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Differential Expression of Syntaxin 1A and 1B by Noradrenergic and Adrenergic Chromaffin Cells*

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The expression and localization of syntaxin isoforms 1A and 1B in adrenergic and noradrenergic chromaffin cells were examined by both immunoblot analysis and confocal immunofluorescence microscopy. Syntaxin 1A was found in higher levels in noradrenergic cells, whereas syntaxin 1B was similarly expressed in most noradrenergic and adrenergic cells. However, some heterogeneity was observed within each catecholaminergic phenotype. Although the majority of adrenergic cells appeared to express low levels of syntaxin 1A, about 7% was strongly stained for syntaxin 1A. A subpopulation of noradrenergic cells, about 17%, expressed greater levels of syntaxin 1B. Syntaxin 1B labeling showed a punctate appearance in the cytoplasm, whereas syntaxin 1A appeared predominantly localized to the plasma membrane. These data show differences in the exocytotic machinery of the two subtypes of chromaffin cells that may underlie some of the distinct characteristics of adrenaline and noradrenaline secretion.

KEY WORDS: Chromaffin cells; exocytosis; syntaxin 1A; syntaxin 1B; adrenaline; noradrenaline.

INTRODUCTION

The secretion of adrenaline and noradrenaline from adrenal chromaffin cells can be differentially regulated by mechanisms not related to the different sets of neurotransmitter receptors expressed by the two main populations of adrenal medulla cells (reviewed in [1]). Adrenaline and noradrenaline release exhibit different sensitivities to Ca^{2+} and depolarizing stimuli (2,3). We have previously reported a greater sensitivity of noradrenaline secretion to botulinum neurotoxin C1 (BoNT/C1) and to BoNT/A, compared to adrenaline secretion (4), which suggested a greater dependence of exocytosis on syntaxin 1 or synaptosomal-associated protein of 25 kDa (SNAP-25) in noradrenergic chromaffin cells. SNAP-25 was already shown to be differentially expressed by noradrenergic and adrenergic chromaffin cells (5). In this work we studied the expression and subcellular localization of syntaxin 1A and syntaxin 1B in the two subtypes of chromaffin cells.

Despite their high homology, the two isoforms of syntaxin 1 may have different functions in the cell (6,7). Syntaxin 1A is involved in the docking and fusion of vesicles with the plasma membrane and in the coupling of the exocytotic machinery to Ca^{2+} channels (8), whereas syntaxin 1B was suggested to be involved in synaptic plasticity (9–11). Many synaptic proteins belong to families with multiple isoforms that are differentially expressed in nervous and neuroendocrine tissues. Synaptotagmin, synapsin, synaptobrevin, SNAP-25, and Rab3 exhibit characteristic patterns of isoform expression (12). Although a redundant role of some isoforms cannot be ruled out, this multiplicity might allow a fine regulation of secretion.

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In the present report we show for the first time that syntaxin 1A is differentially expressed by noradrenergic and adrenergic chromaffin cells. Moreover, we found some heterogeneity in the expression of syntaxin 1 isoforms within each catecholaminergic phenotype, suggesting that noradrenergic and adrenergic cells from adrenal medulla, often considered uniform populations, differ also in essential components of the exocytotic machinery.

EXPERIMENTAL PROCEDURE

Cell Culture. Chromaffin cells were isolated from bovine adrenal glands by digestion with collagenase type B followed by purification on two consecutive gradients of Urografin and Percoll. First, chromaffin cells were separated from endothelial and cortical cells by centrifugation on a discontinuous 7.5%/15% Urografin gradient. The adrenergic and noradrenergic cells were separated on a self-generated Percoll gradient (13). This procedure yielded a denser fraction enriched in adrenergic cells (>90% of PNMT-positive cells) and a less dense fraction enriched in noradrenergic cells (60%–70%). The cells were cultured in a 1:1 mixture of DMEM/Ham's F-12 medium buffered with 15 mM HEPES and 25 mM $NaHCO_3$, supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The populations enriched in either adrenergic or noradrenergic cells were plated in 24-well plates (5×10^5 cells/cm^2) for immunoblot analysis, and on glass coverslips coated with poly-L-lysine (1.25×10^5 cells/cm^2) for immunofluorescence experiments. Cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 and were used 3–4 days after plating. The catecholamine content of chromaffin cells was evaluated in cell lysates by HPLC and electrochemical detection (3).

Western Blotting. Cell extracts were prepared with a lysis buffer containing 50 mM Tris-HCI, 50 mM glicine, 1% SDS (wt/vol), 4% β-mercaptoethanol (vol/vol), and 6 M urea. The plates were scraped and the extracts stored at −20°C. Electrophoresis in a Tris-urea/SDS polyacrylamide gel was carried out as described by Ruiz-Montasell et al. (14), and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P). After blocking for 1 h with 5% (wt/vol) nonfat dried milk in phosphate-buffered saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) containing 0.5% Tween 20 (PBST), the membranes were incubated overnight with the primary antibodies at 4°C. Syntaxin 1A and 1B antibodies were raised against the entire cytosolic region of syntaxin 1 and affinity purified with a cross-absorption step (14). After the incubation with the primary antibodies, the membranes were washed three times, for 15 min each, in PBST containing 0.5% milk powder, followed by 1 h incubation with alkaline phosphatase-conjugated secondary antibodies (1:10.000). Immunoreactive proteins were detected using the enhanced chemifluorescence detection (ECF) kit and quantified by densitometric analysis using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, USA). Protein estimation was performed with the BioRad protein microassay.

Immunofluorescence Staining and Confocal Microscopy. The cells were fixed/permeabilized with methanol/acetone 1:1 for 10 min. The incubation with the primary antibodies was carried out overnight at 4°C, followed by incubation with the fluorochrome-conjugated secondary antibodies at room temperature for 1 h. The preparations were observed in a MRC600 confocal imaging system (BioRad Laboratories, Milan, Italy) linked to a Nikon Optiphot-2 fluorescence microscope. A krypton/argon mixed laser was used in combination with a 488-nm band pass filter (excitation) and a 585-nm long-pass filter (emission) to examine Alexa 488 fluorescence. To detect Cy 5 fluorescence, we used a 647-nm band pass filter (excitation) and a 680-nm long-pass filter (emission).

Materials. Rabbit anti-syntaxin 1A (BR.1A) and rabbit anti-syntaxin 1B (BR.1B) were a generous gift from J. Blasi (Faculty of Medicine, University of Barcelona, Spain). Rabbit anti-dopamine-β-hydroxylase (DβH) was purchased from Affiniti (Exeter, UK), and the sheep anti-phenylethanolamine *N*-methyltransferase (PNMT) antibody was obtained from Chemicon (Temecula, CA, USA). Cy5-conjugated donkey anti-sheep IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and Alexa 488-conjugated goat anti-rabbit IgG was obtained from Molecular Probes Europe. The ECF kit was from Amersham (Buckinghamshire, UK).

RESULTS

To examine the expression of syntaxin 1 in the two main subtypes of chromaffin cells, protein extracts from populations enriched in either noradrenergic or adrenergic cells were subjected to Western blotting (Fig. 1a). Two bands with apparent molecular weight of 38 and 46 kDa were identified using isoform-specific antibodies (14) as the isoforms 1A and 1B of syntaxin 1. The densitometric analysis of blots showed greater levels of both syntaxin 1A and 1B in noradrenergic cell extracts, compared to those in adrenergic cells. To control for protein loading, the blots were also probed for DβH, a protein present in equal amounts in noradrenergic and adrenergic chromaffin cells (15), and all the quantifications were made using the ratios of the densities of syntaxin to DβH bands (Fig. 1b).

Because of the contamination of the isolated subpopulations of chromaffin cells (13), we further examined the expression of syntaxin 1 isoforms by performing double immunofluorescence labeling for syntaxin and for PNMT, a marker of adrenergic cells. Since noradrenergic cells represent a small fraction of bovine adrenal chromaffin cells, equal amounts of noradrenergic-enriched and adrenergic-enriched cell populations were mixed before plating on coverslips, so that both noradrenergic and adrenergic cells could be observed in most microscope fields. Figure 2a shows that the vast majority of cells intensively labeled for syntaxin 1A were noradrenergic cells, because they did not stain for PNMT. Conversely, adrenergic cells were faintly labeled for syntaxin 1A. However, 7.1% of PNMT-positive cells showed a strong staining for syntaxin 1A (left corner of bottom panels of Fig. 2a).

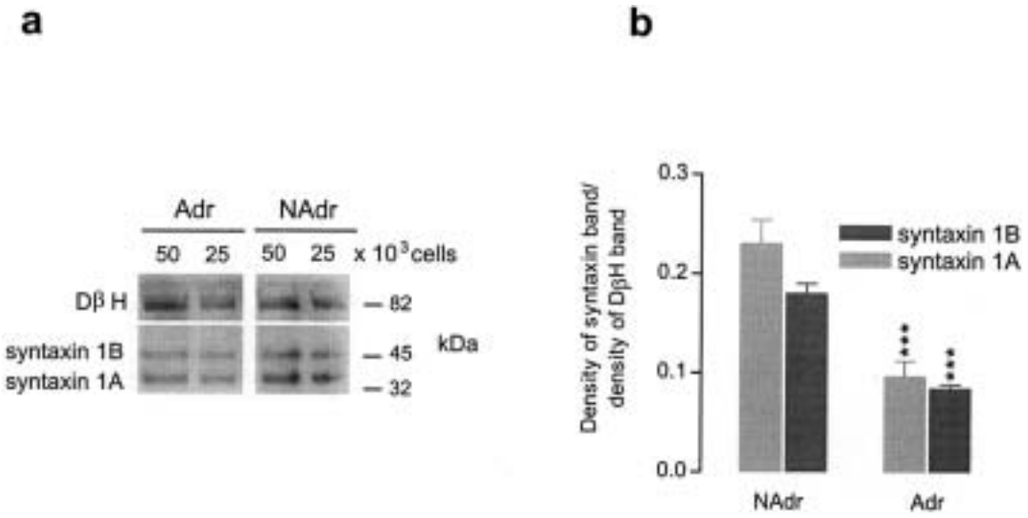

Fig. 1. Immunodetection of syntaxin 1A and 1B in protein extracts from cultures of chromaffin cells enriched in either noradrenergic or adrenergic cells. **a,** Immunoblot probed for syntaxin 1A, syntaxin 1B, and DβH. The volume of protein extract from the indicated number of cells was loaded on each lane. The blot shown is representative of at least five experiments performed with samples from different preparations. **b,** Ratio of the densities of syntaxin 1A or 1B to DβH bands. The values are the mean ± SEM of three experiments. Adr, Adrenergic; NAdr, noradrenergic.

The confocal images also showed a predominant localization of syntaxin 1A to the plasma membrane with a patchy appearance (Fig. 2b).

In contrast to syntaxin 1A, syntaxin 1B labeling was not apparently associated with the plasma membrane (Fig. 3). Syntaxin 1B was distributed throughout the cytoplasm of chromaffin cells, often showing a punctate appearance suggesting its localization in vesicular structures. Most adrenergic and noradrenergic chromaffin cells appeared similarly labeled for syntaxin 1B (cell groups indicated by arrowheads in Fig. 3). However, 17.5% of noradrenergic cells presented a stronger labeling for syntaxin 1B (arrows in Fig. 3). This is consistent with the greater levels of syntaxin 1B observed in the immunoblots of the protein extracts of noradrenergic-enriched populations (Fig 1b).

DISCUSSION

Chromaffin cells were reported to express low levels of syntaxin 1, compared to neurons (16). In the present report we show that adrenergic cells indeed have low levels of syntaxin 1A, but noradrenergic cells express high levels of this protein. Immunofluorescence confocal microscopy confirmed immunoblot data, and showed in addition some heterogeneity of adrenergic cells, because a minority of these cells expressed high levels of syntaxin 1A. The heterogeneous expression of syntaxin 1A in adrenergic cells is unlikely related to differences in the cell developmental stage, because chromaffin cells were isolated from the adult. It may actually reflect adrenergic cell heterogeneity in the gland, as a consequence of regulation by cortical hormones that are known to control the development of the adrenergic phenotype (17) and the expression of SNARE proteins (18). The heterogeneity of adrenergic chromaffin cells was also reported by others, who showed that a small fraction of these cells expressed very low levels of PNMT (19) and secreted both adrenaline and noradrenaline (20).

The distinct subcellular localization of the two syntaxin isoforms that we observed by confocal microscopy agrees with data from subcellular fractionation, indicating that syntaxin 1A is predominantly associated with the plasma membrane fraction (21), whereas syntaxin 1B is associated with chromaffin granule membranes (22). The faint cytoplasmic staining for syntaxin 1A may correspond to protein associated with the granule membranes, which is consistent with the observation of syntaxin 1A in granule fractions from chromaffin cells (23).

The distinct subcellular localization of the two isoforms of syntaxin 1 supports the idea that they carry out different tasks in the cell, in spite of their structural homology. Syntaxin 1A, but not the 1B, was shown to regulate insulin release in the pancreatic βTC3 cell line (7). Expression of syntaxin 1A in cell lines lacking the regulated secretory pathway suggested that

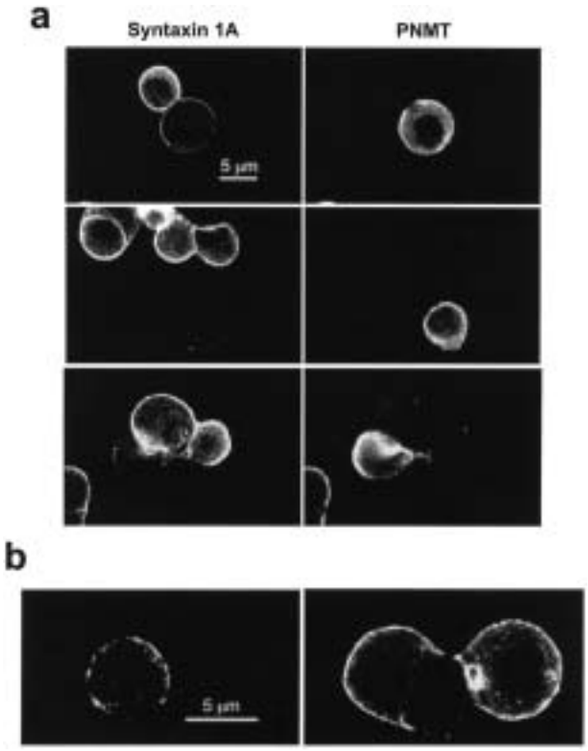

Fig. 2. Confocal immunofluorescence microscopy of chromaffin cells stained for syntaxin 1A and PNMT. **a**, Differential expression of syntaxin 1A in adrenergic (PNMT-positive) and noradrenergic cells (PNMT-negative). **b**, Higher magnification showing nonuniform distribution of syntaxin 1A in the plasma membrane. Immunoreactive syntaxin 1A was detected with Alexa 488-conjugated goat anti-rabbit IgG, and PNMT with Cy5-conjugated donkey anti-sheep IgG.

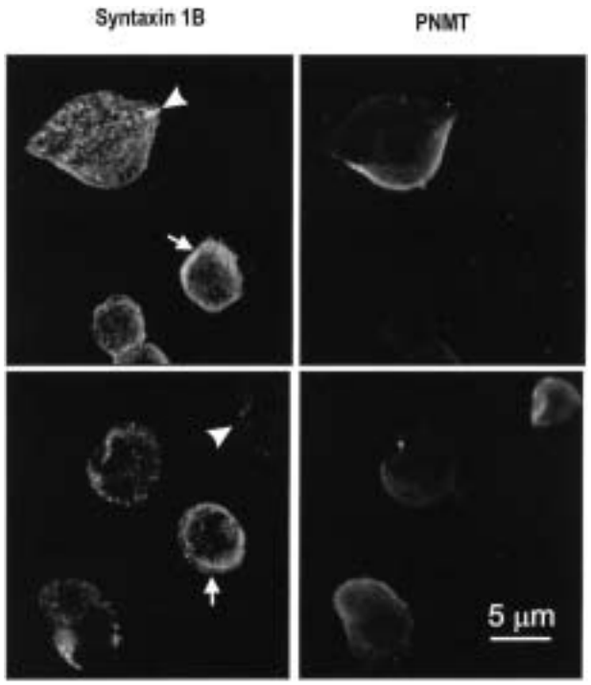

Fig. 3 Double immunofluorescence labeling for syntaxin 1B and PNMT. The arrowheads indicate groups of adrenergic and noradrenergic cells similarly stained for syntaxin 1B, and the arrows indicate noradrenergic cells more intensively labeled. Syntaxin 1B was detected with Alexa 488-conjugated goat anti-rabbit IgG, and PNMT with Cy5-conjugated donkey anti-sheep IgG.

this isoform is involved in the storage of secretory vesicles (6). Changes in syntaxin 1B expression have been related to increases in the efficiency of synaptic transmission and long-term potentiation (9–11). Thus one may suggest that noradrenergic cells expressing high levels of syntaxin 1B might be capable of undergoing plastic changes in the efficacy of secretion.

We have previously shown that noradrenaline release is more sensitive to BoNT/C1 than adrenaline release (4), suggesting a greater dependence of exocytosis on syntaxin 1 and/or SNAP-25 in noradrenergic chromaffin cells. The present report supports this idea and further indicates that the greater sensitivity to BoNT is likely due to the 1A isoform, rather to the 1B isoform, which is similarly expressed by most noradrenergic and adrenergic cells. The function of syntaxin 1A in the final fusion step of exocytosis is likely similar in noradrenergic and adrenergic cells; thus we propose that its higher expression in noradrenergic cells might be related to another role(s) of this syntaxin isoform, for instance, the coupling of the exocytotic machinery to voltage-gated Ca^{2+} channels (8). We previously found evidence suggesting a greater coupling of secretion and voltage-gated Ca^{2+} channels in noradrenergic chromaffin cells, compared to adrenergic cells (3). This hypothesis is also supported by the observation of co-localization of release sites and hotspots of submembrane $[Ca^{2+}]$ in a subpopulation of chromaffin cells, whose phenotype was not identified (24).

Besides syntaxin 1, other neuronal proteins are differentially expressed by the two subtypes of chromaffin cells. GAP-43, a protein that interacts with proteins of the SNARE complex, and calbindin, a Ca^{2+}-binding protein, are expressed only by noradrenergic cells (25,26), whereas the annexin p11 is expressed only by adrenergic cells (2).

CONCLUSION

In conclusion, the present report shows for the first time that syntaxin 1A is differentially expressed by the two main subtypes of adrenal chromaffin cells, whereas the predominant association of syntaxin 1B with a cell

phenotype is not as clear. Moreover, the different cellular localization of the two isoforms suggests that they play different roles in the cell. Another relevant finding of our study is the heterogeneous expression of syntaxin 1 within each catecholaminergic phenotype of chromaffin cells. These data are consistent with the functional heterogeneity often observed when intracellular Ca^{2+} or secretion studies are performed in single cells (27). The present report further supports the idea that the differences in the expression of a number of proteins of the exocytotic machinery may underlie some of the distinct characteristics of noradrenaline and adrenaline secretion from chromaffin cells.

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