Voltage-dependent interaction between the muscarinic ACh receptor and proteins of the exocytic machinery

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1. Release of neurotransmitter into the synaptic cleft is the last step in the chain of molecular events following the arrival of an action potential at the nerve terminal. The neurotransmitter exerts negative feedback on its own release. This inhibition would be most effective if exerted on the first step in this chain of events, i.e. a step that is mediated by membrane depolarization. Indeed, in numerous studies feedback inhibition was found to be voltage dependent.

2. The purpose of this study is to investigate whether the mechanism underlying feedback inhibition of transmitter release resides in interaction between the presynaptic autoreceptors and the exocytic apparatus, specifically the soluble NSF-attachment protein receptor (SNARE) complex.

3. Using rat synaptosomes we show that the muscarinic ACh autoreceptor (mAChR) is an integral component of the exocytic machinery. It interacts with syntaxin, synaptosomal-associated protein of 25 kDa (SNAP-25), vesicle-associated membrane protein (VAMP) and synaptotagmin as shown using both cross-linking and immunoprecipitation.

4. The interaction between mAChRs and both syntaxin and SNAP-25 is modulated by depolarization levels; binding is maximal at resting potential and disassembly occurs at higher depolarization.

5. This voltage-dependent interaction of mAChRs with the secretory core complex appears suitable for controlling the rapid, synchronous neurotransmitter release at nerve terminals.

The mechanism underlying neurotransmitter release has been extensively studied and a large amount of data has been accumulated about molecular (Bennett & Scheller, 1994; Sudhof, 1995; Linial & Parnas, 1996) and physiological aspects (Augustine, Charlton & Smith, 1987; Parnas & Parnas, 1994). However, there is still no detailed description of the molecular mechanism that initiates release, especially in fast responding nerve terminals. As explained below, autoreceptors are suitable candidates for being involved in initiation of release. Autoreceptors regulate neurotransmitter release via feedback inhibition (Kilbinger, 1984; Starke, Gothert & Kilibinger, 1989; Sanchez-Prieto, Budd, Herrero, Vazquez & Nicholls, 1996) and by a voltage-dependent mechanism; inhibition being maximal at low levels of depolarization and lower at higher levels of depolarization (Dolezal & Tucek, 1993; Keith, Horn, Piser & Mangano, 1993; Parnas, Parnas, Ravin & Yudelevitch, 1994; Parnas, Dudel, Parnas & Ravin, 1996).

In addition to modulating secretion (Kilbinger 1984; Starke *et al.* 1989; Sanchez-Prieto *et al.* 1996) autoreceptors may also control neurotransmitter release by a negative feedback loop. According to this scheme, a transmitter inhibits its own release by blocking the first step in the chain of events leading to release (Parnas & Parnas 1994; Parnas *et al.* 1994, 1996; Khanin, Parnas & Segel, 1997). When compared with multi-step biochemical reactions that are inhibited by their end-product, the neurotransmitter is analogous to the end-product. Furthermore, the autoreceptor, the molecular entity which binds the end-product, must be an integral member of the release machinery and, as such, must be involved in controlling activation of release.

It is now well established that the core exocytic machinery is composed of vesicle-associated membrane protein (VAMP)/synaptobrevin on the synaptic vesicle and synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin/HPC-1 on the plasma membrane (the soluble

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NSF-attachment protein receptor (SNARE) proteins; Sollner *et al.* 1993) together with synaptotagmin (Bennett & Scheller, 1994; Linial & Parnas, 1996; Sudhof, 1995). Dolezal & Tucek (1993) showed that the muscarinic-mediated feedback inhibition of ACh release from cerebrocortical cholinergic fibres is voltage dependent. We therefore investigated whether the muscarinic autoreceptor in rat brain synaptosomes interacts with the exocytic apparatus. Furthermore, we monitored whether the interaction between the mAChRs and the exocytic proteins is voltage dependent.

METHODS

Preparation

Male Sprague–Dawley rats (200–250 g) were killed by CO_2 inhalation in accordance with guidelines set out by the National Institute of Health. Following decapitation, the brain was quickly removed and immersed in cold BSS buffer (mM: 10 Hepes–NaOH (pH 7·4), 128 NaCl, 2·4 KCl, 1·2 $MgCl_2$, 1·2 KH_2PO_4 and 10 D-glucose) containing a battery of protease inhibitors (aprotonin, leupeptin and pepstatin; 10 μg ml^{-1}; Boehringer) and 10 mM AEBSF (Calbiochem).

Immunoprecipitation, Western blot analysis

Fresh synaptosomes (fraction P2; Kloog, Michaelson & Sokolovsky, 1980) were solubilized for 45 min at 4 °C in HKA buffer (mM: 10 Hepes–KOH (pH 7·4), 140 potassium acetate, 1 $MgCl_2$ and 0·1 EGTA) containing 2% freshly prepared 3-((3-cholamidopropyl)-dimethylammino)-1-propanesulphonate (CHAPS; Boehringer) and protease inhibitors. For immunoprecipitation, antibodies were first bound to protein G- or protein A-Sepharose beads (Zymed) for 2 h (4 °C) and then ineubated for 10–12 h (4 °C) with 100 μg P2 fraction solubilized in HKA buffer containing 0·1% gelatin and 2% CHAPS. Beads were then washed with solubilization buffer containing only 0·2% CHAPS, and bound proteins were eluted from the beads and separated by SDS–PAGE. Detection was by Western blot analysis as described previously (Linial, Levius, Ilouz & Parnas, 1995) and using an ECL detection system (Amersham). Antibodies used were: polyclonal antibodies against GST-syntaxin1A (Alomone Laboratories); monoclonal against syntaxin (6D2); polyclonal against SNAP-25 and VAMP-2 (kindly provided by M. Takahashi, Tokyo, Japan); monoclonal against SNAP-25 (kindly provided by S. Kozaki, Osaka, Japan); monoclonal HPC-1 against syntaxin (BioMakor, Jerusalem, Israel); anti-SV2 monoclonal antibodies (a kind gift from H. Zimmermann, Frankfurt, Germany); and polyclonal antibodies against the different subtypes of mAChR (M_1–M_5), which were obtained via a collaboration with E. Heldman (Israel Institute for Biological Research, Nes-Ziona, Israel) and were a kind gift of A. I. Levey (Emory University, Atlanta, USA). The amount of antibody used for immunoprecipitation was optimized for each antibody (in the range 0·5–5 μg ml^{-1}). Protein silver staining was done according to the manufacturer's instructions (BioRad). Protein concentration was measured by the Bicinchoninic acid assay (Pierce).

Cross-linking of synaptosomal proteins

P2 fraction (2 mg ml^{-1}) in either DMSO (10%) or 2·5 mM dithiobis succinimidyl propionate (DSP; Pierce) in 10% DMSO were incubated 30 min at 25 °C. The reaction was terminated by addition of 150 mM Tris and synaptosomes were immediately solubilized in 1% SDS (2 h at 25 °C). The undissolved material was discarded following centrifugation (16000 *g*, 15 min) and the soluble fraction was diluted 20-fold (final protein concentration 0·1 mg ml^{-1}) in HKA buffer and CHAPS for immunoprecipitation experiments (as detailed above). Reduction of the thiol groups of DSP was performed using 100 mM dithiothreitol.

Binding of radiolabelled mAChR antagonist

Binding of [3-^{3}H]quinuclidinyl benzilate ([^{3}H]-QNB) to synaptosomes was performed as described (Kloog *et al.* 1980). Briefly, synaptosomes were incubated while shaking with 2 nM [^{3}H]-QNB (45 Ci mmol^{-1}; New England Nuclear, MA, USA) in BSS buffer for 1 h at 25 °C. Synaptosomes were then washed 4 times and filtered through glass fibre filters. Radioactivity retained on protein A- or protein G-Sepharose beads following immunoprecipitation experiments was found to be minimal. The non-specific binding of [^{3}H]-QNB in each experiment was determined by competition with 10^{-5} M atropine, a potent antagonist of the mAChR. Non-specific precipitation of [^{3}H]-QNB binding sites was monitored by immunoprecipitation experiments with various non-relevant antibodies and pre-immune rabbit IgG. The level of [^{3}H]-QNB was minimal in all these control experiments.

Release of glutamate from synaptosomes

Synaptosomes (P2 fraction, 5 mg ml^{-1}) were loaded with neurotransmitter (glutamate) by incubation with [^{3}H]-L-glutamate (60 Ci mmol^{-1}; New England Nuclear) at a final concentration of 0·6 μM for 10 min (37 °C). Labelled synaptosomes were incubated for 2 min (25 °C) in BSS buffer with the indicated concentration of KCl (and a comparable reduction in NaCl) in the presence of Ca^{2+} (1·0 mM) or EGTA (2·0 mM). The Mg^{2+} level was 1·2 mM and was raised to 5 mM in the presence of EGTA. Synaptosomes were then pelleted and radioactivity released to the medium was measured by scintillation counting. A typical value for total labelled glutamate was about 1200 d.p.m. (μg proteins)$^{-1}$ and about 60% of this labelled neurotransmitter was released at 90 mM KCl.

RESULTS

Synaptosomes isolated from rat brain are physiologically active

To analyse the interaction between mAChRs and members of the exocytic apparatus, we used fresh synaptosomes from rat brain and followed the interaction of various synaptic proteins with mAChRs by co-precipitation, using antibodies for mAChRs. We first validated the responsiveness of the synaptosomes to physiological stimuli by monitoring depolarization-dependent neurotransmitter release. Synaptosomes were loaded with radiolabelled glutamate and release was monitored under various stimulation protocols. The results in Fig. 1 show that synaptosomes were sensitive to the level of KCl-induced depolarization. Furthermore, release was strictly dependent on Ca^{2+} since release was inhibited in the presence of EGTA at 90 mM KCl. We conclude that the synaptosomes are functional in terms of their release machinery and suitable for monitoring changes that occur during synapse activation.

Interaction of mAChRs with synaptic proteins

We next investigated possible interactions of mAChRs with components of the release apparatus by immunoprecipitation experiments (Fig. 2). Polyclonal antibodies for mAChRs (specific to each mAChR subtype) were used to immunoprecipitate the interacting proteins. Synapto-

tagmin and VAMP, two integral membrane proteins of the synaptic vesicle, efficiently co-precipitated with mAChRs, while other abundant synaptic vesicle proteins, such as synaptophysin and SV2, were not detected. The two major plasma membrane proteins, syntaxin and SNAP-25, which are essential for docking, priming and fusion of synaptic vesicles (Sollner *et al.* 1993; Bennett, 1995) also co-precipitated with mAChRs (Fig. 2*A*). We performed the reciprocal assay to verify the specificity of the co-precipitation. Synaptosomes were labelled with [^{3}H]-QNB, a mAChR antagonist (Kloog *et al.* 1980), and individual antibodies for synaptic proteins were then used in immunoprecipitation experiments (Fig. 2*B*). All four proteins – VAMP, synaptotagmin, SNAP-25 and syntaxin, which were detected in a complex with the mAChR (Fig. 2*A*) were also able to precipitate [^{3}H]-QNB-bound receptors. Only background levels of [^{3}H]-QNB were obtained following immunoprecipitation with SV2, synaptophysin, actin and non-immune serum (Fig. 2*B*).

Due to the high abundance of all these four proteins in the synapses and the tendency of these proteins to reassemble even following solubilization, we employed several experimental procedures to further ensure the specificity of the immunoprecipitation. We analysed the number of proteins co-precipitated by the mAChR by silver staining (Fig. 2*C*). Although the exact number of precipitated proteins cannot be discerned by silver staining, only a limited number of proteins were specifically precipitated by mAChR antibodies. Furthermore, both syntaxin and VAMP could be visualized as silver-stained bands (marked with asterisks in Fig. 2*C*) and their identity was confirmed by Western analysis (Fig. 2*D*). Synaptotagmin and SNAP-25, which were masked by other proteins (partially by the heavy and light chains on the antibodies) in the silver-stained gel, were also detected by Western analysis (synaptotagmin, Fig. 2*D* and SNAP-25 not shown). The absence of synaptophysin further ensured the specificity of the immunoprecipitation (Fig. 2*A* and *D*).

To ensure that the complex between the SNAREs and synaptotagmin with mAChRs occurs *in vivo* before solubilization, a cross-linking experiment on intact synaptosomes has been performed (Fig. 3). The intact synaptosomes were subjected to a lipid-soluble, homobifunctional cross-linking reagent, DSP, prior to immunoprecipitation with mAChR antibodies. Since the interaction of mAChR with the SNARE proteins is disrupted by SDS (Fig. 3), any interactions that could be formed after solubilization were eliminated by solubilizing the DSP-treated synaptosomes with 1% SDS and by the large dilution volume. Syntaxin (Fig. 3*A*), SNAP-25 and VAMP (Fig. 3*B*) were specifically immunoprecipitated under these conditions and were detected following reduction of the DSP thiol groups. However, in unreduced samples high molecular weight complexes, which were formed by the cross-linking reagent, were too large to enter the SDS gel and thus could not be resolved. In control experiments in which DSP was absent (marked DMSO–1% SDS in Fig. 3), or in which immunoprecipitation was performed on DSP-treated synaptosomes with synaptophysin antibodies, no syntaxin (Fig. 3*A*) or SNAP-25 were detected (in the latter case, a ladder of synaptophysin immunoreactive bands was detected in the DSP-treated reaction, not shown). These experiments corroborate the immunoprecipitation experiments (Fig. 2) and clearly showed that the presynaptic mAChRs indeed comprise an integral part of the *in vivo* exocytic apparatus. We cannot distinguish between complexes composed of mAChR with only one or more of its interacting proteins, but, the very high molecular weight complexes obtained in the cross-linking experiment (Fig. 3) were consistent with all the mAChR interacting proteins being in the same complex.

mAChR–SNAP-25/syntaxin interactions are voltage dependent

Since autoreceptor inhibition of neurotransmitter release is controlled by presynaptic membrane depolarization (Dolezal & Tucek, 1993; Keith *et al.* 1993; Parnas *et al.* 1994; Parnas *et al.* 1996), we examined the effect of depolarization (via changing KCl levels) on the interaction between mAChRs and members of the release apparatus. Experiments were performed in Ca^{2+}-free solutions, so as to uncouple the effect of membrane depolarization from changes in Ca^{2+} concentration due to depolarization. Since a voltage-

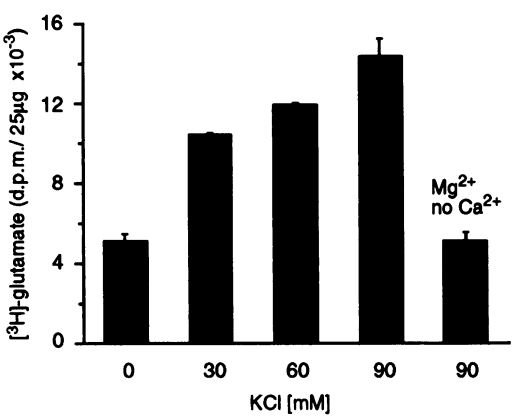

Figure 1. Neurotransmitter release in rat synaptosomes
Synaptosomes (25 µg) were labelled with [^{3}H]-glutamate and release was assayed following depolarization with the indicated KCl concentration. When Ca^{2+} was eliminated (last column), release was abolished even in the presence of 90 mM KCl. Experiments were done in triplicate. Error bars represent S.D.

dependent step must occur at the plasma membrane surface, we focused in the following experiments on the interaction between mAChRs and syntaxin or SNAP-25, which are both plasma membrane proteins of the SNARE complex. To this end, mAChRs were labelled under physiological conditions with [^{3}H]-QNB and the labelled synaptosomes were subjected to varying depolarization levels (by changing KCl concentration and maintaining constant ionic strength by replacing NaCl by KCl). This was followed by immunoprecipitation using antibodies for syntaxin (Fig. 4*A*) or SNAP-25 (Fig. 4*B*). As seen, the dissociation of syntaxin and SNAP-25 from the mAChRs correlates well with the

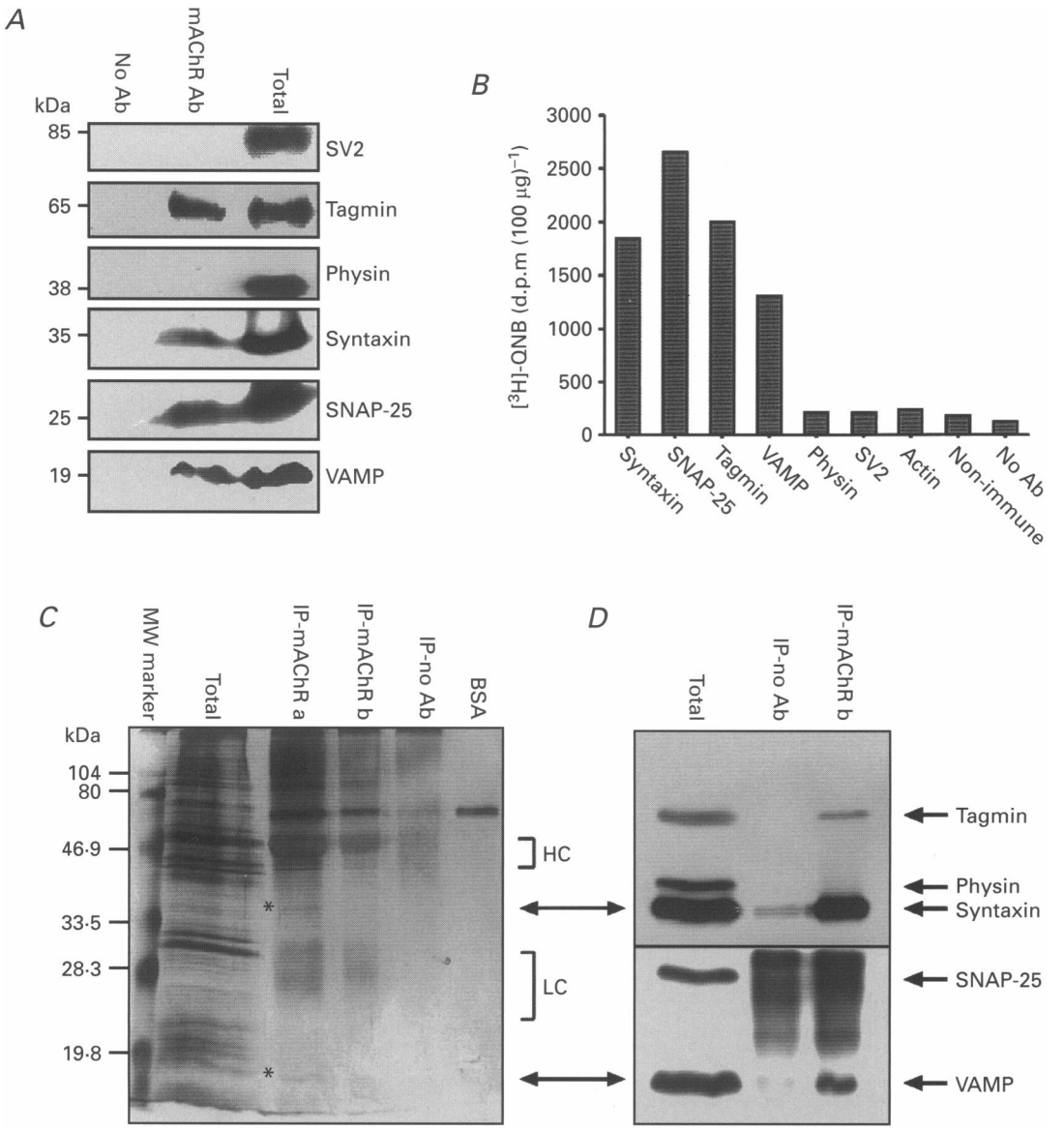

Figure 2. mAChR interacts with proteins implicated in the exocytic release apparatus

A, a mixture of polyclonal antibodies against various mAChRs (raised for subtypes M_1–M_5) was used for immunoprecipitation (IP) with protein A-Sepharose beads. The co-precipitated proteins were separated by SDS–PAGE, blotted and detected by the antibodies indicated. Physin, synaptophysin; Tagmin, synaptotagmin. *B*, co-immunoprecipitation of [^{3}H]-QNB bound to the mAChRs by the indicated antibodies. Each immunoprecipitation experiment was repeated 2–5 times; a typical experiment is shown. *C* and *D*, immunoprecipitation was performed as in *A*, and co-precipitated proteins were separated by SDS–PAGE. Half of the gel was stained by silver (*C*) and half was blotted and processed for Western analysis (*D*). Syntaxin- and VAMP-stained bands are marked by asterisks. In *D*, the heavy staining of the light chain (LC) of the antibodies masks the immunoprecipitated SNAP-25, seen in this experiment before reacting with VAMP antibodies. IP mAChR a and IP mAChR b, loading of $\frac{3}{4}$ and $\frac{1}{4}$ of the immunoprecipitated material, respectively, for quantification of detection level; MW marker, molecular weight marker; no Ab, pre-immune serum; Total, 20 µg of the solubilized proteins. The strong 66 kDa band is residual bovine serum albumin (BSA) used for stabilization of the antibodies.

degree of depolarization. Maximal binding was achieved at low depolarization and binding was reduced at higher levels of depolarization until a plateau was reached. Still higher levels of KCl (up to 120 mM) had no additional effect. However, even at the highest level of depolarization (120 mM KCl), substantial amounts of both syntaxin and SNAP-25 were still bound to the mAChRs (Fig. 4*A* and *B*). Immunoprecipitation showed that varying the Ca^{2+} level had no apparent effect on the binding of the interacting proteins to the mAChRs (not shown).

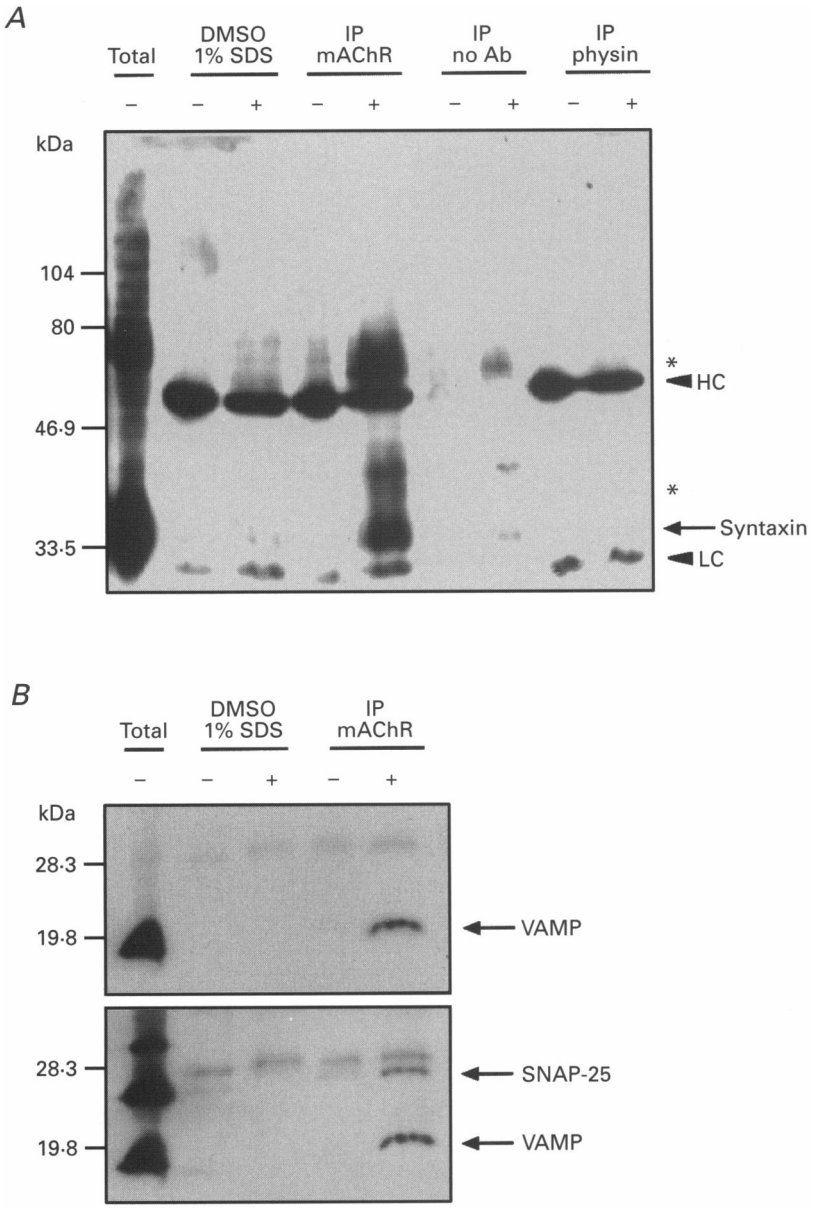

Figure 3. Immunoprecipitation following *in situ* cross-linking of intact synaptosomes

A, immunoprecipitation with antibodies against mAChR (IP mAChR), synaptophysin (IP physin) and without antibodies (IP no Ab) was performed. Each reaction was done with 100 μg of DSP- or DMSO-treated synaptosomes, following solubilization by SDS. Each immunoprecipitation was loaded on an 8·5% SDS gel before (–) or after (+) reduction with 100 mM DTT; Total, as in Fig. 2. The gel was blotted and processed for Western analysis using syntaxin antibodies. Immunoreactivity with syntaxin was only detected following reduction of the DSP-treated synaptosomes. Additional bands (marked by asterisks) obtained by using antibodies against syntaxin are detected as well. These bands (and some additional faint bands) represent products of the incomplete reduction of the cross-linker. The light (LC) and heavy chain (HC) of the antibodies are marked by arrowheads. *B*, an identical immunoprecipitation experiment (as in *A*) was performed, separated on 12·5% SDS gel and reacted with antibodies for VAMP (top panel) and then with SNAP-25 (bottom panel). A non-specific reactive band was detected in all lanes using VAMP antibody. The only immunoreactive bands for VAMP and SNAP-25 are seen following reduction of the cross-linking reagent.

To further demonstrate that the results shown in Fig. 4*A* and *B* are associated with depolarization, we first disrupted synaptosomes by a hypotonic solution which dissipates the ionic gradient (Fig. 4*C*, hypotonic). Under these conditions, where membranes could not undergo depolarization, binding occurred but the degree of binding of mAChRs with syntaxin (Fig. 4*C*, hypotonic) and SNAP-25 (not shown) was independent of KCl concentration. For comparison, results of immunoprecipitation using syntaxin under physiological conditions (similar to Fig. 4*A*) are shown in Fig. 4*C* (isotonic). In the isotonic conditions there is a statistically highly significant reduction in binding of mAChRs to syntaxin. We may therefore conclude that (i) the solubilization procedure and (ii) KCl by itself do not account for the results in Fig. 4*A–C*. Rather, the KCl-dependent depolarization affects the complex stability.

An additional possibility is that binding of the antagonist [^{3}H]-QNB to the mAChRs may be affected by the KCl concentration. We evaluated the number of [^{3}H]-QNB binding sites as a function of KCl concentration. No statistical differences were observed in the binding level of [^{3}H]-QNB in KCl levels ranging from 0 to 120 mM (not shown). Furthermore, the immunoprecipitation procedure was insensitive to KCl, as the amount of [^{3}H]-QNB recovered by the use of a mAChR antibody was identical, irrespective of KCl concentration (0–120 mM). Since the solution was Ca^{2+}-free throughout, we conclude that the effects of depolarization cannot be attributed to depolarization-dependent Ca^{2+} entry and that depolarization *per se* is a prime factor in modulating the interaction between mAChRs and both plasma membrane SNARE proteins.

DISCUSSION

We present here two main experimental results. First, we showed that muscarinic autoreceptors bind to the exocytic machinery. Second, this interaction is voltage dependent – maximal at resting potential and weak at higher depolarization levels.

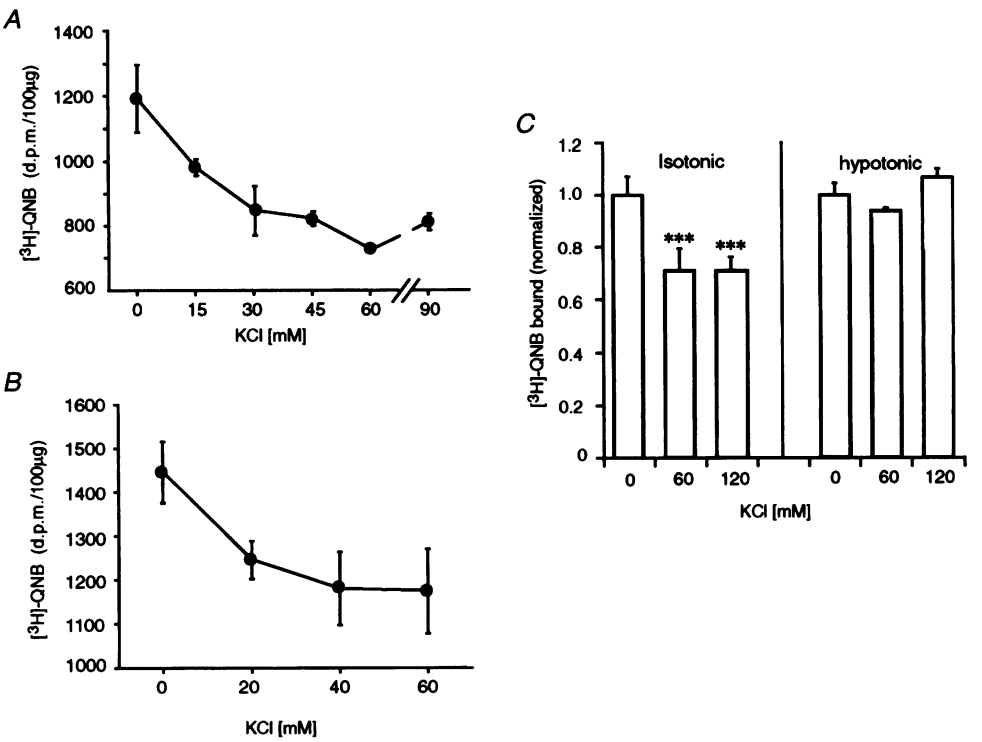

Figure 4. Depolarization-dependent interaction of SNAP-25 and syntaxin with mAChRs

fresh synaptosomes (100 µg) were labelled with [^{3}H]-QNB and subjected to immunoprecipitation as in Fig. 2. All buffers contained 2 mM EGTA and 5 mM $MgCl_2$ throughout. Immunoprecipitation experiments were performed using either syntaxin 1A (*A*) or SNAP-25 (*B*) antibodies. For each data point in *A* and *B* at least 4 independent experiments were done, each experiment was done in duplicate. A typical experiment is shown. The error bars show the mean difference between the duplicated reactions. *C*, depolarization-dependent interaction of syntaxin with mAChRs in physiologically intact and disrupted synaptosomes. Isotonic conditions were identical to *A* and *B* and disruption of synaptosomes in hypotonic conditions were achieved by pre-incubation in a buffer containing 5 mM Tris–HCl (pH 7·4) and EDTA 0·1 mM with or without the indicated amount of KCl (with NaCl replacing KCl). Values are the means of 4–6 independent immunoprecipitation experiments done in duplicate. Variance between duplicates was less then 10% in a single experiment. Statistical significance was calculated using Student's paired *t* test; *** $P < 0.005$.

It could be argued that the observed interactions between the mAChR and SNARE is an artifact, resulting from the high abundance of the SNARE proteins and of synaptotagmin in the synaptosomes and the relatively low concentration of mAChRs. This possibility was taken into consideration by optimizing the experimental procedures in terms of protein and detergent concentration etc. so as to minimize artificial interactions (see details in 'Methods'). Furthermore, the immunoprecipitation experiments (Fig. 2*A* and *B*) showed that an identical set of proteins was precipitated irrespective of the antibody used – mAChR antibodies or any of the SNARE and synaptotagmin antibodies. Furthermore, other abundant proteins in the synaptosomes such as synaptophysin and SV2, each of which interacts with one of the SNARE proteins, were not precipitated. These findings indicate that only a specific subset of the SNARE proteins interact directly with mAChRs. Finally, the protein profile of the immunoprecipitation by mAChR antibodies showed that only a very limited number of proteins were co-precipitated (Fig. 2*C*). A troublesome aspect concerning the SNARE proteins is their tendency to form artificial, but stable, interactions following membrane solubilization. To ensure that the interactions shown here are genuine, we performed an *in situ* cross-linking experiment. The findings (Fig. 3*A* and *B*) that the SNARE proteins co-precipitated with mAChR antibodies under conditions where post-solubilization complexes were disrupted confirm that these interactions were genuine.

Our results enable a rough estimation of the stoichiometry of the interaction of mAChR and the exocytic core proteins. Both syntaxin and VAMP are detected as faint silver-stained bands (marked by asterisks in Fig. 2*C*). Using bovine serum albumin (BSA) for calibration, we estimate each band to contain no more then a few nanograms. These values can be translated to amounts (in moles) using the molecular weight of each protein. The amount (in moles) of mAChR can also be estimated. Specifically, the value of labelled QNB (about 2000 d.p.m. $(100\ \mu g)^{-1}$; Fig. 2*B*) is translated to moles according to the specific activity of [^{3}H]-QNB. The above estimations suggest that in the co-precipitation experiments, all these proteins are in the same molar range.

To isolate the specific effect of membrane potential on the interaction of the autoreceptor with the exocytic machinery we simplified the 'nerve terminal' by eliminating from the experiments two potential candidates that are likely to affect such interactions – Ca^{2+} and neurotransmitter. However, while Ca^{2+} is not essential for the interactions between mAChR and the membranous exocytic proteins, this interaction is affected by the concentration of the mAChR agonist, muscarine, when applied to the synaptosomes (authors' unpublished data).

We may now speculate on the molecular machinery underlying our observations. The interaction between the release apparatus and the voltage-dependent Ca^{2+} channel is well established (Sheng, Rettig, Cook & Catterall, 1996). Assuming that depolarization affects only proteins residing on the membrane surface, we need only consider cross-talk between three key elements: autoreceptors, voltage-dependent Ca^{2+} channels and the exocytic apparatus (specifically SNAP-25 and syntaxin, at the membrane surface). We suggest two alternative models: either the autoreceptor interacts directly with the exocytic apparatus which is linked to the Ca^{2+} channel; or the interaction of the autoreceptor to the exocytic apparatus is indirect and is exerted via its direct interaction to the Ca^{2+} channel. In view of the sensitivity of the interactions to depolarization, it is most likely that the Ca^{2+} channel serves as a voltage sensor. Thus, depolarization-dependent conformational changes of the Ca^{2+} channel weaken the interaction between the autoreceptor and the exocytic apparatus (directly or indirectly), the remaining complex, i.e. the Ca^{2+} channel with the SNAREs either enhancing release (Mochida, Sheng, Baker, Kobayashi & Catterall, 1996) or reducing it (Bezprozvanny, Scheller & Tsien, 1995).

In summary, we have shown that the mAChR is an intrinsic part of the exocytic complex and that this interaction is voltage dependent. These findings fit the notion of the 'first step hypothesis' (Khanin *et al.* 1997). Accordingly, the transmitter inhibits its own release by blocking the first step in the chain of events leading to release. We propose that a voltage-dependent protein–protein interaction, via autoreceptors, is the clamp in controlling fast, synchronous neurotransmitter release in nerve terminals.

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