation with SLO followed a protocol given elsewhere.[31,32] Values are calculated as picomoles per milligram of protein. The uptake in the absence of ATP was 100%.

Secretion was performed from preloaded cells as previously described.[11]

Electrophysiology

For electrophysiological experiments, cells were transferred into a perfusion chamber (4 mL/min) and whole-cell patch-clamp experiments[35] were performed at 37°C. Patch pipettes with pipette resistances of 3–6 MΩ were prepared from borosilicate glass capillaries (Jencons, Leight Buzzard, England). Currents were recorded using a List LM/EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) using the CED (Cambridge Electronic Design, Cambridge, England) interface and software. Voltage-dependent calcium currents were routinely elicited from a holding potential of −80 mV by either 200-millisecond-long voltage pulses to 10 mV or by voltage ramps from −100 to 100 mV (slope, 1 V/s). Calcium

currents were measured as peak inward currents. Leakage currents were determined from the hyperpolarizing part of the voltage ramps (−100 to −80 mV) and subtracted offline. Statistical values are given as means ± SEM.

To isolate inward currents through voltage-dependent calcium channels, sodium and potassium were omitted from the intracellular and extracellular solutions. Barium was used as an extracellular charge carrier. The patch pipettes were filled with a high-cesium pipette solution containing (in mmol/L): CsCl, 120; $MgCl_2$, 1; Mg ATP, 3; HEPES, 10; and EGTA, 10; adjusted to pH 7.4 with CsOH at 37°C. For the measurements of barium inward currents through calcium channels, an external solution was used containing (in mmol/L): D(−)–*N*-methylglucamine, 120; $BaCl_2$, 10.8; $MgCl_2$, 1; CsCl, 5.4; glucose, 10; and HEPES, 10; adjusted to pH 7.4 with HCl at 37°C.

Results

Expression of SNARE Proteins in Pancreatic Neuroendocrine Tumors and Mammalian Cell Lines

Regulated secretion involves the interaction between the vesicular v-SNARE proteins synaptobrevin and plasma membrane t-SNARE proteins syntaxins and SNAP 25. The expression of synaptobrevin, syntaxins, and SNAP 25 were analyzed in serial sections of various neuroendocrine tumors of the pancreas. As an example, a pancreatic gastrinoma from a 33-year-old patient is shown in Figure 1. Antibodies against synaptobrevin, SNAP 25, and the syntaxins immunoreacted only with the tumor cells but not with the surrounding connective tissue. Similar results were obtained in 14 other pancreatic neuroendocrine tumors (Table 1) using monoclonal antibodies against syntaxins and synaptobrevin II (not shown). No immunoreactivity was obtained with an antiserum against synaptobrevin I (data not shown). Table 1 summarizes the expression of SNARE proteins in 14 pancreatic tumors. In normal human pancreatic tissue analyzed for comparison, only the pancreatic islets but not the surrounding exocrine tissue immunoreacted with antibodies against SNAP 25, syntaxins, and synaptobrevin including synaptobrevin II (data not shown). As previously described, marker proteins for either LDCVs such as cytochrome b561 and dopamine β-hydroxylase or SSVs such as synaptophysin and protein SV2 were expressed in these tumors[13] and used for comparison (see Table 1). Similar results were obtained with 16 neuroendocrine tumors derived from the midgut (data not shown).

Mammalian cell lines derived from pancreatic neuroendocrine tumors but not HeLa cells also highly expressed the SNARE proteins necessary for regulated exocytosis, as could also be detected by immunofluorescence microscopy (not shown) and in membranes obtained from postnuclear supernatants (Figure 2).

Functional Analysis of SSV Analogues in Mammalian Neuroendocrine Pancreatic Cell Lines

Because primary cultures from human neuroendocrine tumor tissues were not available, functional studies of SSV analogues were performed in mammalian neuroendocrine pancreatic tumor cell lines.

GABA Uptake Into Intact and Permeabilized Cells

In a first set of experiments, the GABA uptake was characterized using various inhibitors specific for different plasma membrane GABA transporters. GABA uptake in AR42J cells was driven by a low-affinity transporter with a median inhibitory concentration of 200 μmol/L for GABA, betain, and β-alanin. In contrast, GABA uptake into INR and RIN cells with a median inhibitory concentration of 100 μmol/L for GABA was sensitive to β-alanin (median inhibitory concentration, 50 μmol/L for INR and 200 μmol/L for RIN) and almost insensitive to betain, in which millimolar concentrations were required for inhibition of uptake (Figure 3). Similar results were obtained with BON cells (data not shown). diaminobutyric acid, a well-known inhibitor of the neuronal GABA transporter GAT-1[36] that efficiently inhibited GABA uptake into mouse hypothalamic neurons (data not shown), was effective only in millimolar concentrations in the neuroendocrine cell lines (Figure 3).

Transmitter uptake into secretory vesicles is an ATP-dependent process. To prove that GABA is really stored in an intracellular compartment, cells were permeabilized by streptolysin O, washed free of endogenous ATP, and then incubated with radiolabeled GABA in the presence of ATP and various amounts of unlabeled GABA. ATP stimulated GABA uptake 2.5–3-fold (Figure 4) in AR42J and INR cells and twofold in RIN and BON cells (not shown). No ATP-dependent uptake was seen in permeabilized HeLa cells under these conditions (not shown). Adding carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (not shown) or increasing the concentration of unlabeled GABA (Figure 4) inhibited ATP-dependent uptake.

Regulated Secretion of GABA

All four cell lines released GABA in a regulated fashion (Figure 5). GABA secretion could be elicited only by an elevated potassium concentration or by the calcium ionophore A23187 (Figure 5). α-Latrotoxin, a potent

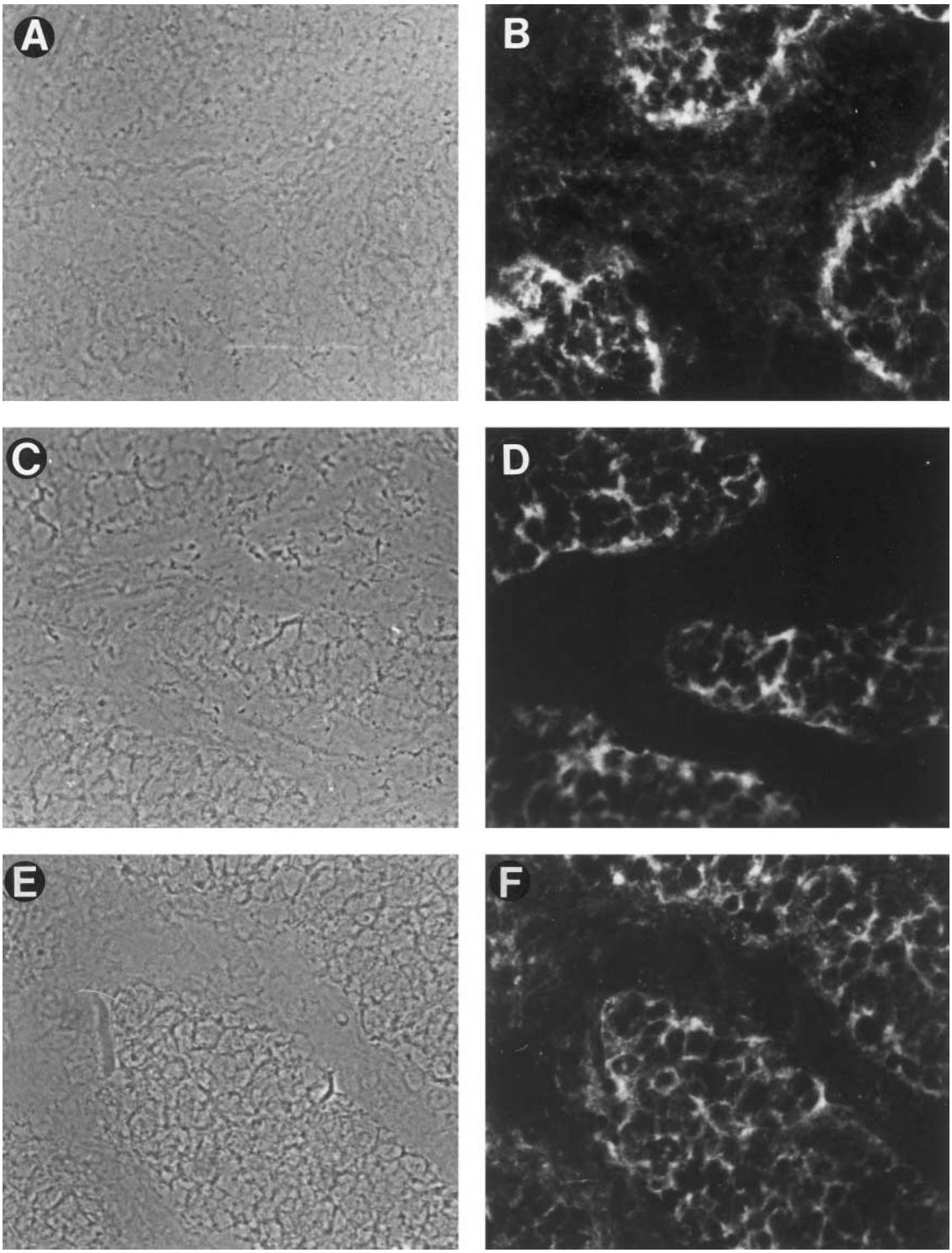

Figure 1. Expression of SNARE proteins in a pancreatic neuroendocrine tumor (gastrinoma). Serial sections were fixed and immunostained as described in Materials and Methods. Immunofluorescence microscopy was performed using a conventional fluorescence microscope. The following antibodies were used: (*A* and *B*) polyclonal antiserum against human synaptobrevin II (dilution, 1:100), (*C* and *D*) monoclonal antibody against SNAP 25 (1:1000), and (*E* and *F*) a polyclonal antiserum against syntaxin (1:100). The antibodies were detected using a Texas Red–labeled goat anti-rabbit or goat anti-mouse antiserum. Immunofluorescence micrographs are shown on the right and corresponding phase contrast images on the left. Note that only the tumor tissue is immunostained, whereas the surrounding connective tissue shows no immunoreactivity.

Table 1. Immunocytochemical Detection of SNARE Proteins and Membrane Proteins of LDCVs and SSVs in Pancreatic Neuroendocrine Tumor Tissue

	LDCVs	SSV analogues	SNARE proteins		
			v-SNAREs	t-SNAREs	
	Cytochrome b561	Synaptophysin	Synaptobrevins	SNAP 25	Syntaxins
Functional					
Insulinoma (n = 1)	++	+++	++	+++	+
Gastrinomas (n = 5)	++	+++	++	+++	+
	5/5	5/5	5/5	5/5	4/5
VIPoma (n = 1)	++	+++	++	++	++
Nonfunctional (n = 7)	++	+++	++	+++	+
	6/7	7/7	7/7	7/7	6/7

NOTE. Besides tumors with hypersecretory symptoms, a second group of pancreatic tumors with neuroendocrine properties lacking these symptoms has been identified using antibodies against secretory vesicle proteins. For clinical diagnostic purposes, these tumors may be classified in general as nonfunctional.[1–3] Immunofluorescence microscopy was performed as described in Materials and Methods. Intensity of the reaction was assessed as follows: +++, very strong; ++, strong; +, less strong.

secretagogue for exocytosis by SSVs from neurons,[37] which stimulated GABA secretion from mouse hypothalamic neurons (not shown), had no effect on GABA secretion from the neuroendocrine cell lines (not shown). Other secretagogues that elicit amylase secretion from AR42J cells such as carbachol[10] did not stimulate GABA secretion from the neuroendocrine cell lines. In the absence of extracellular calcium, no regulated GABA secretion was seen (Figure 6). However, for unknown reasons, the absence of extracellular calcium increased basal release as previously described for AR42J cells.[11]

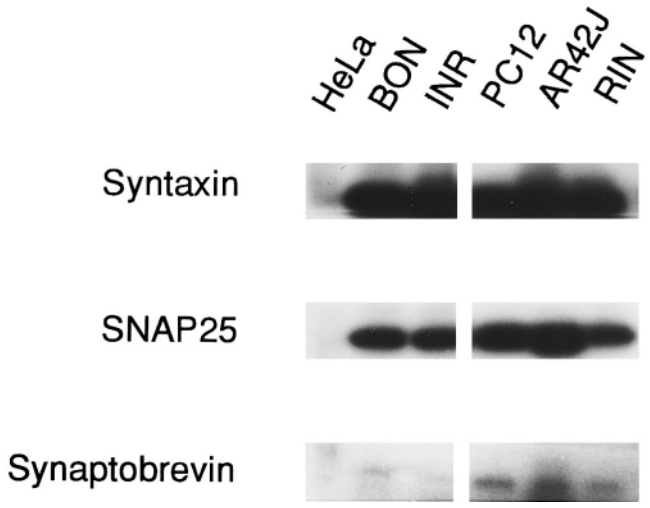

Figure 2. SNARE proteins on membranes of mammalian pancreatic neuroendocrine cell lines. Postnuclear supernatants (40 μg of total protein per slot) of the cell lines indicated were subjected to sodium dodecyl sulfate gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with the antibodies: a polyclonal antiserum against synaptobrevin (dilution, 1:1000), a monoclonal antibody against SNAP 25 (1:2000), and a monoclonal antibody against syntaxin (1:1000). Antibody binding was detected by ^{125}J–protein A. Note that SNARE proteins were detected in neuroendocrine cell lines but not in HeLa cells.

Voltage-Dependent Calcium Channels in Mammalian Neuroendocrine Pancreatic Cell Lines

All neuroendocrine cell lines studied showed voltage-dependent calcium channels. To characterize the different calcium channel subtypes underlying the whole-cell calcium current (I_{Ca}), we used subtype-specific channel inhibitors. Extracellular application of the L-type calcium channel antagonist isradipine (1 μmol/L) inhibited I_{Ca} in INR, RIN, and AR42J cells by 87% ± 4.1% (n = 6), 81.8% ± 3.9% (n = 4), and 71.3% ± 7.8% (n = 5), respectively. During washout, the effect of isradipine reversed completely. Cumulative application of isradipine and the N-type calcium channel blocker ω-conotoxin (3 μmol/L) almost completely (95%–100%) abolished the inward I_{Ca} in INR and RIN cells, whereas in AR42J a cadmium-sensitive I_{Ca} of approximately 29.5% ± 3.6% (n = 4) remained after application of both antagonists (Figure 7). ω-Conotoxin alone reduced the control I_{Ca} by 19.7% ± 7.8% (n = 3), 13.3% ± 3.8% (n = 3), and 36.2% ± 5.2% (n = 4) in INR, RIN, and AR42J, respectively. This indicates the expression of dihydropyridine-sensitive L-type calcium channels and an ω-conotoxin–sensitive I_{Ca} component in all three cell lines.

Because the effects of isradipine and ω-conotoxin were not or only partially additive, we assume ω-conotoxin to probably work on a subset of L-type channels containing the α_{1D} subunit rather than on N-type channels. The α_{1D} subunit found in different neuronal and neuroendo-

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