Intracellular Ca^{2+} channel immunoreactivity in neuroendocrine axon terminals

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Abstract The concentration of neuroendocrine terminals in the neurohypophysis facilitates the identification and localization of Ca^{2+} channel subtypes near neuroendocrine release sites. Immunoblots of rat neurohypophysial tissue identified the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits. Immunofluorescence staining of axon terminal plasma membranes was weak, suggesting that Ca^{2+} channels are dispersed. This contrasts with the highly punctate $\alpha_1$2.2 immunoreactivity in bovine chromaffin cells; the neurohypophysial terminals may therefore lack the specialized release zones found in those cells. Immunofluorescence and immunogold labeling identify dense core granule-like structures in the terminal cytoplasm containing multiple Ca^{2+} channel types. Ca^{2+} channels in internal membranes may play an important role in channel targeting and distribution in neuroendocrine cells.

Key words: Calcium channel; Rat; Neurohypophysis; Secretion; Bovine chromaffin cell

1. Introduction

The axons of the magnocellular neurosecretory cells of the hypothalamus (MNCs) terminate in thousands of swellings and endings in the neurohypophysis. These terminals, which may be extraordinarily large (up to 15 μm), release either vasopressin or oxytocin in response to action potentials generated in their cell somata [1]. The extent of release is finely controlled by the rate and pattern of firing [2], and this control may be mediated in part by the biophysical properties of Ca^{2+} channels in the terminals. Patch-clamp experiments in isolated axon terminals have identified N-, L-, P/Q-, and R-type Ca^{2+} channels [3–6], although not the T-type currents found in MNC somata [7]. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of Ca^{2+} channel genes in single MNC somata confirmed the expression of the $\alpha_1$1.2, $\alpha_1$1.3, $\alpha_1$2.1, and $\alpha_1$2.2, but not $\alpha_1$2.3 subunits [8] (previously referred to as α_{1C}, α_{1D}, α_{1A}, α_{1B}, and α_{1E}; for a description of the new nomenclature see [9]). The functional significance of multiple Ca^{2+} channel subtypes in the terminals is not well understood. The N-type channel blocker ω-conotoxin blocks some but not all electrically stimulated release from isolated neurohypophyses (50–70% [10,11]). Release evoked by long-lasting depolarization with high K^+ may be mediated by influx through any of the Ca^{2+} channel types identified in the terminals, except that the P/Q-type and R-type channels appear to be selectively involved in vasopressin [5] and oxytocin release [6], respectively.

We present here an immunological study of the Ca^{2+} channel α_1 subunits in the neurohypophysial terminals. This study aims first to determine the types of α_1 subunits present, and second to study their distribution on the terminal membrane. Specifically, we test the recent conclusion, based on rapid measurements of voltage-gated Ca^{2+} influx, that Ca^{2+} channels are homogeneously distributed on the terminal membranes [12]. These results were in contrast to those from similar experiments on bovine chromaffin cells (BCCs) suggesting that Ca^{2+} channels are highly clustered [13] and that these sites of influx correspond to specialized release zones [14]. Ca^{2+} channel clustering on the BCC plasma membrane was further supported by the pattern of binding of antibodies directed against N-type channels [15]. It was therefore of interest to see whether immunocytochemical experiments would support a different distribution of Ca^{2+} channels in these two neuroendocrine cell types. The third aim is to study the distribution of Ca^{2+} channels on intracellular membranes. The presence of Ca^{2+} channels in intracellular membranes could be involved in transport of channels to the terminal membranes from their site of synthesis in the MNC somata and could also be involved in a regulated process of channel insertion in response to a physiological signal, as has been demonstrated in *Aplysia* neurons [16,17] and in sea urchin eggs [18].

2. Materials and methods

2.1. Isolation of neurohypophysial terminals

The axon terminals of the neurohypophysis were isolated as previously described [4], with minor modifications as described below. Animal use was reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee. Male Long–Evans rats (150–300 g) were killed by decapitation using a small rodent guillotine (model 51330; Stoelting Company, Wood Dale, IL, USA) following anesthesia with chloroform. The neurohypophysis was extracted and incubated for 90 min at 34°C in 10 ml of an oxygenated (100% O_2) saline containing (in mM): NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] disodium salt (PIPES), 20; D-glucose, 25; pH of 7.1; and containing trypsin (0.7 mg/ml; Sigma type XI, Sigma, St. Louis, MO, USA). The neurohypophysis was then rinsed in trypsin-free oxygenated PIPES saline (up to 2 h), triturated with fire-polished pipettes (0.2–0.5 mm in diameter), and plated onto untreated glass-bottomed Petri dishes. Experiments were performed on terminals isolated using a trypsin incubation to maintain conditions used for previous electrophysiological experiments [4,12]. Similar immunocytochemical results were obtained when terminals were isolated by trituration in the absence of trypsin (data not shown).

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2.2. Preparation and culture of BCCs

Chromaffin cells were isolated from bovine adrenal medullae by enzymatic digestion [15]. Isolated cells were suspended in Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, 10% fetal calf serum, 8 mM 5-fluoro-2′-deoxyuridine, 50 mg/ml gentamicin, 10 mM cytosine arabinofuranoside, 2.5 mg/ml fungizone, 25 U/ml penicillin, 25 mg/ml streptomycin and plated at a density of 100 000–300 000 cells/ml on glass coverslips that formed the bottom of a 35 mm diameter Petri dish. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C for 1–3 days prior to use.

2.3. Western blots

One to three neurohypophyses were rapidly dissected and homogenized on ice using a disposable tissue grinder (Kontes Glass) in a PIPES buffer (pH 7.4) containing the following mixture of protease inhibitors: pepstatin A, leupeptin, and aprotinin (each at 1 mg/ml), phenylmethylsulfonyl fluoride (0.2 mM), benzamidine (0.1 mg/ml), and calpain inhibitors I and II (8 mg/ml each). One fifth volume of 5×Laemmli buffer containing 1% β-mercaptoethanol was added to the sample, which was then heated at 70°C for 10 min, centrifuged, and run on a 6% SDS–PAGE gel (approximately 0.25–0.50 neurohypophysis per lane). The proteins were then transferred to nitrocellulose membranes (Hybond-ECL, Amersham, Little Chalfont, Bucks, UK). Strips were incubated with affinity-purified rabbit polyclonal antibodies (Alomone Labs, Jerusalem, Israel) directed against portions of the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits from rat (1:500 dilutions) for either 1 h at room temperature or overnight at 4°C. Phosphate-buffered saline, pH 7.2 (PBS; Gibco BRL, Gaithersburg, MD, USA) and 0.1% Tween 20 were used in all the blocking, incubation and washing steps. Non-fat milk was present in the blocking (5%) and in the washing solutions (1%). Anti-rabbit or anti-rat horseradish peroxidase-linked antibodies were used at 1/1000 dilutions as the secondary antibodies. The immunoreactive bands were visualized using ECL Western blotting detection reagents (Amersham Pharmacia). To demonstrate the specificity of the antibodies, the membranes were stripped of antibodies by incubating with stripping buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) at 50°C for 30 min, washed twice for 15 min in washing solution, and then exposed as above to antibodies pre-incubated for 1 h with an equal weight of the antigen peptides (Alomone Labs).

2.4. Immunofluorescence

Acutely isolated neurohypophysial terminals (1–3 h following plating) or BCCs (1–3 days following plating) were rinsed twice in PBS, and then fixed for 20 min in 'PLP' fixative (PBS with 4% paraformaldehyde, 0.05% sodium periodate, and 0.34% lysine [19]). Following eight rinses in PBS, the dishes were incubated 30 min in blocking solution (PBS with 4% normal goat serum, and 0.02% Triton X-100), and then left overnight at 4°C in blocking solution containing the indicated antibodies. These included an affinity-purified rabbit polyclonal antibody directed against rat syntaxin 1 A/B (1:100; Alomone Labs), mouse ascitic fluid containing a monoclonal antibody directed against syntaxin 1 (1:20; Sigma), a rat monoclonal antibody directed against the $\alpha_1$2.2 Ca^{2+} channel subunit (cc20, 1:500 [15]) and affinity-purified rabbit polyclonal antibodies directed against the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits from rat (Alomone Labs; 1:100, 1:200, 1:200; and 1:1000, respectively). Following eight rinses in blocking solution, the dishes were incubated for 30 min in the dark with either an anti-rabbit (or anti-rat) secondary antibody from sheep (conjugated with Cy2 or Cy3, diluted 1:100; Amersham) or a biotinylated anti-rabbit antibody followed by a 30 min incubation in streptavidin labeled with Cy2 or Cy3 (1:1000; Amersham). The dishes were then rinsed (four times in blocking solution, four times in PBS), filled with mounting solution (0.1% *p*-phenylenediamine in PBS/glycerol; 1:3), topped with a coverslip, and maintained at 4°C until viewing. For double labeling experiments with two different Ca^{2+} channels, the fixation and labeling procedure was repeated with a different primary antibody and a secondary antibody labeled with a different fluorophore prior to mounting. Control experiments showed that the second fixation effectively prevented binding of the second secondary antibody to the first primary antibody (see Fig. 5B). Samples were viewed on a Zeiss Axiovert microscope using a mercury lamp and a cooled CCD camera (Photometrics, Tucson, AZ, USA), or for the double labeling experiments, a confocal microscope (model LSM 510, Carl Zeiss, Inc., Oberkochen, Germany). No attempt was made to investigate whether Ca^{2+} channel types are differentially distributed on vasopressin and oxytocin terminals, which has been proposed based on differences in sensitivity to antagonists [5,6].

2.5. Immunoelectron microscopy

Neurohypophyses were dissected, fixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 2 h, rinsed in PBS, and dehydrated by incubation with a series of ethanol solutions at progressively lower temperature ending with 80% ethanol at −20°C. The tissue was infiltrated with LR White resin for 24 h, embedded in fresh LR White and polymerized for 2–3 days at 50°C. Thin sections (∼250 nm) were mounted on nickel grids and blocked in PBS containing 0.1% Tween 20 (PBST) and 2% normal goat serum. The indicated Ca^{2+} channel antibodies were diluted 1:20 in PBST and grids were incubated for 3 h at room temperature. Sections were rinsed in PBST and incubated in a secondary antibody conjugated to colloidal gold (diameter 5 nm). After rinsing thoroughly in PBST and water, the colloidal gold was silver enhanced for 3–4 min (using a commercially available kit from Amersham), stained with uranyl acetate and lead citrate, and examined using a Philips CM-10 transmission electron microscope, at 60 kV.

3. Results

3.1. Calcium channels in the neurohypophysis

PCR studies of MNC somata have demonstrated expression of the $\alpha_1$1.2, $\alpha_1$1.3, $\alpha_1$2.1, and $\alpha_1$2.2 Ca^{2+} channel subunit genes in MNC somata [8] and electrophysiological recordings from isolated MNC axon terminals have provided evidence for L-, N-, P/Q-, and R-type Ca^{2+} currents [3–6]. To test for the presence of different α_1 subunit proteins, immunoblot analysis was performed on neurohypophysial homogenates. The neurohypophysis is devoid of neuronal somata and is composed almost entirely of MNC axons and axon terminals [20]. Pituicytes, the glial cells found in the neurohypophysis, are unlikely to express voltage-gated Ca^{2+} channels [21]. Antibodies generated against specific sequences of the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits each recognized specific protein bands (Fig. 1). The $\alpha_1$1.3 antibody labeled a protein of about 170–180 kDa. The antibody against $\alpha_1$2.1 recognized a band of about 170–180 kDa and a band of about 220–240 kDa. The antibody against $\alpha_1$2.2 labeled a band of about 210 kDa, while the $\alpha_1$2.3 labeled a protein band at about 220–240 kDa and a lighter band at about

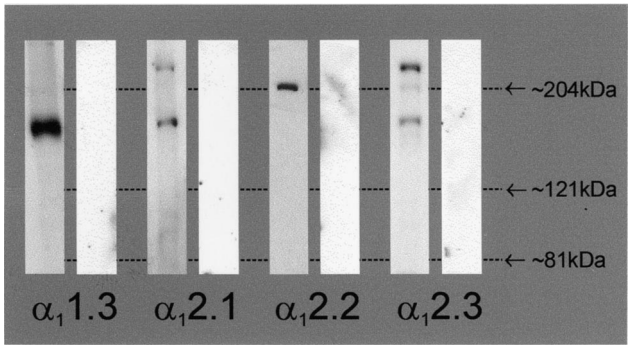

Fig. 1. Immunoblots of neurohypophysial homogenates probed with rabbit polyclonal antibodies raised against the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits. The membranes to the left of each set show the staining of the membranes with the indicated antibodies; membranes on the right show the lack of staining when the antibodies were pre-adsorbed with the peptide fragments of the Ca^{2+} channels against which they were raised. The positions of molecular weight markers run in adjacent lanes are shown for comparison.

170–180 kDa. The presence of multiple isoforms of these channel subunits in these molecular weight ranges is consistent with previous immunological and molecular work [22–33]. Pre-adsorption of the antibodies with the corresponding Ca^{2+} channel peptide completely prevented binding (Fig. 1). Antibodies directed against $\alpha_1$1.2 did not recognize a protein band in immunoblots of neurohypophysial homogenates and were thus not used for immunocytochemical experiments.

3.2. Immunofluorescence

The distribution of Ca^{2+} channel subtypes in isolated neurohypophysial terminals was studied using immunofluorescence. Antibodies against the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 subunits yield similar patterns of immunofluorescence staining (Fig. 2). None of these antibodies yield clear staining of the plasma membrane. Each does, however, show punctate staining within the terminal cytoplasm. Fig. 2 shows the labeling of each of the antibodies at two focal planes in an isolated terminal near the plane of largest cross-sectional area. In all cases there are a large number of structures distributed throughout the terminal cytoplasm that show some fluorescence and a smaller number of structures that stain much more brightly.

The absence of detectable plasma membrane staining would be consistent with a homogeneous distribution of Ca^{2+} channels, if it is assumed that the density is below that detectable by our method. A terminal isolated under these conditions with a diameter of 15 μm might have a peak Ca^{2+} current of about 200 pA in 10 mM Ca^{2+} [12]. If it is assumed that N-type channels in these cells have roughly half the conductance of L-type currents [34], that L-type channels have a conductance of about 0.1 pA at 0 mV in 10 mM Ca^{2+} [35], and that N-type channels constitute one-half or less of the total current [5], it can be calculated that a depolarization would be expected to open about 2000 channels, or about eight channels per μm^2. This corresponds to a mean interchannel distance, assuming that the channels are homogeneously distributed and that all open during a depolarization, of about 350 nm, which might be below the sensitivity of our method to detect.

The presence of syntaxin 1 has been demonstrated in the neurohypophysis using immunoblotting [36], and antibodies against syntaxin 1 were able to co-immunoprecipitate a portion ($\sim$50%) of ω-conotoxin GVIA binding sites in neurohypophysial homogenates [37]. The distribution of syntaxin immunoreactivity on neurohypophysial terminal membranes may therefore reflect in part the distribution of N-type Ca^{2+} channels. The observed pattern of immunofluorescence staining for an antibody against syntaxin 1 is primarily on the plasma membrane and appears to be homogeneous (Fig. 3A). This is in marked contrast to the distribution of syntaxin immunoreactivity in BCCs. Previous experiments with a monoclonal antibody directed against the N-type Ca^{2+} channel have shown that these channels are highly concentrated at a small number of sites on the BCC plasma membrane [15]. These data are consistent with data using pulsed laser Ca^{2+} imaging showing that influx of Ca^{2+} during brief depolarizations of patch-clamped BCCs occurs in a non-homogeneous fashion [13] and that the sites on influx are associated with sites of preferential release [14]. Confocal imaging experiments show that the punctate immunoreactivity for the $\alpha_1$2.2 Ca^{2+} channel subunit in BCCs largely overlaps with a similar punctate distribution for syntaxin (Fig. 3C). This suggests that both $\alpha_1$2.2 and syntaxin are specifically targeted to organized release sites on the BCC membrane. There are also spots of syntaxin immunoreactivity that do not coincide with those of $\alpha_1$2.2, which may be explained by clusters of other types of Ca^{2+} channels that are also associated with clusters of syntaxin. This is consistent with pharmacological experiments showing that some, but not all sites of localized Ca^{2+} influx are blocked by ω-conotoxin [15]. Syntaxin 1 staining has also been shown to be localized at release sites at the frog neuromuscular junction [38], at presynaptic boutons in the cerebellum [39], and at ribbon synapses in cochlear hair cells [40]. The lack of punctate staining for either Ca^{2+} channels or syntaxin 1 on the neurohypophysial terminals is consistent with the hypothesis that these terminals lack large clusters of Ca^{2+} channels on their plasma membrane [12].

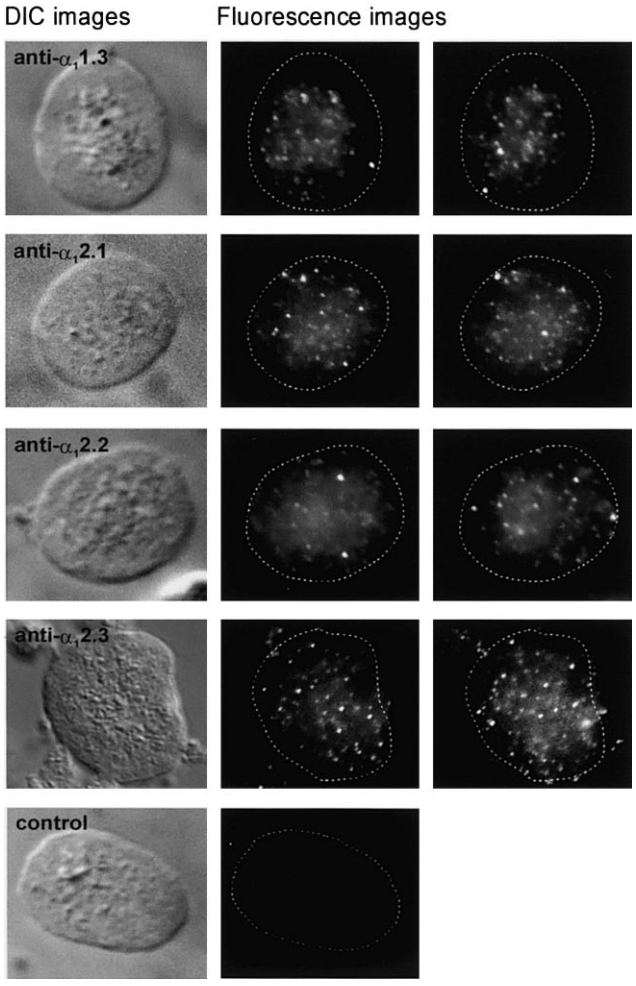

Fig. 2. Immunofluorescence labeling of isolated neurohypophysial terminals using rabbit polyclonal antibodies directed against portions of the $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits. On the left are differential interference contrast (DIC) images of five neurohypophysial terminals and on the right are fluorescence images showing the pattern of labeling with the indicated antibodies, and the lack of staining in the control experiment. For each of the antibodies, fluorescence images are shown at two different focal planes near the widest part of the terminal; the approximate location of the terminal membrane is shown by the dotted line. Note the presence of punctate cytoplasmic staining for each of the antibodies. A control experiment, for which no primary antibody was added, is shown at the bottom.

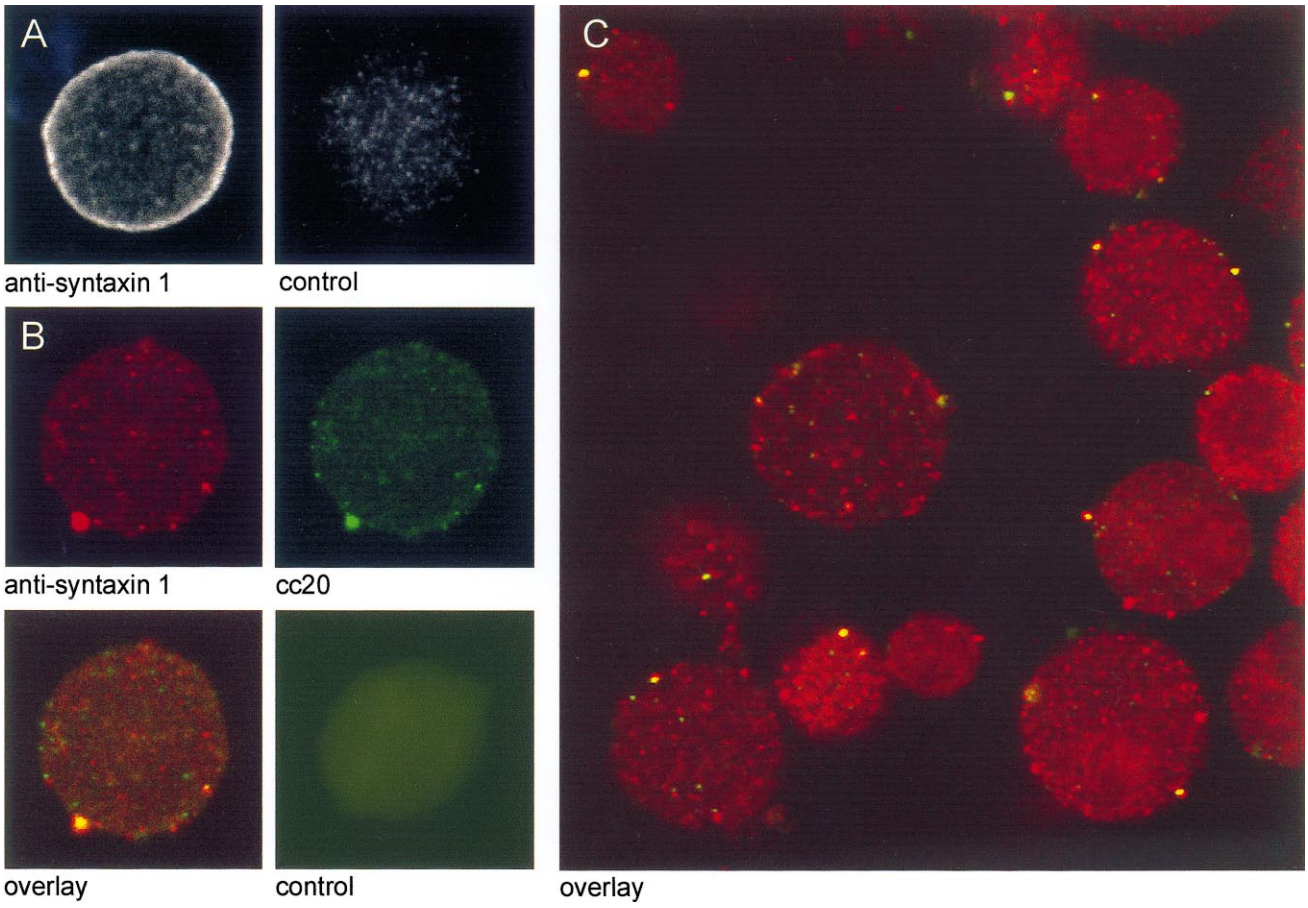

Fig. 3. Immunofluorescence labeling of neurohypophysial terminals and bovine chromaffin cells using a rabbit polyclonal antibody directed against syntaxin 1. A: The pattern of fluorescence obtained in an isolated neurohypophysial terminal. Note the homogeneous membranous staining. The control shows the lack of staining when the primary antibody was omitted. B: Double labeling of an isolated BCC with the anti-syntaxin antibody (red) and a monoclonal rat antibody (cc20) directed against the N-type Ca^{2+} channel (green) shows that both yield a punctate pattern of staining. At this focal plane one intense spot on the cell membrane is visible in both cases. Overlaying the two patterns shows colocalization of the immunoreactivity for the two antibodies (yellow). The control image shows the lack of staining in the absence of primary antibodies. C: Double labeling with cc20 and anti-syntaxin in a field of BCCs. Note that there are several yellow spots indicating colocalization of the immunoreactivity of the two antibodies, and also intense red spots on the membranes suggesting clusters of syntaxin in the absence of N-type Ca^{2+} channels. The cc20 antibody was kindly given by Dr. V.A. Lennon.

3.3. Electron microscopy

To determine whether Ca^{2+} channels are associated with specific intracellular structures, we studied the immunoreactivity of Ca^{2+} channel antibodies at the electron microscopic level. Immunogold labeling of rapidly fixed neurohypophysial tissue with antibodies against $\alpha_1$1.3, $\alpha_1$2.1, and $\alpha_1$2.2 subunits revealed a pattern of staining that was similar to that seen with immunofluorescence (Fig. 4). Although little or no binding of the Ca^{2+} channel antibodies to the terminal plasma membrane was observed, intense labeling was evident in a small number of internal structures similar in size and appearance to dense core granules. The labeling of structures with this appearance was heterogeneous; some structures were labeled with many gold particles whereas the majority was labeled with few or none. This observation supports the hypothesis that a population of internal structures similar to dense core granules is involved in the trafficking of Ca^{2+} channels from their site of synthesis in the MNC somata to the terminal membrane.

3.4. Colocalization of Ca^{2+} channel subtypes

Double staining with antibodies directed against $\alpha_1$2.1 and $\alpha_1$2.2 shows that the immunoreactivity for the two antibodies is partly overlapping (Fig. 5A). Terminals were fixed, incubated with one Ca^{2+} channel antibody and then a labeled secondary antibody (e.g. Cy2) and then fixed again and incubated with a second Ca^{2+} channel antibody and a differently labeled secondary antibody (e.g. Cy3). Shown are two sets of confocal images (separated by 0.5 μm) that show staining for $\alpha_1$2.1 (red), staining for $\alpha_1$2.2 (green), and an image showing the combination of the two, in which areas of colocalization appear yellow. Several bright yellow structures are visible in the two images, indicating the presence of both Ca^{2+} channel subtypes. Similar results were obtained for double staining with $\alpha_1$2.1 and $\alpha_1$1.3 and with $\alpha_1$2.2 and $\alpha_1$1.3 (not shown). In control experiments, the procedure was repeated, but without incubation with the second primary antibody. The resultant image is dominated by the fluorescence of the first secondary antibody (red) and shows little binding of the second

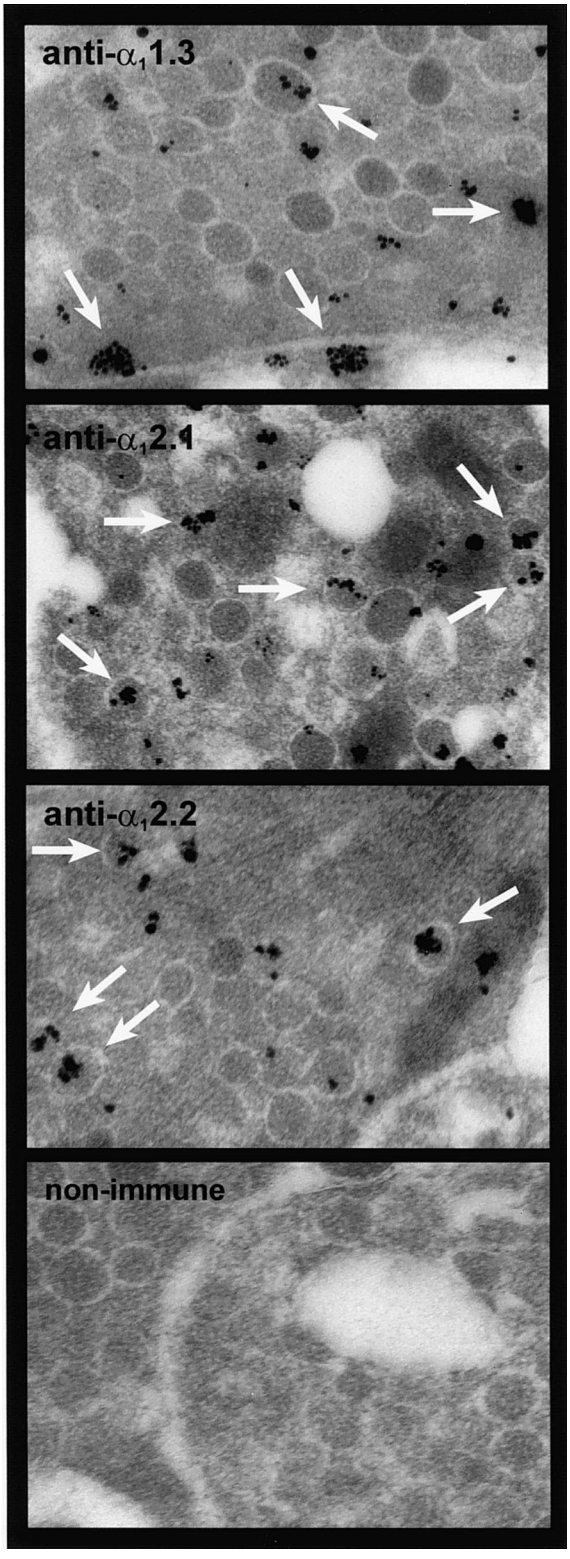

Fig. 4. Immunogold labeling of neurohypophysial terminals using rabbit polyclonal antibodies directed against portions of the $\alpha_1$1.3, $\alpha_1$2.1, and $\alpha_1$2.2 Ca^{2+} channel subtypes. Fixed and embedded thin neurohypophysial sections were incubated with the indicated primary antibody followed by incubation with a secondary antibody conjugated to 5 nm gold particles. In each case, a subset of granule-sized membranous structures were labeled (some examples are indicated by the white arrows). When the sections were treated with non-immune serum rather than primary antibodies, there was no labeling with gold particles.

secondary antibody (which would appear in green, Fig. 5B). This demonstrates that the second fixation effectively prevented interaction between the first primary antibody bound to the sample and the second secondary antibody. When the second primary antibody was the same as the first, the fluorescence from the two secondary antibodies overlapped almost completely. These results suggest that the membranous structures contain more than one type of channel, and may be involved in the trafficking of Ca^{2+} channels to the terminal membrane.

4. Discussion

The identification of $\alpha_1$1.3, $\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$2.3 Ca^{2+} channel subunits in the neurohypophysial terminals is consistent with pharmacological and biophysical experiments that identified P/Q-, N-, L-, and R-type Ca^{2+} currents [3–6]. RT-PCR experiments on single MNC somata were able to amplify genes for the $\alpha_1$1.2, $\alpha_1$1.3, $\alpha_1$2.1, and $\alpha_1$2.2 Ca^{2+} channel subunits, but were unable to find detectable expression of the $\alpha_1$2.3 subunit [8]. Pharmacological evidence for R-type currents in oxytocin-releasing terminals [6], however, as well as the evidence presented here, supports the presence of $\alpha_1$2.3 in the neurohypophysis. The functional significance of multiple Ca^{2+} channel types in these terminals is not clear. N-type currents are important in mediating neuropeptide release, since ω-conotoxin GVIA can block 50–70% of electrically evoked release [10,11]. Blockers of P/Q- and R-type currents can inhibit the K^+-evoked release of oxytocin [5] and vasopressin [6], respectively. The role of L-type currents in evoking release is unclear. Dihydropyridines, which block L-type currents, can inhibit K^+-evoked vasopressin release [10,41,42], but have a small effect if any on electrically evoked secretion [10,11,43]. The presence of immunoreactivity for an antibody directed against the $\alpha_1$1.3 subunit supports the earlier immunohistochemical observation of $\alpha_1$1.3 in the supraoptic nucleus of the hypothalamus [44], where many of the MNC somata are found. The terminals may or may not express the $\alpha_1$1.2 subunit as well. The presence of $\alpha_1$1.3 is consistent with the low threshold of activation (−50 mV) of L-type currents in MNC somata [7], since these channels have similar thresholds both in cochlear hair cells [45] and when they are expressed in oocytes [29]. While $\alpha_1$1.3 subunit labeling is generally not found at synaptic terminals in the CNS [30], L-type currents constituted from the $\alpha_1$1.3 subunit are associated with release from pancreatic β-cells [46] and cochlear hair cells [45].

The lack of punctate staining for Ca^{2+} channels or for syntaxin suggests that the terminals do not have the specialized release sites found in the BCCs, although this conclusion must be viewed with caution in the absence of a clear pattern of staining on the terminal membrane. This interpretation is consistent with rapid measurements of voltage-gated Ca^{2+} influx into these terminals [12] and has implications for the mechanism of secretion in the terminals. Ca^{2+} channels and the exocytotic apparatus are closely colocalized at synaptic terminals [47,48], which allows a close temporal relationship between Ca^{2+} influx and evoked release [49]. This relationship allows the rapid processing of information upon which brain function depends. Release from endocrine and neuroendocrine systems, however, is generally much slower [50–53] suggesting that the relationship of Ca^{2+} channels and the release

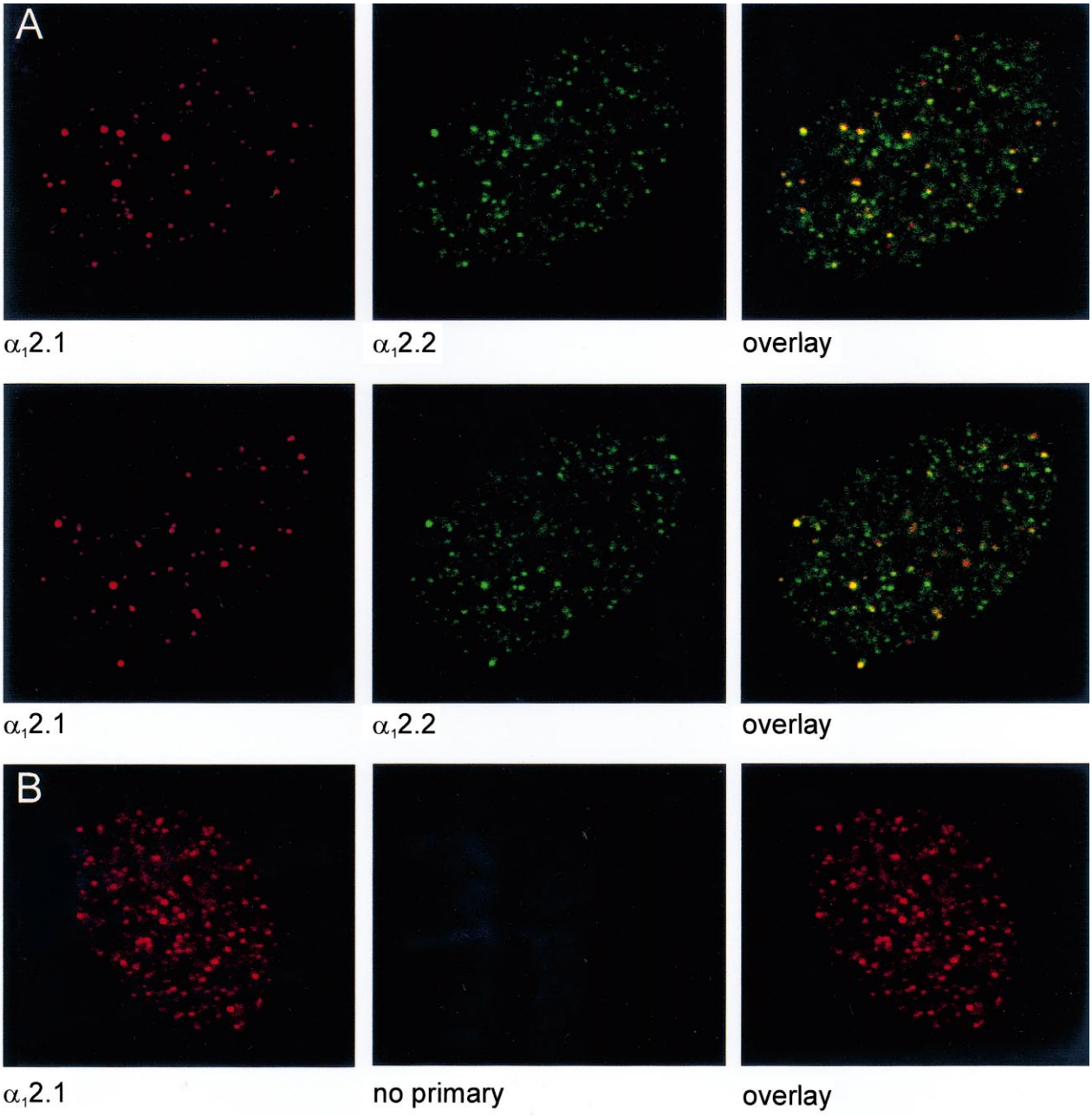

Fig. 5. Colocalization of immunofluorescence labeling of isolated neurohypophysial terminals using rabbit polyclonal antibodies directed against portions of the $\alpha_1$2.1, and $\alpha_1$2.2 Ca^{2+} channel subtypes. A: Two focal planes of a terminal double-labeled with antibodies directed against the two antibodies. Terminals were fixed, incubated with an antibody directed against $\alpha_1$2.1 and then a Cy3-conjugated secondary antibody. They were then fixed again, incubated with an antibody directed against $\alpha_1$2.2, and then a Cy2-conjugated secondary antibody. Labeling with $\alpha_1$2.1 is shown in red, and labeling with $\alpha_1$2.2 is shown in green. The overlay shows that there is a small number of cytoplasmic structures intensely labeled by both antibodies. B: In a control experiment a similar procedure was followed except that the second primary antibody was omitted (i.e. the antibody directed against $\alpha_1$2.2 was not added). The absence of fluorescence from Cy2 indicates that the second fixation effectively prevents binding of the second secondary antibody to the first primary.

apparatus is not as close. Such release is largely blocked by the slow Ca^{2+} chelator EGTA, which suggests that Ca^{2+} must diffuse from its site of entry to its site of action [54]. The slow rate of endocrine and neuroendocrine release therefore suggests that there is no direct association between channels and releasable granules, and that release may be triggered by a general increase in sub-membranous Ca^{2+}, caused by the summation of influx through widely dispersed Ca^{2+} channels. Thus a dispersed distribution of Ca^{2+} channels on the neurohypophysial membrane would be consistent with the relatively slow rate of exocytotic release observed in single cells following depolarization [49,55–58] and the fact that it is largely blocked by the inclusion of EGTA in the patch pipette [50,56,58]. This model is also consistent with observations that release of neuropeptide is optimized by bursts of action potentials interspersed between periods of quiescence [2], which mimics patterns of electrical behavior seen in vivo [1].

Rapid measurements of voltage-gated Ca^{2+} influx in BCCs,

in contrast, suggest that Ca^{2+} channels in these neuroendocrine cells are highly clustered [13]. By combining pulsed laser Ca^{2+} imaging and amperometric measurement of catecholamine release it was shown that these Ca^{2+} channel clusters represent organized release sites [14]. Immunocytochemical analysis supports the clustering of N-type channels on the chromaffin cell membrane [15] and suggests that channel clusters are associated with clusters of syntaxin 1 (Fig. 3). Zones of preferential release are supported with independent data on amperometric localization of release sites [59], and on the localization of fusion of visualized granules [60]. Organized release sites may mediate the rapid phase of catecholamine release seen in response to brief depolarizations of chromaffin cells, including single action potentials [61,62]. Ca^{2+} channel clustering in the BCCs may therefore represent a specialization for rapid release that is not present in neurohypophysial terminals.

The sequestration of voltage-gated Ca^{2+} channels in internal membranes has been observed in neuroendocrine cells in *Aplysia californica* [17]. In these cells, two types of Ca^{2+} channels were found to have a different subcellular distribution – one type of channel was found primarily on internal membranes, while a second channel type was found primarily on the plasma membrane. The translocation of the channel found in the internal membranes to the plasma membrane may underlie the appearance of a new species of channel on the cell surface that occurs in response to treatment with protein kinase C activators [16] and that results in an enhancement of Ca^{2+} influx during the episodic release of egg-laying hormone from these cells [63]. Internal storage of N-type Ca^{2+} channels has been demonstrated in neuroblastoma and PC12 cell lines [64,65]. Channels were found to be enriched in cellular fractions containing chromogranin B-containing secretory granules and were translocated to the plasma membrane by exposure to various secretagogues [65]. Regulated mobilization of channels from internal stores has also been demonstrated in the mammalian collecting duct and the amphibian bladder where water channels are inserted in response to vasopressin [66]. The internal stores of Ca^{2+} channels in the neurohypophysial membranes may therefore serve as a reserve pool that could be inserted into the plasma membrane to enhance the release of neuropeptides when the physiological need is high.

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