SNAP-25 is differentially expressed by noradrenergic and adrenergic chromaffin cells

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Abstract This study examines chromaffin cell expression of the synaptosomal-associated protein SNAP-25 in the adrenal medulla by immunoblotting, immunocytochemistry and PCR. Both mRNAs coding for the SNAP-25 isoforms a and b were detected and SNAP-25 was found to be present in all chromaffin cells in adult rat adrenal gland sections. It was essentially restricted to a zone close to the cytoplasmic face of the plasma membrane in the majority of cells, but located extensively throughout the cytoplasm in a chromaffin cell sub-population, identified by double immunofluorescence labelling to have a noradrenergic phenotype. This differential SNAP-25 expression may reflect different stages in the phenotypic development of the sympathoadrenal lineage and be related to an additional functional role in noradrenergic chromaffin cells not associated with secretion.

Key words: SNAP-25; Chromaffin cell; Noradrenergic phenotype; Immunocytochemistry; PCR; Rat adrenal gland

1. Introduction

Neurones transport their secretory vesicles to their terminal cell surface where they are docked before releasing the contents into the extracellular space, in response to specific chemical or electrical stimuli in a regulated manner [1]. It has been suggested that N-ethylmaleimide sensitive fusion protein (NSF) and a number of soluble NSF attachment proteins (SNAPs) together constitute the neurotransmitter vesicle docking release apparatus and that the NSF-SNAP complex forms a bridge between SNAP receptors (SNAREs) in vesicular and target membranes [2,3], although the exact function of NSF in the fusion process has recently been questioned [4]. The fusion complex includes at least three SNAREs, including SNAP-25 (synaptosomal-associated protein of 25 kDa) thought to act as one of the target SNAREs on the plasma membrane [5–7]. The central role of SNAP-25 in the exocytosis step of neurotransmission was first suggested by studies on the action on nerve terminals of certain bacterial neurotoxins, for which it is a specific substrate [8–13]. Apart from its putative role in secretory mechanisms however, SNAP-25 in neurones has also been suggested to be implicated in processes associated with cell plasticity, in particular those concerned with neurite growth [14–16].

In contrast to neurones, relatively few data are currently available on the potential role of SNAP-25 in neuroendocrine cells, in particular adrenal medullary chromaffin cells, which have been employed for many years as an experimental model to study regulated exocytosis [17]. Such cells transport their neuromodulators in large secretory granules with dense cores, but small clear vesicles similar in appearance to classical synaptic vesicles are also present, although the precise function of such microvesicles in these cells remains to be elucidated [18]. SNAP-25 has been shown to be present in PC12 tumour cells derived from adrenal medulla [19] and its presence in cultured chromaffin cells was reported while the present study was in progress [20]. In addition a possible role of SNAP-25 in secretion in chromaffin cells has been proposed [21,22].

The present study aimed to shed light on the likely function of SNAP-25 in neuroendocrine cells, given their ambiguity regarding the presence of both large dense core secretory granules and more classical small synaptic type vesicles. SNAP-25 and its corresponding mRNA was detected in the rat adrenal gland, in cultured bovine chromaffin cells and in PC12 cells by immunoblots and reverse transcriptase polymerase chain reaction (RT-PCR). Its cellular localisation in chromaffin cells within the adrenal gland in situ, and in culture, was determined by both immunofluorescence and immunoperoxidase cytochemistry with a monoclonal antibody [23] and compared with the distribution of the granule membrane marker, dopamine β-hydroxylase (DBH) by double immunofluorescence labelling to test whether SNAP-25 might be associated with secretory granules. SNAP-25 expression was also correlated with chromaffin cell phenotype, determined with antibodies against phenylethanolamine *N*-methyltransferase (PNMT), an enzyme characteristic of adrenergic cells.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody SMI 81, originally produced by immunising with rat hypothalamic tissue and previously named 02-29 [23], was obtained from Sternberger Monoclonals Incorporated, Baltimore, MD, USA. Rabbit polyclonal antibodies directed against a synthetic C-terminal SNAP-25 peptide [24] were generously supplied by Dr. M.C. Wilson (Scripps Institute, La Jolla, CA, USA). The rat polyclonal anti-DBH antibodies were those described previously [17]. Rabbit polyclonal anti-PNMT antibodies, generously donated by Dr. L. Denoroy (INSERM U 52, Lyon, France) were previously characterised on chromaffin cell cultures [25]. All secondary antibodies were affinity-purified and pre-adsorbed; tetramethyl rhodamine conjugated goat anti-mouse and donkey anti-rabbit IgGs, 5-{4,6-dichlorotriazin-2-yl} amino-fluorescein conjugated donkey anti-rabbit IgG and horseradish peroxidase conjugated goat anti-mouse IgG were purchased from Euromedex (Strasbourg, France). Alkaline phosphatase conjugated goat anti-mouse IgG and anti-rabbit IgG were from BioRad (Ivry sur Seine, France).

2.2. Cell cultures

Bovine chromaffin cells were isolated and plated for immunofluorescence on collagen coated coverslips as previously described [25] and studied at 3–7 days of culture. The rat phaeochromocytoma PC12 cell line was purchased from ATCC (France) and grown in suspension in RPMI 1640 supplemented with 5% foetal calf serum and 10% horse serum (BRL-Gibco, Eragny, France).

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2.3. Immunoprecipitation

Rat cerebellar homogenates were solubilised in 'immunoprecipitation' buffer (50 mM Tris, 190 mM NaCl, 5 mM EDTA, 2.5% Triton X-100, 0.1% SDS, 2 mM phenylmethanesulphonyl fluoride pH 7.4). To reduce non-specific binding, the extract was first cleared by centrifugation and treated with protein A-Sepharose beads (BioRad) preincubated in non-immune serum. After a second centrifugation, the supernatant was incubated overnight at 4°C with protein A-Sepharose beads bound to SMI 81. Beads were subsequently washed in immunoprecipitation buffer without detergent, before solubilising bound proteins for SDS-PAGE.

2.4. Immunoblots

Protein concentrations were determined by the Bradford method (BioRad). After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Hybond C-Extra, Amersham, Les Ulis, France) and SNAP-25 was detected with either SMI 81 (1:1000) or the polyclonal anti-SNAP-25 antibodies (1:1000). Blots were then incubated with appropriate secondary antibodies conjugated to alkaline phosphatase (1:1500) and developed with nitroblue tetrazolium-sodium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, Saint Quentin Fallavier, France).

2.5. Analysis of SNAP-25 mRNA by RT-PCR

Amplification of DNA complementary to SNAP-25 mRNA was performed firstly using oligonucleotide primers with sequences common to both forms; the 5′ primer (nucleotides 171–191) was on exon 2 and the 3′ primer (nucleotides 759–739) was on exon 8 (EMBL X51673). To distinguish between the messengers coding for the two isoforms, a and b, produced by alternative splicing of exon 5 [26], the common 3′ primer was replaced by the 3′ primer 5a corresponding to nucleotides 295–262 (exon 5a, Genebank L19760) or 3′ primer 5b corresponding to nucleotides 370–337 (exon 5b, EMBL X51673). The reverse transcription step was performed on total RNA with oligo-dT using a first strand synthesis kit (BRL-Gibco). For amplification, cDNA corresponding to 50 ng of total RNA was used. dNTP was from Boehringer Mannheim (France) and Taq polymerase and reaction buffer was from Appligene (Strasbourg, France) and 30 cycles were employed. Aliquots (10 μl) of each PCR were run in parallel on 2% agarose gels. The common PCR product was characterised by restriction endonuclease digestion with *Pst*I, *Hin*dIII, *Sma*I (Boehringer Mannheim, France). Sizes of products obtained were compared to those predicted by the cDNA sequence [24].

2.6. Immunocytochemistry

Cell cultures were fixed in phosphate buffered formaldehyde (4%, 0.12 M, pH 7.2, 30 min) and permeabilised in the same solution containing Triton X-100 (0.2%, 10 min) before immunolabelling [27] with SMI 81 (1:1000). Some cultures were also double labelled for SNAP-25 (SMI 81, 1:1000) and the granule marker DBH (1:500) after stimulation for 10 min with 10 μM nicotine or 59 mM KCl [17] and fixation. For adrenal glands, adult Wistar rats were perfusion fixed, under anaesthesia, with the same fixative (without detergent, 2 h); vibratome or cryostat sections were cut and labelled as previously described [27]. For immunoperoxidase labelling, endogenous peroxidase activity was quenched by pretreatment with hydrogen peroxide (0.1% in phosphate buffered saline, 10 min).

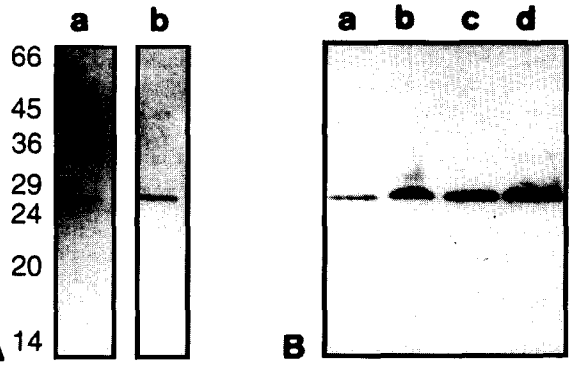

Fig. 1. (A) Immunoblots of the immunoprecipitate obtained with monoclonal antibody SMI 81 from rat cerebellum. Lane (a) detected with SMI 81, lane (b) the same immunoprecipitate detected with rabbit anti-SNAP-25 polyclonal antibody. Both antibodies detect a single band migrating identically, with an apparent molecular mass of 25 kDa. (B) Immunoblot of soluble protein fractions of rat adrenals (a), PC12 cells (b), bovine chromaffin cells (c) and rat cerebellum (d), detected with SMI 81. Molecular weights (kDa) are indicated on the left.

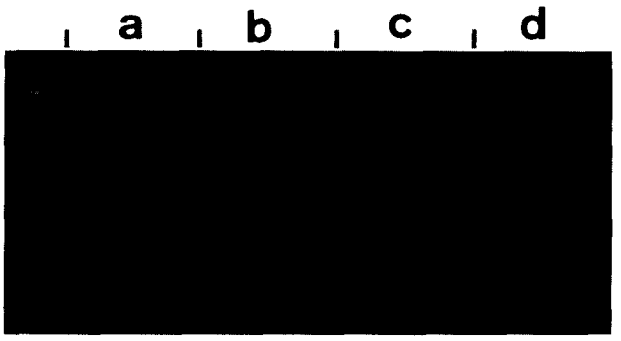

Fig. 2. RT-PCR amplification of SNAP-25 mRNA: ethidium bromide stained gels of the PCR products obtained from (a) rat adrenal, (b) bovine chromaffin cells, (c) PC12 cells, (d) rat cerebellum, using the common 3′ primer (exon 8) for the 591 bp SNAP-25 sequence (first lane for each tissue) and the specific 3′ primers for exon 5a or 5b for the 201 bp SNAP-25 a and b isoform sequences (second lane for each tissue isoform a, third lane isoform b). On the left, the 600 bp band in the 100 bp molecular weight ladder is more intense than the other bands.

2.7. Confocal microscopy

Serial optical sections (0.2 μm thick) of cultures were examined from approximately the central plane of the cell. Images were acquired using a Zeiss LSM 410 inverted laser scan confocal microscope using the 543 nm He/Ne laser as excitation source for rhodamine and 488 nm argon laser for fluorescein. Emission signals were filtered with a Zeiss 515–565 nm filter (fluorescein) or with a 595 nm filter (rhodamine). Images were recorded digitally, stored and later down-loaded to a FOCUS digital image recorder.

3. Results and discussion

3.1. SNAP-25 in the adrenal gland

The monoclonal antibody used here, produced several years ago using rat hypothalamic tissue as immunogen [23], has long been considered, from its cerebellar staining pattern, to recognise a synapse-associated antigen. Its specificity was first verified here by immunoprecipitation experiments on rat cerebellar homogenates. SMI 81 detected on immunoblots of electrophoresed samples prepared by immunoprecipitation with this antibody a single 25 kDa protein. An identical band was recognised in the same preparations by previously characterised [24] polyclonal anti-SNAP-25 peptide antibodies (Fig. 1A). Thus SMI 81 immunoprecipitates a protein, identified as SNAP-25, that runs as a single band on SDS-PAGE. In addition, immunoblots with SMI 81 of cerebellar extracts were identical to those obtained with polyclonal anti-SNAP-25 antibodies and SMI 81 detected no bands on immunoblots of heart, lung and spleen extracts (as would be expected of tissues where SNAP-25 is absent; data not shown). Moreover the immunocytochemical labelling of rat cerebellum with SMI 81 was the same as that reported for SNAP-25 (data not shown). SMI 81 was therefore considered here to be a specific

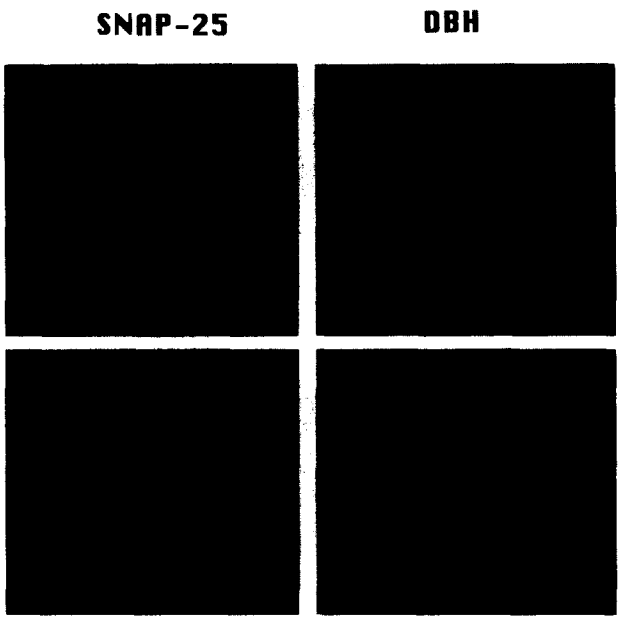

Fig. 3. Laser confocal microscope images of single optical sections of unstimulated (a,b) or K^+ stimulated chromaffin cells (c,d), fixed before immunofluorescence labelling for SNAP-25 with SMI 81 (a,c) or DBH (b,d). SNAP-25 labelling is peripheral, DBH labelling is cytoplasmic. Bar = 5 μm.

anti-SNAP-25 antibody, in agreement with the conclusions from other laboratories [28,29].

Immunoblotting with SMI 81 demonstrated SNAP-25 expression in rat adrenal gland (Fig. 1B) and also confirmed, using a different antibody than employed elsewhere, its presence in PC12 and cultured chromaffin cells, as recently reported [19,20]. Its electrophoretic migration was identical to that of the band detected in cerebellar extracts (lane d; Fig. 1B), suggesting the likely conservation of its primary structure and antigenicity.

A RT-PCR product with the size predicted from the SNAP-25 sequence (591 bp) was obtained from extracts of rat adrenal gland, chromaffin cells, PC12 cells and rat cerebellum using the SNAP-25 primers common to both isoforms (Fig. 2). Endonuclease digestion of this PCR product with *Pst*I, *Hin*dIII and *Sma*I produced the digestion products predicted from the reported sequence [24]: 310 and 280 bp with *Hin*dIII and approximately 490 and 400 bp for the larger fragments obtained with *Pst*I and *Sma*I respectively (data not shown). These data confirmed that the product amplified from these extracts was indeed from SNAP-25 mRNA. When sequence-specific 3′ primers for either SNAP-25 a or b isoforms were employed, PCR products of the expected size (201 bp) were obtained from each tissue extract (Fig. 2), but the relative proportions of each appeared to vary. In contrast to the adrenal tissues, the SNAP-25 isoform b was particularly predominant in the cerebellum, providing further evidence that the major brain form is SNAP-25 [16,26].

3.2. *Immunocytochemistry of SNAP-25 in adrenal chromaffin cells*

Immunofluorescence of cultured bovine chromaffin cells for SNAP-25 (Fig. 3a) demonstrated that it was located mainly in a region close to the surface membrane; very little cytoplasmic labelling was observed. However, it did not appear to be uni-

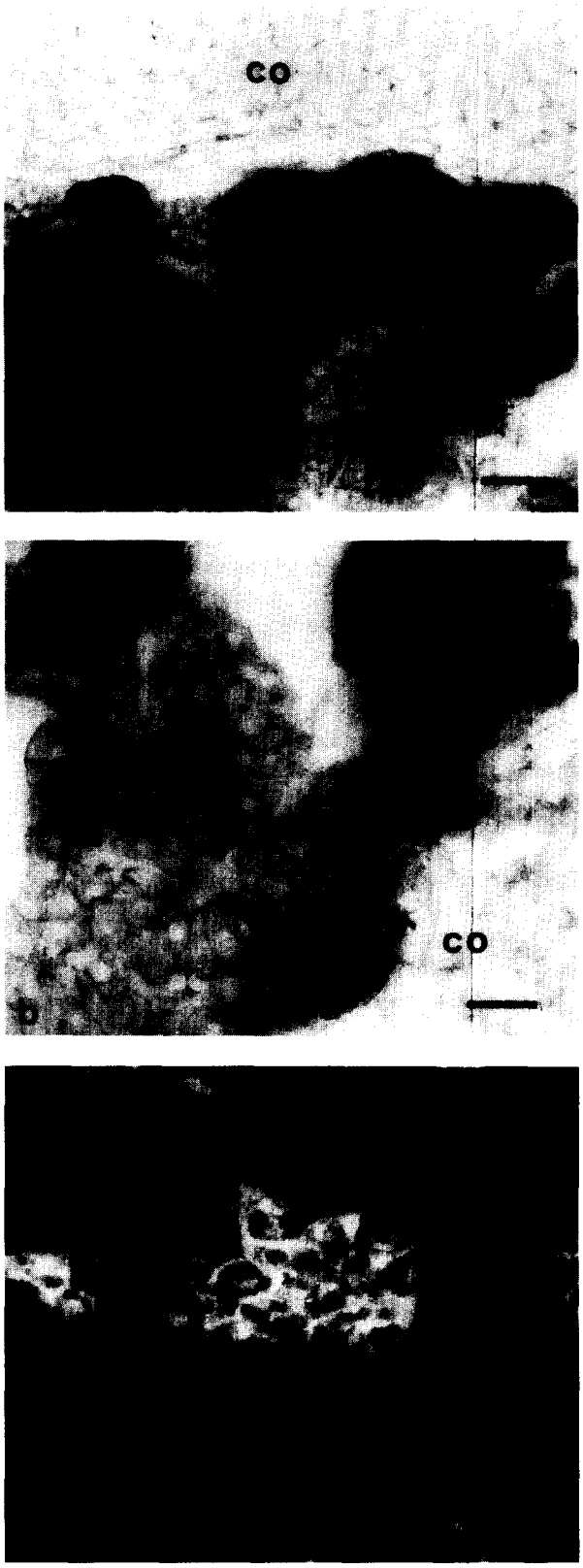

Fig. 4. Bright field micrographs of vibratome (a,b) or cryostat (c) rat adrenal gland sections, immunoperoxidase (a,b) or immunofluorescence (c) labelled for SNAP-25 with SMI 81. Cortical cells (CO) are unstained; most medullary chromaffin cells are just surface labelled, but cytoplasmic labelling is seen in cells in more intensely stained groups (b). Nerve fibres (arrow) and nerve terminals (arrowheads) are heavily stained. An identical labelling pattern was obtained with the immunofluorescence technique (c). Bar = 40 μm for (a), 20 μm for (b) and (c).

formly distributed around the cell periphery and differences in labelling intensity between different cells or groups of cells were also evident. A similar pattern, particularly evident in approximately 1 μm thick epoxy resin embedded sections, was obtained with the immunoperoxidase method (data not shown). This contrasted with the more extensive cytoplasmic staining obtained for the secretory granule marker DBH (Fig. 3b). When cells were stimulated with either nicotine or potassium before fixation and immunolabelling, no obvious changes in labelling patterns for either SNAP-25 (Fig. 3c) or DBH (Fig. 3d) compared to controls were observed. Thus no general colocalisation of these two proteins was evident in resting or stimulated cells. It may be concluded that SNAP-25 is not apparently associated at detectable levels with secretory granules distributed throughout the cytoplasm.

In adrenal gland sections, nerve terminals and nerve fibre bundles were, as expected, intensely labelled for SNAP-25 by the immunoperoxidase method (Fig. 4a,b), but an unexpected staining pattern of chromaffin cells was found. Two sorts of cell groups could be distinguished by their SNAP-25 labelling. Cells in most groups had only peripheral staining, while cells in some groups, often located more peripherally in the medulla, were stained throughout their cytoplasm (Fig. 4a,b). An identical differential SNAP-25 staining pattern between groups of chromaffin cells was also detected by immunofluorescence on either cryostat (Fig. 4c) or vibratome sections, excluding the possibility that such differential staining represents an artefact either due to diffusion of peroxidase reaction product or due to the sectioning method employed.

3.3. SNAP-25 expression and chromaffin cell phenotype

The phenotype of chromaffin cells with cytoplasmic SNAP-25 staining was identified by double immunofluorescence labelling with anti-PNMT antibodies, which label only adrenergic cells [25]. The chromaffin cells with cytoplasmic labelling for SNAP-25 were found to be unlabelled with anti-PNMT antibodies (Fig. 5), leading to the conclusion that chromaffin cells with SNAP-25 distributed throughout their cytoplasm are noradrenergic and those with only membrane-associated labelling are adrenergic. This novel finding that SNAP-25 distribution and level of expression is dependent on chromaffin cell phenotype correlates with our present data showing SNAP-25 apparently only in a sub-plasmalemmal location in cultures of bovine chromaffin cells, since these consist mainly of adrenergic cells [25]. In another study, published while this work was in progress [20], also no differential staining pattern in such cultures was reported.

The cytoplasmic localisation found in noradrenergic cells was unexpected, since it has been described as essentially associated with membranes [30], even though we found SNAP-25 to have a cytoplasmic distribution in rat cerebellar granule cells (unpublished data). It is thought to anchor to membranes by palmitoylation of clustered cysteine residues in a sequence that varies between the two isoforms [26]. While it is likely that the monoclonal antibody employed here recognises an epitope present in both isoforms, the localisation we found in adrenergic chromaffin cells is that of a plasma membrane-associated protein, probably SNAP-25b [26]. In the present study RT-PCR clearly demonstrated the presence of messenger coding for both isoforms in adrenal gland, PC12 and chromaffin cell culture extracts (Fig. 2). Palmitoylation of the SNAP-25 is considered to be a dynamic event, regulating

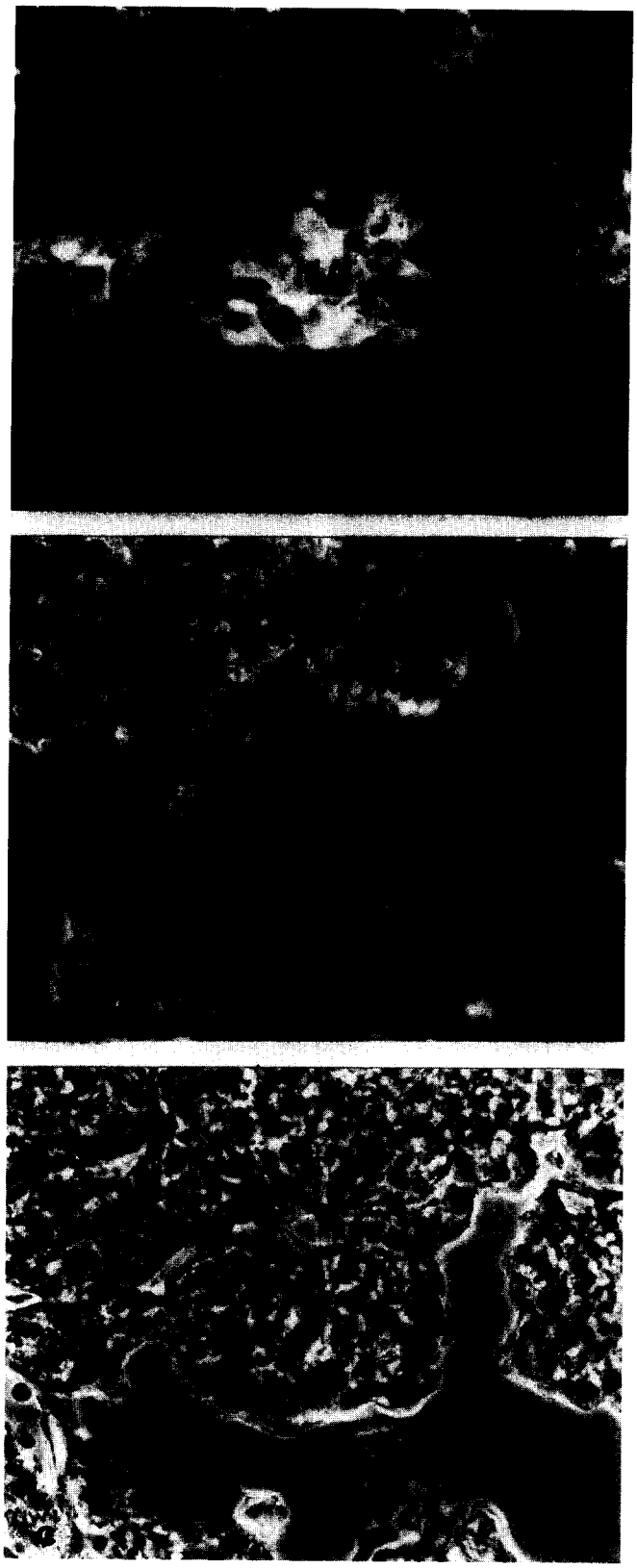

Fig. 5. Cryostat section of rat adrenal gland, double immunofluorescence labelled for SNAP-25 with SMI 81 (a) and PNMT (b); phase contrast optics (c). Cells with intensely immunofluorescent cytoplasm in (a) are unlabelled for PNMT (i.e. noradrenergic, NA) while in adrenergic (A), PNMT-positive chromaffin cells SNAP-25 labelling is confined to the cell periphery. V indicates a blood vessel. Bar = 20 μm.

the extent of the attachment of the protein to the cell membrane. It thus seems likely that the SNAP-25 widely distributed throughout the cytoplasm of noradrenergic cells lacks this post-translational modification, and may be the SNAP-25a isoform, which associates less readily with membranes [16,26].

3.4. SNAP-25 and function

SNAP-25 is currently considered to be a receptor protein on the plasma membrane permitting synaptic vesicle docking before membrane fusion [7,31]. Whether SNAP-25 performs a similar role in the secretion of chromaffin granules has yet to be proved, but the results reported here clearly demonstrate for the first time that SNAP-25 is present in these cells in situ, and establish that in adrenergic chromaffin cells it is limited to a region close to the cytoplasmic face of the plasma membrane, a localisation compatible with its putative function as a plasma membrane-associated SNARE. The apparent absence of SNAP-25 on un-docked secretory granules of these cells, i.e. those distributed throughout the cytoplasm, demonstrated here by double labelling with a granule marker (Fig. 3), also fits with such a role in granule docking.

While at the present time, reasons for the unexpected differential SNAP-25 expression between the two major chromaffin cell phenotypes are not clear, the cytoplasmic localisation in noradrenergic cells may suggest that in these cells SNAP-25 could also be involved in processes other than regulated exocytosis, such as those associated with morphological plasticity, as has been previously proposed [14-16,32]. The subcellular localisation of SNAP-25 in neurones reported from several groups is not consistent with an exclusively exocytotic role for this protein [24,28,32,33]. Also in a parallel study we found at the ultrastructural level that splanchnic nerve terminal synaptic vesicles in adrenal medulla were strongly labelled for SNAP-25, a localisation unexpected for a plasma membrane SNARE (unpublished data). In this regard, it is interesting to note that the SNAP-25 isoform considered to be involved in morphological plasticity, a well documented phenomenon in noradrenergic chromaffin cells [34-36], is the more broadly distributed form [16] with modified palmitoylation efficiency [26]. Expression of several other proteins has been found to differ between adrenergic and noradrenergic chromaffin cells in adult rat adrenal tissue. Two proteins also initially described as neurone-specific, the cell adhesion molecule L1 and the growth associated protein GAP-43 have both been found only in the noradrenergic chromaffin cell phenotype [27,37,38]. Moreover one of these, GAP-43, like SNAP-25, associates with membranes by palmitoylation [14] and its expression has been linked with cellular plasticity [34,39]. The expression of all these proteins may reflect the fact that noradrenergic chromaffin cells are phenotypically closer to neurones than adrenergic cells [40], which represent the terminal phenotype in this lineage, and supports the hypothesis that adrenergic and noradrenergic chromaffin cells represent two distinct lines within the sympathoadrenal lineage.

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