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N-type Ca^{2+} Channels Are Present in Secretory Granules and Are Transiently Translocated to the Plasma Membrane during Regulated Exocytosis*

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An intracellular pool of N-type voltage-operated calcium channels has recently been described in different neuronal cell lines. We have now further characterized the intracellular pool of N-type calcium channels in both IMR32 human neuroblastoma and PC12 rat pheochromocytoma cells. Intracellular N-type calcium channels were found to be accumulated in subcellular fractions where the chromogranin B-containing secretory granules were also enriched. ^{125}I-ω-Conotoxin GVIA binding assays on fixed and permeabilized cells revealed that intracellular N-type calcium channels translocate to the plasma membrane in cells exposed to secretagogues (KCl, ionomycin, and phorbol esters). The kinetics, Ca^{2+} and protein kinase C dependence, and brefeldin A insensitivity of N-type calcium channels translocation were similar to the regulated release of chromogranin B, while no correlation was found with the constitutive secretion of a heparan sulfate proteoglycan. A PC12 subclone deficient in the regulated but not in the constitutive pathway of secretion had a small intracellular pool of N-type calcium channels, and no secretagogue-induced translocation occurred in these cells. Calcium channel translocation was accompanied by a stronger response of Fura-2-loaded cells to depolarizing stimuli, suggesting that the newly inserted channels are functional.

Multiple voltage-operated calcium channel (VOCC)[1] subtypes, with different biophysical and pharmacological properties, have been characterized in vertebrate secretory cells (1–3). Among these, the N-type is selectively blocked by the marine snail toxin ω-conotoxin GVIA (ω-CTx), and is expressed in many neurons and endocrine cells (2). The N-type VOCC plays a crucial role in the control of neurotransmitter release (4), although its involvement in other processes, such as neuronal migration (5) and neurite outgrowth and retraction (6, 7), has also been described. Consistent with their function in regulated exocytosis, N-type VOCCs were found clustered in the presynaptic active zone of frog neuromuscular junctions (8), where vesicle fusion is known to occur under physiological conditions, and at the synaptic sites in cultured hippocampal neurons and ciliary ganglia (9, 10). At the molecular level, the interaction of N-type VOCCs with syntaxin (11) and, indirectly, with other proteins of both the presynaptic plasma membrane and the secretory vesicles (12), strengthens the idea that this channel plays a crucial role in secretory events. Clinical evidence, showing that anti-N-type VOCC autoantibodies are present in a human disorder of neurotransmission (the Lambert-Eaton myasthenic syndrome; Ref. 13), are also consistent with this channel subtype having a major role in Ca^{2+}-dependent release.

Given their importance, it is not surprising that N-type VOCCs represent the target of various forms of modulation of both their gating properties and their actual expression. G protein-mediated modulation of the gating of N-type VOCCs by hormones and neurotransmitters has been characterized extensively in several cell types (14–19). Recent studies have also addressed the regulation of the actual number of N-type VOCCs expressed by cells. Exposure of neuronal cells to differentiating agents (20, 21), or transfection with specific immediate early genes like c-*fos* or c-*jun* (22), has been found to stimulate N-type VOCC expression on the plasma membrane over a time scale of days.

In contrast, few data are available on the biosynthesis and intracellular trafficking of N-type VOCCs in neuronal and endocrine cells. Using biochemical and pharmacological methods, we recently studied the turnover rates of plasma membrane N-type VOCCs in undifferentiated neuronal cells and found that it varied between 15 and 18 h (23); furthermore, in all the cell types studied (IMR32, PC12, SH-SY5Y, and F11), cell differentiation was accompanied by an increase in surface N-type VOCCs due to their stabilization in the membrane, *i.e.* a slowing down in their internalization and degradation rates (23, 24). During these studies we also found that most of the neuronal cells analyzed contain a large intracellular pool of N-type VOCCs (25), and that these intracellular channels can be recruited to the cell surface (over a time scale of several hours) if the cells are exposed to ω-CTx (25).

In this paper, we have further investigated the presence, localization, and regulated translocation to the plasma membrane of the intracellularly located N-type VOCCs. The cell models used were the IMR32 human neuroblastoma cell line, which can acquire the regulated secretory pathway after differentiation (26) and the PC12 pheochromocytoma cell line, in which the regulated and constitutive secretory pathways have been extensively characterized (27, 28). We have found that

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[1] The abbreviations used are: VOCC, voltage-operated calcium channel; BFA, brefeldin A; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CgB, chromogranin B; Fura-2, Fura-2 acetoxymethyl ester; hsPG, heparan sulfated proteoglycan; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ω-CTx, ω-conotoxin fraction GVIA; BSA, bovine serum albumin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; D-PBS, Dulbecco's modified phosphate-buffered saline.

N-type VOCCs (revealed as ^{125}I-ω-CTx binding sites) are enriched in subcellular fractions corresponding to the secretory granules and that different agents stimulating the Ca^{2+}- and protein kinase C (PKC)-dependent exocytosis of these granules stimulate, in parallel, the translocation of N-type VOCCs to the plasma membrane.

The translocation here described may represent an important cellular pathway regulating N-type VOCC expression, and may be relevant to the potentiation of Ca^{2+}-dependent events in neuronal cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation

The human neuroblastoma IMR32 cell line (ATCC CCL1277) was obtained from the American Type Culture Collection (Rockville, MD) and grown and differentiated as described previously (26). The cells were used after 4 days of differentiation achieved by the addition of 1 mM dibutyryl-cAMP and 2.5 mM 5-bromodeoxyuridine (Sigma) to the culture medium.

PC12-251 cells were used as a model of normally secreting neuroendocrine cells (27), while PC12-27 cells, kindly provided by Dr. E. Clementi, were chosen because they lack regulated secretion (29). Both types of PC12 cells were grown in Dulbecco's modified minimum essential medium supplemented with 10% horse serum and 5% fetal calf serum in 10% CO_2 as described previously (27). The cells, plated at a concentration of $5 \times 10^5/cm^2$ in plastic culture Petri dishes, were used 4–5 days thereafter.

Subcellular Fractionation

Subcellular fractionation by velocity and equilibrium gradient centrifugation was performed as described (28, 30) with minor modifications. All steps were performed at 4 °C. PC12-251 cells, detached from 80 15-cm Petri dishes, were homogenized in an homogenization buffer (0.25 M sucrose, 1 mM $Mg(CH_3COO)_2$, 1 mM EDTA, 10 mM Hepes, pH 7.4 with KOH) plus protease inhibitors (10 μg/ml aprotinin, 2 μg/ml leupeptin,2 μg/ml pepstatin A), and a post-nuclear supernatant was prepared. The post-nuclear supernatant was centrifuged at 150,000 × *g* for 30 min. The cytosol was collected, and the pellet was resuspended in the same homogenization buffer supplemented with 10 mM EDTA, for 10 min. This suspension was loaded on the top of a sucrose gradient (0.3–1.2 M) and centrifuged at 110,000 × *g* for 30 min (velocity gradient). Fractions (1 ml each) were automatically collected from the top of the gradient. Fractions 5–9 of this first gradient, which are enriched in secretory granules (see Ref. 30 and below), were pooled, applied to a cushion of 2 M sucrose and centrifuged at 220,000 × *g* for 1 h; the band visible at the cushion interface was collected and loaded on a second sucrose gradient (1.1–2.0 M) and centrifuged at 110,000 × *g* for 18 h (equilibrium gradient). Fractions (1 ml each) from both gradients were further processed for either Western blotting or ^{125}I-ω-CTx binding.

In some experiments intact PC12-251 cells were incubated with a high (1 μM) concentration of unlabeled ω-CTx for 1 h at 4 °C before being washed and homogenized as above. By this procedure it was possible to eliminate any contribution of surface binding sites in the subsequent ^{125}I-ω-CTx binding assays.

Western Blotting

Aliquots of each fractions from both the velocity (100 μl) and equilibrium (200 μl) gradients were precipitated overnight at −20 °C using 80% acetone as described (31). The pellets were solubilized in Laemmli sample buffer, the proteins separated by SDS-PAGE in 10% polyacrylamide gels, and transferred to nitrocellulose membranes for 18 h at 120 mA. The blots were blocked at 4 °C for 12 h with Tris-buffered saline (TBS) containing 8% dried milk and incubated at room temperature for 2 h with appropriate concentrations of the specific antibodies in TBS plus 8% milk and 0.3% Tween 20. After extensive washing, the blots were incubated with anti-mouse IgG rabbit antibodies (1 μg/ml) for 1 h. After further washing, the blots were incubated for 45 min with ^{125}I-protein A (177.000 cpm/ml) diluted in the same buffer, washed again with TBS containing 0.3% Tween 20 and autoradiographed at −80 °C for variable period of times. For quantitation, the relevant bands were cut from the nitrocellulose paper and counted in a Packard Cobra γ counter. Background radioactivity was determined from unrelevant pieces of the blots as described (32).

Antibodies

The characteristics of the monoclonal and polyclonal antibodies against rat chromogranin B (CgB) were described (27, 33). Mouse monoclonal antibodies against synaptophysin were from Boehringer (Mannheim, Germany) and the monoclonal antibody 6H against the α_1 subunit of the Na^+/K^+ ATPase (34) was kindly provided by Dr. G. Pietrini (CNR Cellular and Molecular Pharmacology Center, Milan, Italy). The primary antibodies were used for Western blotting at a 1:500 dilution.

^{125}I-ω-CTx Binding

Intact Cells—^{125}I-ω-CTx binding to intact adherent cells was performed as described recently (23, 25). Briefly, in 35-mm Petri dishes, parallel groups of control cells and cells stimulated with the different agents for the indicated times (in Krebs-Ringer-Hepes buffer (KRH), containing 125 mM NaCl, 5 mM KCl, 12 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES-NaOH, pH 7.4) were washed twice in Dulbecco's modified phosphate-buffered saline (D-PBS), and then incubated for 90 min at room temperature with 25 pM ^{125}I-ω-CTx (Amersham International, United Kingdom) dissolved in D-PBS supplemented with 0.1% bovine serum albumin (D-PBS-BSA). The binding buffer also contained 0.02% NaN_3 in order to block exo-endocytosis during the 90 min of toxin incubation. At the end of the incubation the cells were washed three times with D-PBS-BSA, extracted in 1 ml of 1 N NaOH, and bound radioactivity was determined by means of a Packard Cobra γ counter. Each point was evaluated in triplicate (unless otherwise specified), and nonspecific ^{125}I-ω-CTx binding was evaluated for every group by means of the parallel incubation of three dishes in the presence of an excess (84 nM) of unlabeled toxin (Bachem, Bubendorf, Switzerland). Under these conditions, nonspecific ^{125}I-ω-CTx binding was 15–50% of total binding.

Fixed and Permeabilized Cells—The same buffers, toxin concentrations, washes, and radioactivity counting procedure as those described above were also used on fixed cells. Cells were fixed and permeabilized as described in Ref. 25 by using 1% paraformaldehyde (20 min at 20 °C) and 0.1% Triton X-100 (5 min at 20 °C), respectively.

With this technique we could determine separately the total cellular binding of ^{125}I-ω-CTx, the surface binding only (in cells fixed but not permeabilized), or the intracellular binding only (in cells fixed, with the surface channels presaturated with unlabeled ω-CTx, and then permeabilized). For each group of dishes, nonspecific binding was determined as described above.

In some experiments the cells were preexposed to the secretagogue agents for the indicated times at 37 °C and thoroughly washed before being fixed and processed for ^{125}I-ω-CTx binding.

Stock solutions of brefeldin A (BFA) (Epicentre Technologies, Madison, WI) (in ethanol), ionomycin (Calbiochem), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and calphostin C, (Sigma), (in dimethyl sulfoxide) were diluted in KRH buffer at the indicated concentrations.

Solubilized Channels—In order to determine the amount of N-type VOCCs in each gradient fraction utilizing a ^{125}I-ω-CTx binding assay, two sets of problems had to be addressed. First, each fraction contained a different concentration of sucrose. In separate experiments we found that sucrose, in the range of concentrations found in the fractions, inhibited ^{125}I-ω-CTx binding in a dose-dependent manner. This was solved by diluting each fraction to the lowest concentration of sucrose (0.3 M) before the binding assay.

Second, since the toxin binding sites face the lumenal part of intracellular organelles, it was necessary to solubilize the membranes and perform a ^{125}I-ω-CTx binding assay on soluble channels. This was done by incubating the diluted fractions with 1% each of Triton X-100 and CHAPS, for at least 6 h at 4 °C. The binding was then performed with the same buffers and toxin concentrations as above, but in plastic tubes. At the end of the incubation period (30 min at 37 °C), the samples were filtered through GF/B filters presoaked in 1% polyethyleneimine. After extensive washing, the radioactivity remaining on the filters was counted in the γ counter.

Metabolic Labeling and Stimulation of Release

To investigate the kinetics of release from secretory granules, PC12-251 cells were labeled overnight with 200 mCi/ml [^{35}S]sulfate (SJS.1, Amersham), chased for 2 h, and then incubated for 5 or 30 min in a medium containing 5 or 55 mM KCl, or 100 nM TPA, in the presence or absence of 2.2 mM Ca^{2+}. In some experiments, BFA (2.5 μg/ml) was added during depolarization. CgB was then quantitatively immunoprecipitated from the different media using polyclonal antibodies directed against rat CgB (27, 31). The immunoprecipitates were either analyzed by SDS-PAGE followed by fluorography or quantified by scintillation

counting. To study the kinetics of release of heparan sulfate proteoglycans (hsPG) from constitutive secretory vesicles, PC12 cells were pulse-labeled for 15 min with 500 μCi/ml [^{35}S]sulfate and then incubated for 5 and 30 min in the same media described above. To test the effect of BFA on constitutive secretion, the cells were labeled for 30 and 90 min with 200 μCi/ml [^{35}S]sulfate in the presence or absence of the drug. Aliquots of the total media were analyzed by SDS-PAGE followed by fluorography.

Fura-2 Measurements

IMR32 and PC12-251 cells were loaded for 15 min at 37 °C with 2.5 mM Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) in KRH buffer. At the end of the loading period, some of the cells were diluted 1:5 in the same buffer for another 15 min, and others were diluted in a buffer containing either 55 mM KCl or 100 nM TPA. After this treatment, the cells were centrifuged, washed and resuspended in normal buffer at a concentration of 3×10^6/ml, and transferred to a cuvette; the levels of $[Ca^{2+}]_i$ before and after depolarization with 60 mM KCl were determined as described previously (35).

RESULTS

Subcellular Localization of N-type VOCCs

We have shown previously that cultured neuronal cell lines contain an intracellular pool of N-type VOCCs (25), a finding now extended to PC12-251 cells (see below). This intracellular pool of N-type VOCCs is large and accounts for 60–80% of the total cellular channels depending on the cell line (see Ref. 25 and below).

In order to identify the intracellular organelle(s) where these channels are accumulated, we subjected PC12-251 cells to subcellular fractionation on sucrose gradients and detected, in parallel, the distribution of different organelle markers and the distribution of ^{125}I-ω-CTx binding sites. This toxin is a highly specific and irreversible ligand for N-type VOCCs (2, 35).

Following described procedures for secretory granule purification from PC12 cells (28, 30), we performed two subsequent sucrose gradients: the first a velocity gradient, and the second an equilibrium gradient. Aliquots of each collected fraction were processed for either Western blotting or ^{125}I-ω-CTx binding as described under "Experimental Procedures."

Fig. 1*A* shows the distribution of three different organelle markers in the various fractions obtained from the first velocity gradient. The *top line* shows the distribution of both the α subunit alone and the α and β subunit dimer of the Na^+/K^+ ATPase, a marker of the plasma membrane. Consistent with the distribution of other plasma membrane markers (30), the Na^+/K^+ ATPase is also distributed rather homogeneously throughout the gradient.

The *middle line* of Fig. 1*A* shows the distribution of synaptophysin, an integral membrane protein of both small synaptic-like microvesicles and endosomes in neuroendocrine cells (36). Clearly, synaptophysin is enriched in the fractions containing the smallest organelles.

The *third line* of Fig. 1*A* shows the distribution of CgB, a major soluble protein of secretory granules. The distribution of CgB was bell-shaped but quite broad, with a peak in fraction 6. Fig. 1*C* summarizes quantitatively the averaged distribution pattern of the three markers obtained in several independent velocity gradient experiments.

Other markers such as mannosidase II for the the Golgi complex and ribophorin for the endoplasmic reticulum had their typical distribution in the velocity gradient, with mannosidase II being accumulated in the last fractions and ribophorin being distributed rather homogeneously (Ref. 30 and data not shown).

In order to achieve an even better separation of the different organelles, we pooled fractions 5–9 of the velocity gradient, performed an intermediate concentration step by centrifugating this pool of fractions on a cushion of 2 M sucrose, and loaded the recovered material on the second equilibrium gradient.

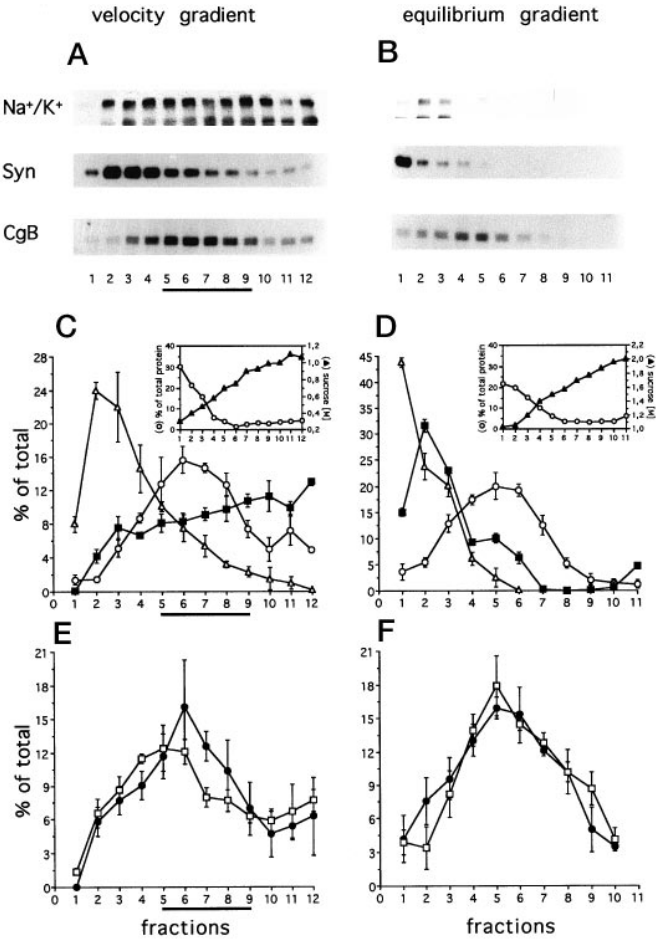

FIG. 1. **Subcellular fractionation reveals the presence of ^{125}I-ω-CTx binding sites in fractions enriched in secretory granules.** PC12-251 cells were homogenized and subcellular fractionation on sucrose gradients was performed as described under "Experimental Procedures." *Panels A*, *C*, and *E* show the data obtained from the velocity gradients. The *black line* underlines the fractions that were pooled, concentrated, and reanalyzed on the equilibrium gradients (*panels B*, *D*, and *F*). In *panels A* and *B*, representative Western blots are shown, which demonstrate the different distribution of the plasma membrane marker (α subunit of the Na^+/K^+ ATPase (*Na^+/K^+*)), the small synaptic-like microvesicle and endosome marker (synaptophysin (*Syn*)) and the secretory granule marker (chromogranin B (*CgB*)). The quantified and averaged values obtained in four independent experiments (*bars* representing S.E.) are shown in *panels C* and *D*, where ■ indicates the α subunit of the Na^+/K^+ ATPase, △ synaptophysin, and ○ chromogranin B. The *insets* in *panels C* and *D* report the molar concentration of sucrose in each fraction as well as the relative distribution of the total cellular proteins. *Panels E* and *F* show the relative distribution of ^{125}I-ω-CTx binding sites in the different fractions. The experiments were performed as described under "Experimental Procedures," starting from control cells (●) or from cells with surface binding sites presaturated with unlabeled toxin (□). The values represent the average of three independent experiments, each performed in quadruplicate, with the *bars* representing the S.E.

As can be seen in the Western blots of Fig. 1*B*, at equilibrium, both the plasma membrane marker Na^+/K^+ ATPase (*top line*) and the small synaptic-like microvesicles and endosomes marker synaptophysin (*middle line*) were highly concentrated in the first fractions containing the less dense organelles. On the other hand CgB, marker of the more dense secretory granules, again had a bell-shaped distribution (*bottom line*) with a peak in fractions 5–6. In agreement with Stinchcombe and Huttner (30), we found that fractions 7, 8, and 9 from these second gradients were almost pure in secretory granules with no contamination by the plasma membrane or small

vesicles. Fig. 1*D* shows the quantified and averaged results of the marker distributions in several equilibrium gradient experiments.

The ^{125}I-ω-CTx binding distribution was very similar to the distribution of CgB, in both the first and second gradient (Fig. 1, *E* and *F*). The distribution was bell-shaped and broad, with a peak in fractions 5–6 and 5 in the first and second gradient, respectively.

^{125}I-ω-CTx binding sites are normally expressed on the cell surface. However, the distribution of ^{125}I-ω-CTx in our gradients did not superimpose with the distribution of the plasma membrane marker, suggesting that most of the cellular ^{125}I-ω-CTx binding sites are present not on the cell surface, but in intracellular organelles (see also below).

To further demonstrate that the membrane component of binding sites did not compromise our data, we performed fractionation experiments and sucrose gradients on cells in which surface binding sites were saturated by a preincubation with unlabeled ω-CTx. Since ω-CTx binds irreversibly to the channels, after fractionation of the cells only intracellular binding sites should be revealed. Although the total recoverable ^{125}I-ω-CTx binding was reduced by around one-fourth (data not shown), the relative distribution of ^{125}I-ω-CTx binding in presaturated cells (Fig. 1, *E* and *D*, *open squares*) was not significantly different from the control (Fig. 1, *E* and *F*, *filled circles*).

The fact that we did not see a significant difference in ^{125}I-ω-CTx binding distribution, regardless of whether the surface component was present or not, is in line with an even distribution of the plasma membrane throughout the velocity gradients and the small amount of plasma membrane loaded on the equilibrium gradients. Together, these data suggest that most of the cellular ^{125}I-ω-CTx binding in PC12-251 cells is intracellular and, specifically, that it is concentrated in the membrane of CgB-containing secretory granules.

Comparison of Secretagogue-induced ^{125}I-ω-CTx Binding Site Translocation with CgB and hsPG Release

High Potassium Stimulations—The presence of ^{125}I-ω-CTx binding sites in the membrane of secretory granules implies that these binding sites should be exposed to the cell surface when the cells are stimulated to exocytose. This was indeed the case. Cell depolarization with KCl dose-dependently stimulated an increase in the number of surface ^{125}I-ω-CTx binding sites in both IMR32 and PC12-251 cells (Fig. 2, *A* and *B*). With 55 mM KCl, ^{125}I-ω-CTx binding was increased, after 10 min of incubation, to 204 ± 3.6% (n = 7) and 212 ± 9.8% (n = 7) that of controls in PC12-251 and IMR32 cells, respectively. Saturation experiments demonstrated that the K_d of ^{125}I-ω-CTx binding was similar in control and after depolarization (10–18 pM) in both cell lines. Under identical experimental conditions, there was no increase in surface ^{125}I-α-bungarotoxin binding to the nicotinic receptor ion channel (data not shown).

The time course of the KCl-stimulated increase in surface N-type VOCCs is shown in Fig. 2 (*C* and *D*). Even after only a 5-min incubation, there was a significant increase in surface ^{125}I-ω-CTx binding, and the peak effect occurred within 10 min in both cell types. In the continuous presence of KCl, the increase in surface binding showed a transient kinetic (Fig. 2, *C* and *D*); furthermore, the removal of KCl after maximal stimulation (10 min with 55 mM KCl) was followed by a return to basal surface binding levels within 3 h (data not shown). Stimulating the cells with high KCl in a Ca^{2+}-free medium, or in a medium containing 100 μM Cd^{2+} to prevent Ca^{2+} influx through the VOCCs, did not induce any increase in surface ^{125}I-ω-CTx binding (Fig. 2, *C* and *D*).

The rapid KCl-stimulated recruitment of ^{125}I-ω-CTx binding sites to the cell surface shares several similarities with the KCl-stimulated release of CgB from PC12-251 cells (Fig. 2*E*, *panel 1*). The peak of release of radiolabeled CgB occurred after 5 min of incubation with 55 mM KCl; the level of released CgB increased to 392 ± 14.72% (n = 3) that of control and no further release was observed after a 30-min incubation (395 ± 12.57%, n = 3). Very little CgB was released in the presence of high KCl, in the absence of extracellular Ca^{2+} (126 ± 7.68% and 115 ± 12.55% of control after 5 and 30 min, respectively). On the other hand, the release of hsPG, a marker for the constitutive pathway of secretion (28, 31), had different kinetics. The levels of radiolabeled hsPG increased slowly in the medium, reached a plateau after 30–40 min, and were only slightly stimulated by KCl in a Ca^{2+}-independent manner (Fig. 2*E*, *panel 2*).

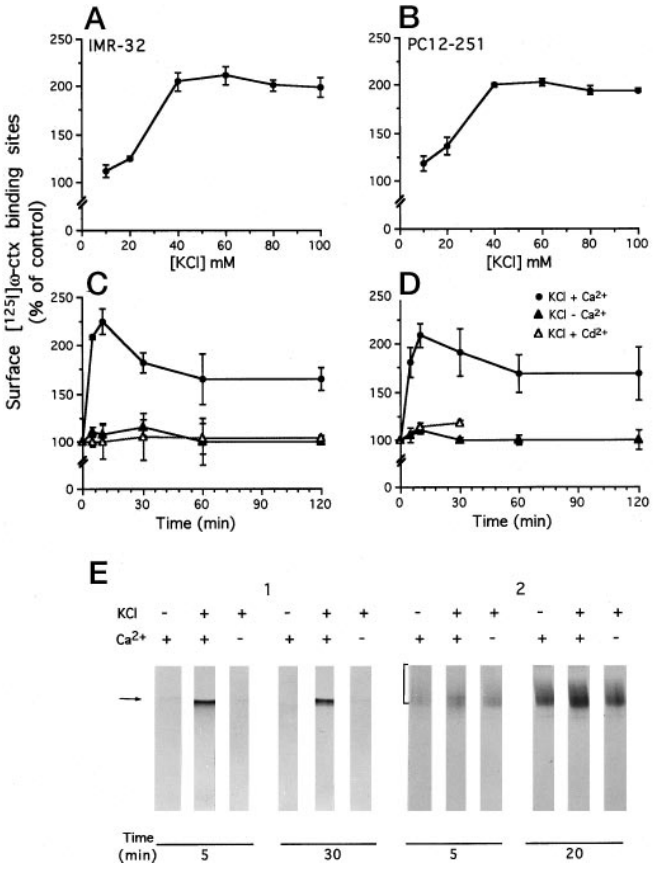

FIG. 2. **^{125}I-ω-CTx binding site recruitment is stimulated by cell depolarization in parallel to regulated but not constitutive secretion.** IMR32 (*A*) and PC12-251 (*B*) cells were incubated with the indicated concentrations of KCl for 10 min at 37 °C, and then surface ^{125}I-ω-CTx binding was performed as described under "Experimental Procedures." Each value represents the average ± S.E. of seven independent experiments. In *panels C* and *D*, the time course of ^{125}I-ω-CTx binding site recruitment is shown for IMR32 and PC12-251 cells, respectively. The cells were exposed to a fixed concentration (55 mM) of KCl in the presence (●) or absence (▲) of external Ca^{2+} or in the presence of 100 μM Cd^{2+} (△) for the indicated times at 37 °C. Surface ^{125}I-ω-CTx binding was then measured. Each value represents the average ± S.E. of seven independent experiments. *Panel E*, *part 1*, shows the time- and Ca^{2+}-dependent release of CgB. PC12-251 cells were labeled overnight with [^{35}S]sulfate, chased for 2 h, and then incubated with 5 mM KCl (−) or 55 mM KCl (+) in the presence of 2.2 mM $CaCl_2$ (+) or with 55 mM KCl (+) in the absence of 2.2 mM $CaCl_2$ (+). The media obtained after the indicated times were subjected to immunoprecipitation using an antibody directed against rat CgB, followed by SDS-PAGE and fluorography. *Panel E*, *part 2*, shows the time-dependent, Ca^{2+}-independent release of hsPG. PC12-251 cells were labeled for 20 min with [^{35}S]sulfate and then incubated in depolarizing or non-depolarizing medium as described above. The media obtained after the indicated times were subjected to SDS-PAGE and fluorography.

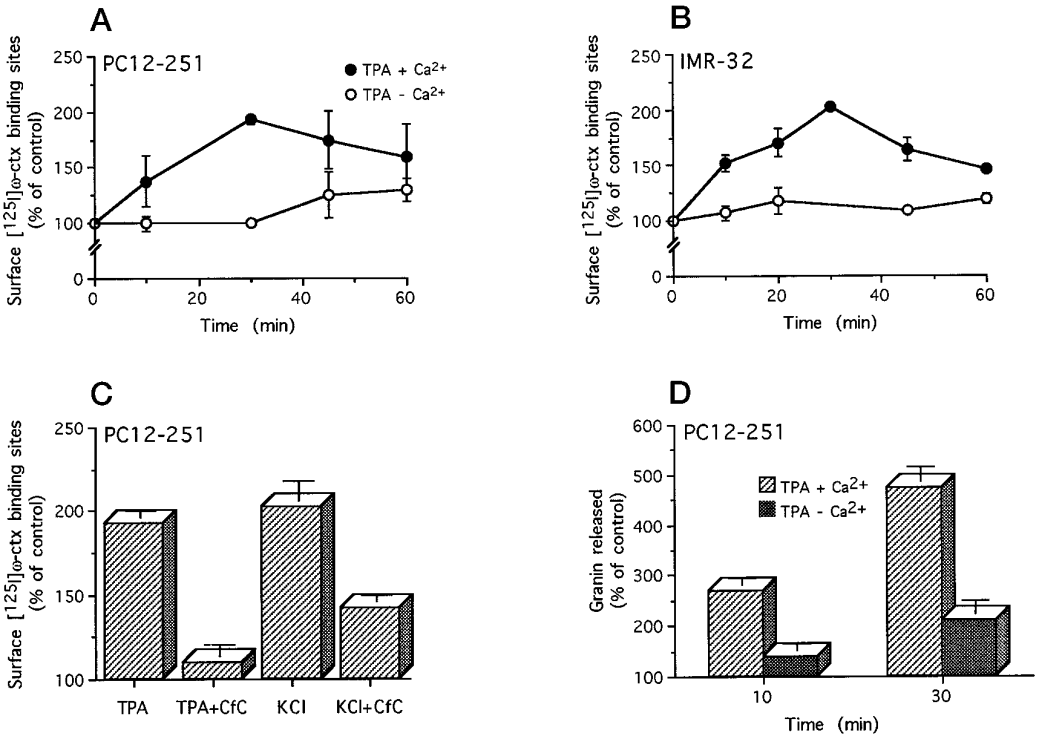

FIG. 3. **Kinetics of ^{125}I-ω-CTx binding site recruitment and CgB release during exposure to TPA.** PC12-251 (*A*) and IMR32 (*B*) cells were incubated with 100 nM TPA at 37 °C for the indicated times, in the presence (●) or absence of Ca^{2+} (○); thereafter, surface ^{125}I-ω-CTx binding was determined as described under "Experimental Procedures." Each value represents the average ± S.E. of five independent experiments. In *C* it is shown that calphostin C (*CfC*), a selective PKC antagonist, completely prevents TPA-stimulated, and partly inhibits KCl-induced, ^{125}I-ω-CTx binding site recruitment. The data represent the average ± S.E. of four experiments. *Panel D* shows that TPA stimulates the regulated secretion of CgB with the same time and Ca^{2+} dependence as ^{125}I-ω-CTx binding site recruitment (▨, TPA + Ca^{2+}; ▩, TPA − Ca^{2+}). ^{35}S-Labeled PC12-251 cells were incubated for 10 and 30 min with or without 100 nM TPA in the presence or absence of extracellular calcium. CgB was immunoprecipitated from the media and quantified by scintillation counting. Values are expressed as percent of the control. *Bars* represent the S.E. obtained from three experiments.

Stimulations with Ionomycin—The above experiments utilized KCl to depolarize the cells and stimulate Ca^{2+} influx through the VOCCs. However, VOCC activation was not a necessary step in order to stimulate ^{125}I-ω-CTx binding site recruitment.

Stimulating Ca^{2+}-dependent secretion with the Ca^{2+} ionophore ionomycin (100 nM) was equally effective in stimulating a large increase in surface ^{125}I-ω-CTx binding. After 15 min of incubation at 37 °C, surface ^{125}I-ω-CTx binding increased in both PC12-251 (198 ± 10.5% of control, n = 3) and IMR32 cells (210 ± 17.0% of control, n = 5).

Stimulations with TPA—When PC12-251 and IMR32 cells were incubated with another secretagogue, the PKC-activating phorbol ester TPA, a dose-dependent recruitment of surface ^{125}I-ω-CTx binding sites was observed in both PC12-251 (199 ± 9.5% of control, n = 4) and IMR32 cells (218 ± 27.6% of control, n = 4). This recruitment was slower than that stimulated by KCl and reached a plateau only after 30 min (Fig. 3, *A* and *B*). Like KCl-induced recruitment, TPA-induced recruitment was also prevented in a Ca^{2+}-free medium (Fig. 3, *A* and *B*). The K_d of ^{125}I-ω-CTx binding also remained similar (10–15 pM). The effects of TPA and KCl were not additive (210% (n = 2) increase with TPA; 208% (n = 2) increase with KCl; 220% (n = 2) increase with both), suggesting a partially common mechanism of action. This is also supported by the fact that the selective PKC inhibitor, calphostin C (1 μM) completely prevented TPA effects but also substantially inhibited KCl-induced recruitment (Fig. 3*C*). CgB release was stimulated by TPA (100 nM) with a slow kinetic that was similar to that of ^{125}I-ω-CTx binding site recruitment (Fig. 3*D*). In addition, in the absence of external Ca^{2+} the rate of TPA-induced CgB release was also greatly reduced (Fig. 3*D*). Therefore, there is a strong correlation between ^{125}I-ω-CTx binding site recruitment and the stimulation of the regulated secretory pathway by TPA as well as by the other secretagogues.

Effects of Brefeldin A on ^{125}I-ω-CTx Binding Site Recruitment and CgB or hsPG Secretion

BFA is known to block the exit of secretory proteins from the trans-Golgi network, but does not inhibit the exocytosis of already formed secretory granules (31, 37). This drug is thus expected to be acutely ineffective on regulated exocytosis and to affect constitutive secretion to a greater extent. This was found to be the case in our experiments; BFA did not affect the secretion of CgB prepackaged in secretory granules (Fig. 4*B*, *panel 1*), but completely blocked the release of hsPG (Fig. 4*B*, *panel 2*). We found that BFA did not inhibit KCl-induced ^{125}I-ω-CTx binding site recruitment in either PC12-251 (Fig. 4*A*) or IMR32 cells (data not shown). This confirmed that the recruitable pool of ^{125}I-ω-CTx binding sites is accumulated in vesicles of the regulated secretory pathway downstream from the trans-Golgi network.

^{125}I-ω-CTx Binding Site Recruitment Does Not Occur in the PC12-27 Subclone Deficient in the Regulated Secretory Pathway

A variant clone of PC12 cells (PC12-27) that has recently been isolated lacks secretory vesicles of the regulated secretory pathway (29) but sustains constitutive secretion.[2] We found

[2] N. Corradi, E. Clementi, J. Meldolesi, and P. Rosa, unpublished results.

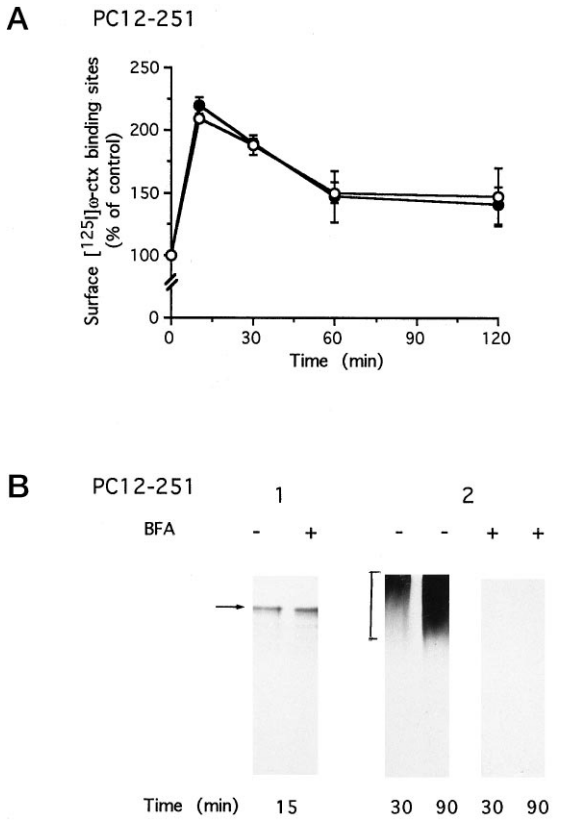

FIG. 4. **Effects of brefeldin A on ^{125}I-ω-CTx binding site recruitment and CgB secretion.** PC12-251 cells were stimulated with 55 mM KCl in a Ca^{2+}-containing medium for the indicated times in order to stimulate ^{125}I-ω-CTx binding site recruitment (*A*). Parallel dishes were stimulated in the absence (●) or presence (○) of 10 μM BFA, and surface ^{125}I-ω-CTx binding was then evaluated. No difference between the two groups was found. Each value represents the average ± S.E. obtained from seven independent experiments, each performed in quintuplicate. The lack of effect of BFA on the regulated release of CgB (55 mM KCl, 15 min, at 37 °C) is also shown in *panel B*, *part 1*. PC12-251 cells were labeled overnight with [^{35}S]sulfate, chased for 2 h, and then incubated 15 min with 55 mM KCl in the presence (+) or absence (−) of BFA. Radiolabeled CgB was then immunoprecipitated from the media. The block by BFA of the constitutive secretion of hsPG is shown in *panel B*, *part 2*. PC12-251 cells were labeled 30 or 90 min with [^{35}S]sulfate in the presence (+) or absence of BFA (−). Total media were then analyzed by SDS-PAGE. The gels shown are from one representative experiment, reproduced three times.

TABLE I
Absence of ^{125}I-ω-CTx binding site recruitment in the secretory deficient PC12–27 cell subclone

^{125}I-ω-CTx binding to intact living cells was performed as described under "Experimental Procedures." The data are expressed as cpm per dish and represent the average ± S.E. of the number of experiments, each performed in quintuplicate, shown in parentheses.

	^{125}I-ω-CTx binding sites
	cpm/dish
PC12–27 cells (control)	963 ± 2.6 (4)
PC12–27 cells pretreated with 55 mM KCl (15 min.)	863 ± 8.0 (4)
PC12–27 cells pretreated with 100 nM TPA (30 min.)	910 ± 12.8 (4)
PC12–27 cells pretreated with 100 nM ionomycin (15 min.)	916 ± 16.2 (2)

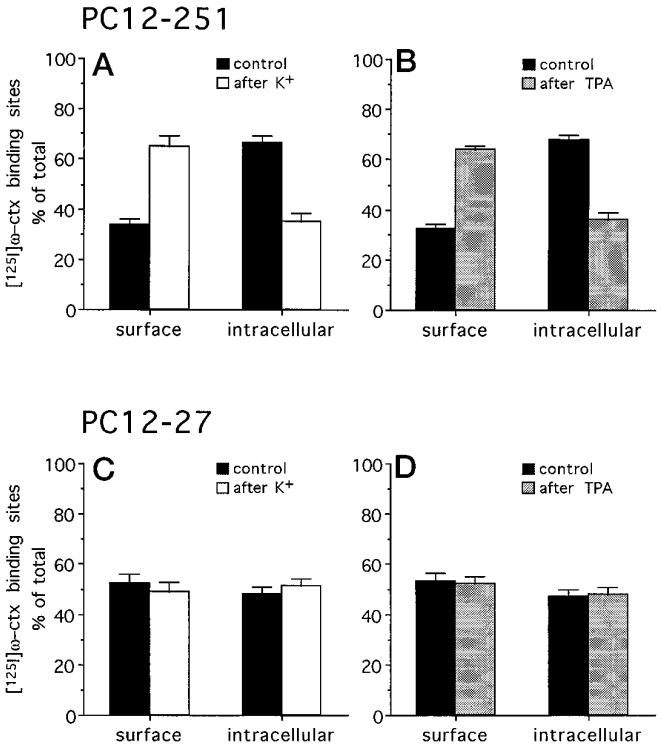

FIG. 5. **Translocation of ^{125}I-ω-CTx binding sites to the cell surface from intracellular compartments.** Surface and intracellular ^{125}I-ω-CTx binding sites were determined in fixed and permeabilized cells as described under "Experimental Procedures." *Panels A* and *B* show that in PC12-251 cells, under basal conditions, only one third of the ^{125}I-ω-CTx binding is on the cell surface. After exposure to 55 mM KCl (*A*) or 100 nM TPA (*B*), there is an increase in surface binding, which is paralleled by a reduction in the intracellular binding. *Panels C* and *D* show that in PC12-27 cells, under basal conditions, the percentage of intracellular binding is significantly lower, and exposure to 55 mM KCl (*C*) or 100 nM TPA (*D*) does not induce any ^{125}I-ω-CTx binding site translocation. Values, obtained from three independent experiments, each performed in quintuplicate, are expressed as percent of total cellular ^{125}I-ω-CTx binding sites, with the *bars* representing the S.E.

that this PC12 subclone expressed surface ^{125}I-ω-CTx binding sites at a comparable level to normal PC12 cells. However, exposure to KCl, ionomycin or TPA did not stimulate any surface ^{125}I-ω-CTx binding site recruitment in these cells (Table I).

^{125}I-ω-CTx Binding Site Recruitment Is Due to Translocation to the Plasma Membrane of the Intracellular Pool of Binding Sites

To further demonstrate that the increase in surface ^{125}I-ω-CTx binding sites (recruitment) in response to the various agents described above is really due to a translocation of the binding sites from the internal pool to the cell surface, occurring during regulated exocytosis, and not to possible modifications of channels preexisting in the plasma membrane, we performed ^{125}I-ω-CTx binding studies on fixed and permeabilized cells.

This protocol confirmed that as for IMR32 and other neuronal cell lines (25), PC12-251 cells contain a large intracellular pool of ^{125}I-ω-CTx binding sites, which is even larger than the surface component (Fig. 5, *A* and *B*). Furthermore, after exposure of PC12-251 cells to either KCl or TPA, there is a large increase in the proportion of surface ^{125}I-ω-CTx binding, which is paralleled by a reduction in intracellular binding (Fig. 5, *A* and *B*).

We studied again the secretory deficient PC12-27 subclone with these fixation/permeabilization protocols and found two interesting and complementary results; not only was the ratio of intracellular *versus* surface binding much less than in PC12-251 cells, but no translocation to the cell surface occurred when

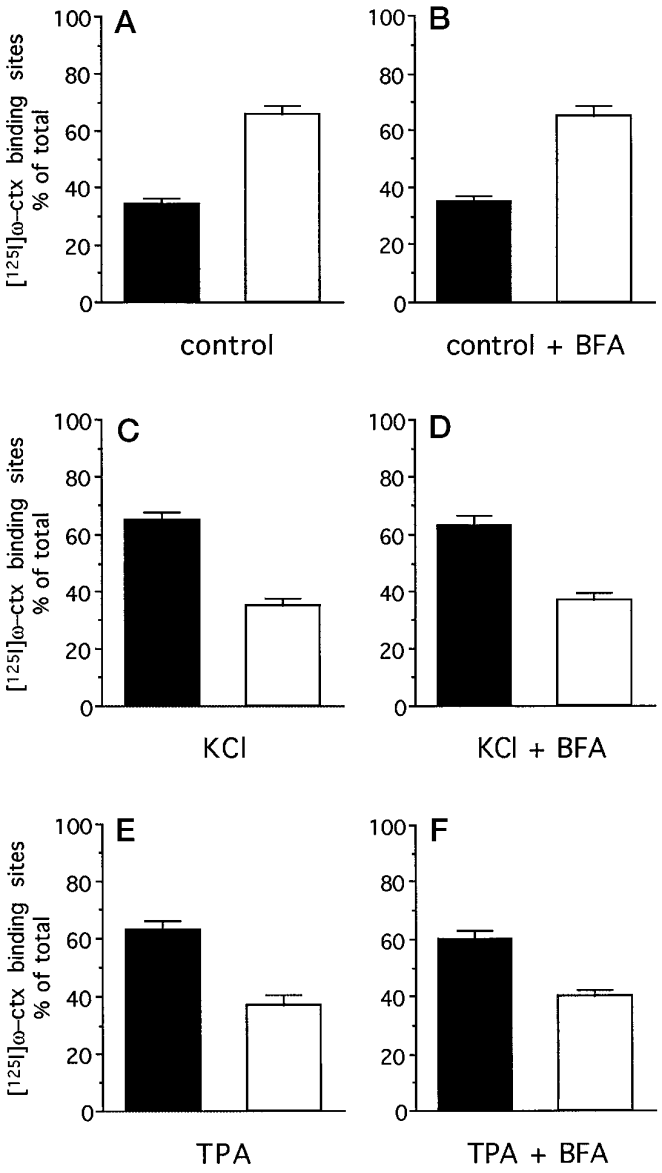

FIG. 6. **Brefeldin A does not affect ^{125}I-ω-CTx binding site translocation.** Fig. 6 shows, in fixed and permeabilized PC12-251 cells that blockade of constitutive secretion with BFA does not affect ^{125}I-ω-CTx binding site distribution under control conditions (*B versus A*) nor the translocation of ^{125}I-ω-CTx binding sites to the cell surface induced by KCl (*D versus C*) and TPA (*F versus E*). Fixation, permeabilization, and ^{125}I-ω-CTx binding were performed as described under "Experimental Procedures." Values, obtained from three independent experiments, each performed in quintuplicate, are expressed as percent of total cellular ^{125}I-ω-CTx binding sites, with the *bars* representing the S.E.

these cells were exposed to either KCl (Fig. 5*C*) or TPA (Fig. 5*D*).

We then confirmed, with the fixation/permeabilization protocol, the result with BFA reported above in time course experiments; in PC12-251 cells BFA alone did not influence the steady-state distribution of ^{125}I-ω-CTx binding sites between the intracellular pool and the plasma membrane, and it did not affect the translocation of ^{125}I-ω-CTx binding sites stimulated with either KCl or TPA (Fig. 6).

These results confirm that control PC12-251 cells translocate, during regulated exocytosis, an intracellular pool of ^{125}I-ω-CTx binding sites present in secretory granules to the cell surface and that a cell lacking secretory granules lacks also the recruitable pool of ^{125}I-ω-CTx binding sites.

TABLE II
Recruitment of functional VOCCs after KCl or TPA pretreatment

Values are expressed in nanomolar $[Ca^{2+}]_i$ concentrations and represent the average ± S.E., obtained from the number of experiments indicated in parentheses. IMR32 and PC12–251 cells were loaded (15 min at 37 °C) with Fura 2, after which they were diluted in normal KRH (control) or in KRH buffer containing either 55 mM KCl or 100 nM TPA (15 min at 37 °C). At the end of these treatments, the cells were stimulated with 60 mM KCl and the increase in $[Ca^{2+}]_i$ determined as described under "Experimental Procedures."

	Basal $[Ca^{2+}]_i$	KCl (60 mM) $[Ca^{2+}]_i$	Δ
	nM	*nM*	%
IMR32 cells (control)	96.4 ± 3.6 (7)	163 ± 12 (7)	69
IMR32 cells pretreated with 55 mM KCl	113 ± 15.1 (4)	338 ± 33.6 (4)	201
IMR32 cells pretreated with 100 nM TPA	84 ± 6.0 (4)	225 ± 11.7 (4)	167
PC12–251 cells (control)	116 ± 2.5 (2)	189 ± 2.0 (2)	62
PC12–251 cells pretreated with 55 mM KCl	96 ± 3.8 (2)	245 ± 28.0 (2)	155
PC12–251 cells pretreated with 100 nM TPA	80 ± 12.1 (2)	192 ± 11.2 (2)	140

Recruited ^{125}I-ω-CTx Binding Sites Are Functional Channels

In order to check whether the recruited ^{125}I-ω-CTx binding sites represent functional VOCCs, Fura-2 measurements of the depolarization-dependent increase in $[Ca^{2+}]_i$ were made in both control cells and in cells prestimulated with the various secretagogues (Table II). Basal $[Ca^{2+}]_i$ levels were found to be similar in the different groups of cells, but the increase in $[Ca^{2+}]_i$ in response to cell depolarization (60 mM KCl) was much higher in the cells pretreated for 30 min with 55 mM KCl or 100 nM TPA, than in the control cells (Table II). These data suggest that the recruited binding sites correspond to functional channels.

DISCUSSION

VOCCs are multimeric plasma membrane proteins (3), and, as is the case of most plasma membrane integral proteins, they can be expected to reach the cell surface via the constitutive secretory pathway (38). This may be the case under "basal" conditions, where the number of surface VOCCs is mainly regulated by their turnover rate (23), but we have shown recently (25) that cultured neuronal cells contain a large intracellular pool of ^{125}I-ω-CTx binding sites that can be transported to the cell surface in response to different experimental manipulations. In this paper, we describe the novel finding that ^{125}I-ω-CTx binding sites are present in subcellular fractions of PC12-251 cells enriched in secretory granules and that they can be translocated to the plasma membrane via a process, which, given that it is stimulated by cell depolarization, Ca^{2+} influx, and PKC activation and is insensitive to BFA, has all of the characteristics of a regulated secretion. Its time course is also strictly parallel to that of regulated, but not constitutive, release. Preliminary experiments showing a nocodazole sensitivity of the translocation event also suggest a possible involvement of microtubules in this transport.[3]

Regulated translocation of plasma membrane proteins is not a novel finding, especially in the field of transporters and ion channels, the glucose transporter being one of the most thoroughly studied. Some confusion still exist, however, on the

[3] M. Passafaro and E. Sher, unpublished results.

nature of the vesicles responsible for the translocation event. Glucose transporters have been transfected in PC12 cells by two groups, but, whereas one described its accumulation in a new type of vesicles (39), the other showed its accumulation in the secretory granules (40). These are, however, transfection experiments that do not necessarily represent the situation *in vivo*. Our results, instead, suggest that endogenous N-type VOCCs are present in the membrane of secretory granules. Noteworthy is the complete discordance between synaptophysin and ^{125}I-ω-CTx binding sites localization. From these preliminary experiments, we cannot exclude that some ^{125}I-ω-CTx binding sites could "travel" through either endosomes or synaptic-like microvesicles before reaching their dominant accumulation sites, *i.e.* the secretory granules and the plasma membrane. However, at steady state, no accumulation of ^{125}I-ω-CTx binding sites was detectable in these organelles.

A "regulated" translocation of voltage-dependent Na^+ channels (41), nerve growth factor receptors (42), and acetylcholinesterase (43) has been reported. However, to our knowledge this represents the first report of a regulated translocation of VOCCs in neuronal cells.

Conflicting results have been reported concerning the effects of cell depolarization with high KCl on the expression of neuronal VOCCs; continuous exposure to high KCl for several days causes a reduction in surface Ca^{2+} channels in cultured rat myenteric neurons (44), but short, daily stimulations with high KCl cause an increase in Ca^{2+} channels in cultured rat hippocampal neurons (45). The effects described here are quite different. The former effects of KCl occur on a time scale of days, require protein synthesis, and are difficult to correlate with secretory events.

The effect of TPA are also intriguing; they are probably mediated by PKC activation (since they are blocked by calphostin C), but the exact target of PKC action is unknown. The α_1 subunit of the N-type VOCC itself has been shown to be a substrate for PKC-mediated phosphorylation (46); however, although Ca^{2+} channel gating properties can be affected by phosphorylation, the fact that we are measuring an increase in the *number* of surface channels and the parallelism with CgB release both support the idea that the TPA-induced N-type VOCC recruitment is also related to a stimulation of a regulated secretory pathway. In line with this, the effects of both TPA and KCl required the presence of extracellular Ca^{2+}. It is possible that the inhibition of K^+ channels induced by TPA-activated PKC depolarizes the cells, and thus stimulates the opening of VOCCs, Ca^{2+} influx, and subsequent exocytotic release, as has been shown previously in the case of pancreatic β cells (47). In this respect, the effects of KCl and TPA could be considered very similar, although a direct stimulatory effect of PKC on the secretory apparatus is also possible.

PKC-mediated modulation of VOCCs, as well as PKC-mediated recruitment of "covert" VOCCs has been reported previously (48–50). Our present data support the hypothesis that at least in same of these preparations, VOCCs recruitment could be also due to a regulated secretion of VOCC-containing vesicles.

The form of N-type VOCC recruitment described here (fast, depolarization- and Ca^{2+}-dependent, and PKC-mediated) is not only different from the constitutive pathway of secretion (see "Results"), it is also different from another form of N-type VOCC recruitment we described recently, which was stimulated by exposing the cells to either ω-CTx or Cd^{2+} (25). The present form of recruitment due to translocation during granule exocytosis is faster, occurring over minutes rather than hours. Furthermore, the two types of recruitment are readily discriminated by BFA, which does not affect the present form, but almost completely inhibits ω-CTx-induced VOCC recruitment. Another difference, which is related to the previous point, is the fact that the overall extent of VOCC recruitment is much larger during ω-CTx treatment than during the stimulation of regulated secretion (5–6-fold *versus* only 2-fold). A further difference is also that ω-CTx-induced VOCC recruitment is mostly prevented at 20 °C, whereas the translocation events here described are only slowed down at this temperature.[3] Further studies are needed in order to define better the secretory pathways utilized by the two recruitment processes.

What could be the functional significance of N-type VOCCs recruitment during secretion? In all biochemical schemes of the secretory apparatus, the VOCCs are placed in a "static" position on the cell surface, with all the "dynamism" attributed to the so-called vSNAREs (proteins of the vesicles) and tSNAREs (proteins of the target membrane). However the exact contribution of each single protein to the secretory machinery is still controversial. For example syntaxin, a protein that is considered a typical tSNARE, and therefore believed to be present mainly, if not only, on the plasma membrane, was recently shown to be present also in the membrane of secretory granules (51). Interestingly, syntaxin is one of the few proteins shown to modulate VOCC gating in the plasma membrane (52), probably through a direct physical interaction (53). It might not be a case, therefore, that both syntaxin (51) and *bona fide* N-type VOCCs (this paper) are present together in the membrane of the secretory granules.

The rapid and presumably localized insertion of new VOCCs during exocytosis may underlie different forms of facilitation of stimulus-secretion coupling reported in the literature. For example, in rat neurohypophyseal terminals, specific patterns of stimulations have been shown to facilitate both Ca^{2+} uptake and hormone release (54). More recently Wojtowicz *et al.* (55) have shown that the long term facilitation of neurotransmitter release, which occurs at the crustacean neuromuscular junction following repetitive stimulation, is accompanied by a remodeling and by an increase in the number of VOCC-containing active zones, in strong agreement with the evidence of an exocytosis-dependent insertion of new VOCCs here reported. As mentioned above, ω-CTx-sensitive N-type VOCCs are known to participate in the "synapto-secretosome," a multimolecular protein complex composed of both plasma membrane and vesicular proteins that is responsible for the fast and localized release of neurotransmitters (12). Our present data, showing a regulated insertion of Ca^{2+} channels in the plasma membrane, further support the evidence that this complex and its function is highly regulated (56).

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N-type Ca^{2+} Channels Are Present in Secretory Granules and Are Transiently Translocated to the Plasma Membrane during Regulated Exocytosis

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