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A complex of rab3A, SNAP-25, VAMP/synaptobrevin-2 and syntaxins in brain presynaptic terminals

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Two monoclonal antibodies (SPM-1 and SPM-2) immunoprecipitate brain N-type calcium channels. On immunoaffinity chromatography of digitonin extracts of bovine brain membranes on SPM-1- and SPM-2-Sepharose, proteins of 36 (syntaxins A and B), 28 and 19 kDa are specifically retained by both columns. Here we show that the 19 and 28 kDa bands contain VAMP/synaptobrevin-2, and rab3A/smg25A and SNAP-25, respectively. Since SPM-1 and SPM-2 recognize only syntaxins and the 28 kDa band (rab3A/smg25A and SNAP-25), respectively, the results indicate that all these proteins form a complex. Our results suggest tight linkage between the components involved in neurotransmitter release.

Rab3A/smg25A; Syntaxin; VAMP/synaptobrevin-2; SNAP-25; Neurotransmitter release

1. INTRODUCTION

The influx of Ca^{2+} into nerve terminals through calcium channels triggers the fusion of synaptic vesicles with the presynaptic membrane and then their exocytosis. Söllner et al. [1] found that receptors for the soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment proteins (SNAPs) in the brain contain syntaxins [2,3], VAMP/synaptobrevin-2 [4,5] and SNAP-25 [6]. Syntaxins [2,7] and SNAP-25 [6] are present in the presynaptic membrane, and VAMP/synaptobrevin-2 [4,5] is present in synaptic vesicles. These findings suggest the involvement of these proteins in synaptic vesicle fusion with the presynaptic membrane. We have identified two proteins of 36 and 28 kDa [8] associated with ω-conotoxin-sensitive (N-type) calcium channels that are involved in neurotransmitter release at some of the nerve terminals in the brain [9]. Subsequently we found that the 36 kDa protein consists of several isoforms (collectively named synaptocanalins) including syntaxins A (HPC-1) and B [10]. Here we show that the 28 kDa protein contains rab3A/smg25A and SNAP-25. Our results indicate that rab3A/smg25A, SNAP-25, VAMP/synaptobrevin-2 and syntaxins (synaptocanalins) exist as a complex in brain presynaptic terminals in the apparent absence of NSF and SNAPs.

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2. MATERIALS AND METHODS

2.1. *Partial sequence analyses*

The eluates from SPM-1- and SPM-2-Sepharose were fractionated by SDS-PAGE in urea [10]. The 19b, 28a and 28b components (see section 3) were electroeluted with 25 mM Tris/192 mM glycine/0.1% SDS, and SDS was removed as described previously [10]. The 19b and 28a proteins were treated with 0.15 M CNBr in 70% formic acid for 24 h at room temperature. The 19b component cleaved with CNBr was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). Material in the major band (about 2 kDa) was applied to a protein sequencer (Model 470A, Applied Biosystems). The 28a component cleaved with CNBr was fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a C18 column (octadecyl-NPR, Tosoh) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Components in major peaks (A to E) of the absorbance at 214 nm were subjected to sequence analysis. The 28a component was digested with a protease as follows. The 28a band excised from SDS-gel was equilibrated with an equal volume of 0.1 M pyridine-acetate buffer pH 6.5, containing 0.1 mM $CaCl_2$ at 37°C for 1 h and then digested with 3.3 μg Staphylococcal V8 protease (Wako) for 3 h at 25°C, and subjected to SDS-PAGE. The three major fragments (25, 24 and 15.5 kDa) blotted onto PVDF membranes were sequenced by a protein sequencer. The partial sequence of the 24 kDa fragment was contained in that of the 25 kDa fragment, starting at the fourth residue of the latter. The 28b protein extracted from SDS-gel was digested with papain essentially by the method of Cleveland et al. [11].

Briefly, the 28b protein (5 μg) dissolved in 140 μl of 125 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.1% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% Bromophenol blue (BPB) was loaded on the stacking gel. Fifty ng of papain (Sigma) in 20 μl of 125 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.1% SDS, 10% glycerol, 0.01% BPB was layered on the 28b solution, and SDS-PAGE was performed by the method of Horigome et al. [12]. Then the fractionated papain digest was transferred to a PVDF membrane. One major fragment (about 10 kDa) was applied to a protein sequencer.

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2.2. *Preparation of antibodies*

Peptides were synthesized using a Milligen peptide synthesizer (9050 PepSynthesizer) and purified by HPLC on a Waters μBondapak C18 column. Each purified peptide (2 mg) was conjugated overnight with 12 mg of keyhole limpet hemocyanin (Calbiochem) in 0.1 M sodium phosphate buffer pH 7.4, 0.06% glutaraldehyde, and dialyzed against 20 mM sodium phosphate buffer pH 7.4, 0.15 M NaCl. The conjugate (corresponding to 0.3 mg of the original peptide) was emulsified with an equal volume of Freund's complete (1st injection) or incomplete adjuvant (subsequent injections) and injected subcutaneously into a rabbit at two week intervals. All antisera used for immunoblotting were diluted 500–1,000 times.

2.3. *GTPγS binding.*

GTPγS binding to the eluates from SPM-1- and SPM-2-Sepharose was detected by a modification of the method of Lapetina and Reep [13]. All procedures were performed at room temperature. The nitrocellulose membranes with transferred proteins were rinsed briefly with one of the following binding buffers. Buffer 1: 20 mM HEPES-NaOH pH 8.0, 0.1 M NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.05% Tween 20; Buffer 2: Buffer 1 without DTT; Buffer 3: Buffer 1 without $MgCl_2$. The membranes were incubated with competing substrates (1 μM GTP, GDP, ATP, CTP, UTP) (in Buffer 1) or with 10 mM *N*-ethylmaleimide (NEM) (in Buffer 2) or without Mg^{2+} (in Buffer 3) for 1 h. They were then incubated with 0.3 nM [^{35}S]GTPγS (50 TBq/mmol, Dupont-NEN Research Products) for 1.5 h, washed six times with 5 ml of the same buffer for 1.5 h and air-dried. GTPγS binding was detected by autoradiography using Ultrofilm ^{3}H (Baxter Healthcare Corporation).

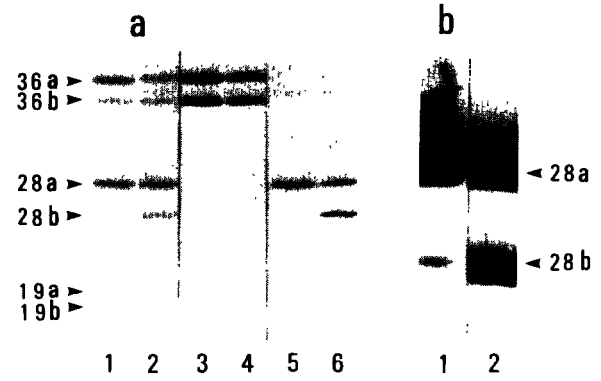

Fig. 1. Fractionation by SDS-PAGE of proteins bound to SPM-1- and SPM-2-Sepharose. The lysed P2 fraction from bovine brain was extracted with digitonin and subjected to immunoaffinity chromatography on SPM-1- and SPM-2-Sepharose, and the bound materials were eluted with SDS [8]. The eluates were analysed by SDS-PAGE in the presence of urea [10]. (a) Lanes 1 and 2, staining of blots with Amidoblack 10B; lanes 3 and 4, immunoblots with SPM-1; lanes 5 and 6, immunoblots with SPM-2. Lanes 1, 3 and 5, eluates from SPM-1-Sepharose; lanes 2, 4 and 6, eluates from SPM-2-Sepharose. Positions of bands are shown on the left. (b) Presence of the 28b component in the eluates from SPM-1-Sepharose (lane 1) and SPM-2-Sepharose (lane 2). In this case, immunoblotting was carried out using biotinylated anti-mouse IgG and alkaline phophatase–streptavidin.

3. RESULTS

The monoclonal antibodies SPM-1 and SPM-2, which recognize proteins of 36 kDa (syntaxins) and 28 kDa, respectively, immunoprecipitate significant fractions of N-type calcium channels solubilized from chick and bovine brain [8]. The proteins of 36, 28 and 19 kDa are major brain membrane components and specifically bound to SPM-1- and SPM-2-Sepharose [8]. None of these proteins bind to normal mouse IgG-Sepharose [8]. Since only the 36 and 28 kDa proteins are recognized by SPM-1 and SPM-2, respectively [8], all these proteins must be associated with each other. On SDS-PAGE in the presence of urea, the 36 kDa protein is separated into two major bands (36a and 36b), corresponding to syntaxins B and A, respectively (Fig. 1a), as described previously [10]. Material in the 19 kDa band is also separable into two components (19a and 19b) (Fig. 1a). A CNBr fragment (approximately 2 kDa) of 19b gave a partial sequence that exactly corresponded to amino acid residues 48–79 of bovine and rat brain VAMP/synaptobrevin-2 (Fig. 2a). The amino acid sequence of the corresponding part of VAMP/synaptobrevin-1 is almost identical, but it does not contain methionine-46 [5,14] and therefore should not be cleaved by CNBr at the position. Threonine-79 is also replaced by serine. Moreover, 19b, but not 19a, was recognized by antisera against two partial sequences of VAMP/synaptobrevin-2 (Fig. 2a). Thus 19b is probably identical to VAMP/synaptobrevin-2. The 19a protein differs from the 19b protein, but its identity remains to be determined.

The 28 kDa band from SPM-2-Sepharose also contained two components (28a and 28b), which both reacted with SPM-2 (Fig. 1a). The 28b component was not detected in the SDS-eluate from SPM-1-Sepharose in immunoblots using alkaline phosphatase-anti-mouse IgG, but was detected using the more sensitive, avidin–biotin complex system (Fig. 1b). The 28a component was cleaved with Staphylococcal V8 protease and CNBr, and partial sequences of its fragments were determined. All the exact partial sequences obtained were contained in the sequence of SNAP-25 (Fig. 2b). Furthermore, antisera raised against two partial sequences of SNAP-25 reacted specifically with 28a, but not with 28b (Fig. 2b). These results indicate that the 28a component is SNAP-25. The 28b component was resistant to V8 protease and CNBr, but was digested with papain. A major fragment (about 10 kDa) had an identical sequence to that of a small GTP-binding protein, rab3A/smg25A [15,16] (Fig. 2c). This sequence is unique to rab3A/smg25A and differs from those of the corresponding parts of the two close homologs rab3B/smg25B [15,17] and smg25C [15]. The 28b component, but not the 28a component, reacted with antibodies against a partial sequence unique to rab3A/smg25A (Fig. 2c). The 28b component in the SDS-eluates from both SPM-1- and SPM-2-Sepharose bound GTPγS in a similar manner as purified rab3A/smg25A [18] (Fig. 3). Furthermore, GTPase activity was detectable in SPM-1- and SPM-2-Sepharose bound to these proteins (data not shown). These results indicate that the 28b component is rab3A/smg25A.

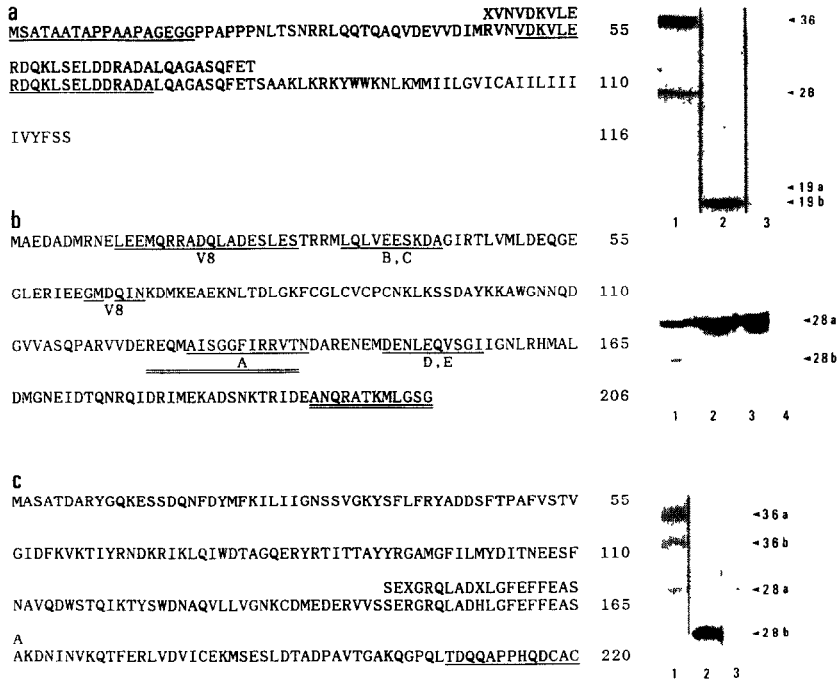

Fig. 2. Identities of the 19b, 28a and 28b components with VAMP/synaptobrevin-2, SNAP-25 and rab3A/smg25A, respectively. (a) The 19b component. The partial sequence obtained is shown above the corresponding sequence of bovine VAMP/synaptobrevin-2 [4]. X denotes an unidentifiable residue. Numbers of amino acid residues are given at the right of each line. Immunoblots of the SDS-eluate from SPM-1-Sepharose with antisera against two partial sequences (underlined) of bovine brain VAMP/synaptobrevin-2 are shown on the right. In these immunoblots, SDS-PAGE (12.5% acrylamide) was carried out in the absence of urea, as blots after SDS-PAGE in urea gave considerable background staining with the antisera against VAMP/synaptobrevin-2. The 19 kDa band was separable into two components (19a and 19b) even in the absence of urea. Lane 1, Amidoblack 10B staining; lanes 2 and 3, immunoblots with antisera against residues 50–69 and 1–18 of VAMP/synaptobrevin-2, respectively. The 19b component in the eluate from SPM-2-Sepharose also reacted with these antisera. (b) The 28a component. Partial sequences obtained are shown with the sequence of mouse SNAP-25 [6]. Underlines show partial sequences obtained by CNBr cleavage (fragments A to E) or by V8 protease digestion (marked V8). Double underlines show sequences used for obtaining antibodies. Immunoblots of the eluate from SPM-2-Sepharose with antibodies against SNAP-25 are shown on the right. Lane 1, SPM-2; lanes 2 and 3, antisera against residues 124–138 and 195–206 of SNAP-25, respectively; lane 4, nonimmune rabbit serum. The 28a component in the eluate from SPM-1-Sepharose also reacted with these antisera (data not shown). (c) The 28b component. The partial sequence obtained is shown above the corresponding sequence of bovine rab3A/smg25A [15]. Immunoblots of the eluate from SPM-2-Sepharose with the antibodies against a partial sequence (residues 208–220, underlined) of rab3A/smg25A are shown on the right. Lane 1, Amidoblack 10B staining; lane 2, antiserum against rab3A/smg25A; lane 3, nonimmune rabbit serum. The 28b component in the eluate from SPM-1-Sepharose also reacted with the antiserum (data not shown).

4. DISCUSSION

Our results indicate that syntaxins, rab3A/smg25A, SNAP-25, VAMP/synaptobrevin-2 and the 19a component form a complex in brain presynaptic terminals. None of these proteins are releasable from SPM-1- or SPM-2-Sepharose with high salt (T.A., unpublished observations), suggesting that the proteins form a firm complex. The molar ratio of the proteins in the complex was difficult to assess, because not all the molecules of the 36 kDa and 28 kDa proteins were present in the complex; they were only partially retained by SPM-2- and SPM-1-Sepharose, respectively [8]. Only a small amount of rab3A/smg25A was present in the eluate from SPM-1-Sepharose. The protein dissociates from synaptic vesicles during their exocytosis [19,20]. It is also releasable from synaptic vesicles and membranes in vitro by interacting with smg25A GDP dissociation inhibitor [21]. Thus rab3A/smg25A seems to be in dynamic equilibrium between membrane-bound and dissociated states and only a small amount of the protein may be associated with the complex in vivo. Alternatively, most rab3A/smg25A might have dissociated from the complex during our experimental procedures.

Söllner et al. [1] postulated that 20 S fusion particles containing NSF and SNAPs attach vesicles to a target membrane by binding to SNAP receptors in vesicles and the membrane. Thus these SNAP receptors may specify targeting of fusion of vesicles and membranes. In our previous [8,10] and present experiments, no significant amounts of known SNAPs (α, β and γ) (33–36 kDa) [22] or NSF (76 kDa) [23] were detected in SDS eluates from either column, as judged from SDS-PAGE patterns. The molar ratio of α-SNAP to the sum of all of the SNAP receptor species identified in the study of Söllner et al. (syntaxins, SNAP-25 and VAMP/synaptobrevin-2) was about 2 [1]. The amount of γ-SNAP was about 1/5 of that of α-SNAP [1]. Thus the SNAPs could have easily been detected. Our procedures for preparing the lysed P2 fraction and/or for extraction of membrane

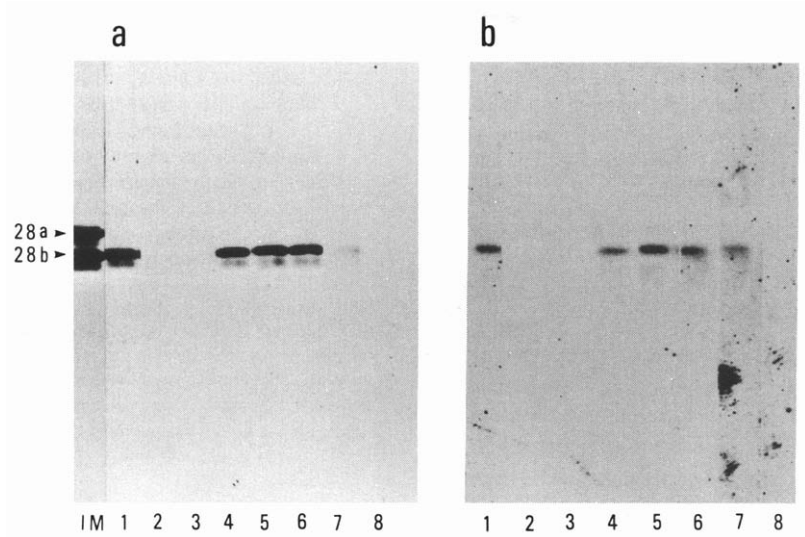

Fig. 3. GTPγS binding to the 28b component from SPM-2- (a) and SPM-1-Sepharose (b). For both a and b, lane 1, in the absence of other nucleotides; lanes 2–6, in the presence of 1 μM GTP, GDP, ATP, CTP and UTP, respectively; lane 7, in the presence of 10 mM NEM; lane 8, in the absence of Mg^{2+}. The left lane in a (marked IM) is an immunoblot with SPM-2.

proteins may dissociate SNAPs and NSF from the complex, but our results indicate that rab3A/smg25A, SNAP-25, VAMP/synaptobrevin-2, syntaxins and the 19a component form a complex even in the apparent absence of SNAPs and NSF.

Rab3A/smg25A and VAMP/synaptobrevin-2 exist in synaptic vesicles. In addition, syntaxins bind another synaptic vesicle protein, synaptotagmin [2,24,25], though the latter dissociates from the former in digitonin ([2] and T.A. et al., unpublished observations). Thus synaptic vesicles located at the release site are probably associated with the presynaptic membrane by binding between these synaptic vesicle proteins and presynaptic membrane proteins such as syntaxins and SNAP-25. These synaptic vesicles should be situated very close to N-type calcium channels, as syntaxins [2,8] and the 28 kDa protein (rab3A/smg25A and/or SNAP-25) [8] are associated with N-type calcium channels. Thus the present results suggest tight linkage of components probably participating in synaptic neurotransmitter release [1,2,26]. This presynaptic complex may represent an essential machinery for the extremely rapid synaptic vesicle exocytosis (within 0.2 ms of Ca^{2+} entry) [27].

Our study suggests that the action of rab3A/smg25A in presynaptic terminals is mediated at least partly by its interaction with synaptic SNAP receptors (SNAP-25, syntaxins and VAMP/synaptobrevin-2) present in the complex. A brain membrane protein (86 kDa) has been suggested to be a target for rab3A/smg25A [28]. The relationship between this protein and the complex of proteins demonstrated in the present study remains to be clarified.

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