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Mechanism of acetylcholine release: Possible involvement of presynaptic muscarinic receptors in regulation of acetylcholine release and protein phosphorylation

(muscarinic acetylcholine receptors/calcium uptake/*Torpedo*)

DANIEL M. MICHAELSON, SOFIA AVISSAR, YOEL KLOOG, AND MORDECHAI SOKOLOVSKY

The Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

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ABSTRACT **Acetylcholine (AcCho) release from purely cholinergic *Torpedo* synaptosomes was evoked by K^+ depolarization in the presence of Ca^{2+}. Activation of muscarinic receptors, present in the synaptosomal fraction, by the agonist oxotremorine resulted in the inhibition of AcCho liberation. This inhibition was abolished by the muscarinic antagonist atropine, which by itself has no effect. These findings suggest that the muscarinic receptor, present in the electric organ of *Torpedo*, is presynaptic and that its physiological function is to regulate AcCho release by negative feedback. The mechanism of presynaptic muscarinic inhibition was investigated by examining the effect of muscarinic ligands on synaptosomal $^{45}Ca^{2+}$ uptake and on the level of phosphorylation of specific synaptosomal proteins. Ca^{2+}-dependent K^+ depolarization-induced synaptosomal AcCho release was accompanied by $^{45}Ca^{2+}$ uptake and by a marked increase in the phosphorylation of a specific synaptosomal protein (band α) of approximately 100,000 daltons. Activation of the muscarinic receptor by the agonist oxotremorine had no detectable effect on synaptosomal $^{45}Ca^{2+}$ uptake but resulted in the concomitant inhibition of AcCho release and of phosphorylation of band α. The muscarinic antagonist atropine abolished the inhibitory effect of oxotremorine both on AcCho liberation and on phosphorylation of band α. These findings suggest that phosphorylation of band α may be involved in regulation of the presynaptic processes that underly AcCho release and that activation of the muscarinic receptor by agonists may inhibit AcCho release by blocking the phosphorylation of band α.**

It has been suggested that some cholinergic synapses contain presynaptic muscarinic receptors which regulate the extent of acetylcholine (AcCho) release by feedback inhibition (1–5). The molecular mechanisms underlying AcCho liberation, and in particular its modulation by muscarinic receptors, are not known.

Protein phosphorylation and dephosphorylation is now recognized as a ubiquitous regulatory mechanism of enzymatic and cellular activities (for review, see ref. 6). Hence it is plausible that AcCho liberation, triggered *in vivo* and *in vitro* by an increased level of free Ca^{2+} in the nerve ending's cytoplasm (7–9), is accompanied by the phosphorylation of specific proteins. Krueger *et al.* (10) have shown that agents which increase Ca^{2+} transport into intact rat brain synaptosomes stimulate the incorporation of $^{32}P_i$ into synaptosomal proteins of apparent subunit molecular weights of 80,000 and 86,000. DeLorenzo (11) and Hershkowitz (12) used a preparation of rat brain synaptosomes and [γ-^{32}P]ATP and found Ca^{2+}-dependent phosphorylation of specific proteins in the range of 60,000, 50,000, and 42,000 daltons. These findings show that Ca^{2+} can modulate protein phosphorylation of rat brain synaptosomes. Brain synaptosomes contain a mixture of nerve endings, and these endings use various neurotransmitters. Consequently, the phosphorylation observed in such preparations represents that of an average population of different types of nerve endings, of which the cholinergic nerve endings are only a minor constituent.

We have recently found (13) that the *Torpedo* electric organ contains a muscarinic cholinergic receptor that can be separated from the classical nicotinic postsynaptic receptors and that synaptosomes can be isolated from the *Torpedo* electric organ which release AcCho upon Ca^{2+} stimulation (9) and which are enriched in muscarinic receptors (13). These findings prompted the present investigation of the physiological function of muscarinic receptors in *Torpedo*. This report discusses experiments in which the effects of muscarinic ligands on Ca^{2+}-dependent AcCho release, on $^{45}Ca^{2+}$ uptake into *Torpedo* synaptosomes, and on the level of phosphorylation of specific synaptosomal proteins were investigated.

EXPERIMENTAL

Materials. Live *Torpedo ocellata* were caught off the coast of Tel Aviv. Hepes and ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) were from Sigma. Acrylamide was from Merck and *N,N'*-methylene-bisacrylamide and sodium dodecyl sulfate ($NaDodSO_4$) were from BDH. Phospholine iodide was from Ayerst Laboratories. $^{32}P_i$ (carrier-free) was from Israel Nuclear Research Center-Negev, and $^{45}CaCl_2$ (10–40 Ci/mg Ca; 1 Ci = 3.7×10^{10} becquerels) was from Amersham. All other chemicals were of reagent grade.

Preparation of *Torpedo* Synaptosomes. Synaptosomes were purified from the electric organ of *Torpedo ocellata* by differential and density gradient centrifugation (9). Synaptosomal protein content was determined by the method of Lowry *et al.* (14); AcCho content was measured with the guinea pig ileum bioassay (15).

Phosphorylation of *Torpedo* Synaptosomes. The *Torpedo* synaptosomes were diluted 1:5 with 0.8 M glycine/1 mM EDTA, pH 7.0, and pelleted by a 15-min centrifugation at $17,500 \times g$. The synaptosomes were resuspended, at 1.8 mg of protein per ml, in modified Krebs–Ringer buffer (modified KRB) which contained (in mM): NaCl, 250; KCl, 4.8; $MgSO_4$, 2.4; EGTA, 0.1; D-glucose, 10; sucrose, 260; Hepes, 20. The pH was adjusted to 7.4 with NaOH and the buffer was oxygenated with O_2 prior to use. The synaptosomal suspension was purged with O_2 and incubated at 25°C for 10 min; then, 0.5 mCi of $^{32}P_i$ was added per ml. The suspension was incubated at 25°C for 30 min in oxygenated, sealed tubes. After preincubation, 0.2 ml of the synaptosomes was stimulated by a 1:2 dilution with

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Abbreviations: AcCho, acetylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; modified KRB, modified Krebs–Ringer buffer.

either modified KRB, in the presence or absence of $CaCl_2$, or with K^+-modified KRB which was similar to modified KRB except that it contained 255 mM KCl and no NaCl. When the effect of muscarinic ligands on protein phosphorylation was examined, they were added 4 min (atropine) or 2 min (oxotremorine) prior to stimulation. The reaction was terminated at the indicated time intervals by the addition of 0.2 ml of buffer containing $NaDodSO_4$, 2-mercaptoethanol, EDTA, and KH_2PO_4 to yield final concentrations of 3%, 15%, 5 mM, and 3 mM, respectively. The samples were then boiled for 2 min. After boiling, the samples were dialyzed at room temperature for 16 hr against 20mM Tris·HCl which contained 190 mM glycine/2 mM EDTA/10 mM KH_2PO_4/5% 2-mercaptoethanol/0.1% $NaDodSO_4$ at pH 8.3. After dialysis the samples were boiled again for 2 min and tracking dye (0.1 mg/ml) was added. Polyacrylamide slab gel electrophoresis (16) and autoradiography were carried out essentially as described by Krueger *et al.* (10).

Measurement of AcCho Release from *Torpedo* Synaptosomes. Synaptosomal AcCho release was determined by measuring the levels of endogenous AcCho retained within the synaptosomes after stimulation (9). The experiments were performed at 25°C as described (9). The synaptosomes were suspended in modified KRB and depolarized by a 1:2 dilution with K^+-modified KRB in the presence or absence of 2 mM $CaCl_2$. When the effect of muscarinic ligands was to be studied, they were added 4 min (atropine) or 2 min (oxotremorine) prior to depolarization. The concentration of synaptosomes during the experiment (0.2–0.4 mg of protein per ml) corresponded to an AcCho concentration of 20–30 μM and thus ensured that the muscarinic ligands did not interfere with the determination of AcCho by the bioassay (15).

$^{45}Ca^{2+}$ Uptake into *Torpedo* Synaptosomes. The synaptosomes (1.8 mg of protein per ml) were incubated in modified KRB for 5 min at 25°C, after which $^{45}Ca^{2+}$ was added by a 1:2 dilution with either modified KRB or K^+-modified KRB, both of which contained 2 mM $CaCl_2$ and about 2 μCi of $^{45}Ca^{2+}$ per ml. When the effect of muscarinic ligands was examined, they were added 4 min (atropine) or 2 min (oxotremorine) prior to the addition of $^{45}Ca^{2+}$. After the designated incubation time, 0.2 ml of the synaptosomal suspension was rapidly filtered on glass fiber filters (Whatman, GF/C) and washed at 4°C (five times, 3 ml each) with modified KRB. Total filtering time was about 10 sec. The filters were dried, placed in vials containing 5 ml of scintillation liquid [330 ml of Triton X-100, 660 ml of toluene, 5.5 g of 2,5-diphenyloxazol (Packard), and 0.1 g of dimethyl 1,4-bis[2(5-phenyloxazolyl)]benzene (Merck)], and maintained at 25°C for 30 min. Radioactivity was assayed by liquid scintillation spectrometry (Packard Tri-Carb model 2002). The counting efficiency of $^{45}Ca^{2+}$ was 45% as determined by applying standard amounts of $^{45}Ca^{2+}$ to blank filters washed with medium. Results are given as nmol of $^{45}Ca^{2+}$ per mg of synaptosomal protein.

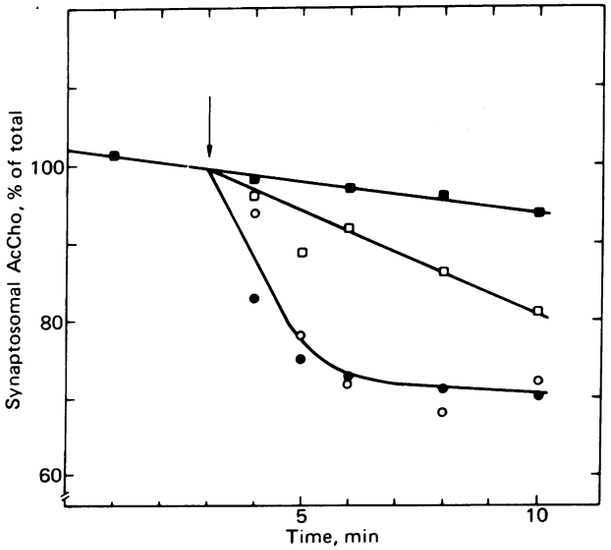

FIG. 1. Effects of muscarinic ligands on Ca^{2+}-dependent K^+-depolarization-induced AcCho release from *Torpedo* synaptosomes. ■, Synaptosomes in modified KRB; ●, synaptosomes K^+-depolarized in the presence of 1 mM Ca^{2+} (at arrow); □, similarly treated preparation except that the buffer also contained 2 μM oxotremorine; ○, synaptosomes K^+-depolarized in the presence of Ca^{2+}, 2 μM oxotremorine, and 50 nM atropine. The results presented are means of three experiments; SEM was <10%.

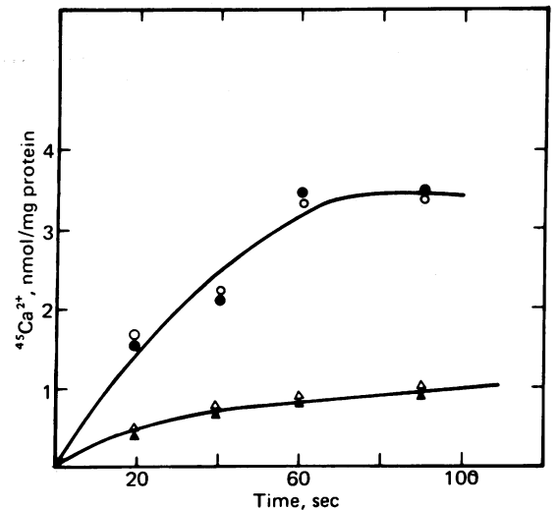

FIG. 2. The effect of oxotremorine on $^{45}Ca^{2+}$ uptake into *Torpedo* synaptosomes. At time zero, the synaptosomes were diluted into modified KRB (△,▲) or K^+-modified KRB (○,●), both of which contained $^{45}Ca^{2+}$. These treatments were so treated in the presence (●,▲) or absence (○,△) of 2 μM oxotremorine. The results presented are means of three experiments; SEM was <15%.

RESULTS

Effect of Muscarinic Activation on Synaptosomal AcCho Release. The physiological role of muscarinic receptors in *Torpedo* was investigated by examining the effect of muscarinic ligands on synaptosomal AcCho release. The muscarinic agonist oxotremorine markedly decreased the amount of AcCho liberated after Ca^{2+}-dependent K^+-depolarization-induced AcCho release (Fig. 1). The effect of oxotremorine was detected at 0.5 μM and was maximal at about 5 μM.

Ca^{2+}-dependent synaptosomal AcCho release can also be mediated by the ionophore A23187 (9). The effect of oxotremorine on Ca^{2+}-dependent (1-mM) AcCho liberation mediated by ionophore A23187 (1 μg/ml) was similar to that observed with the K^+-depolarized synaptosomes—i.e., 0.5 μM oxotremorine inhibited ionophore-mediated AcCho release only slightly, whereas maximal inhibition was observed at about 5 μM. Oxotremorine had no effect on synaptosomal AcCho levels in the absence of stimulation by Ca^{2+} plus K^+ or Ca^{2+} plus A23187.

The muscarinic agonist arecoline (10 μM) also inhibited Ca^{2+}-dependent K^+-induced AcCho release whereas the nicotinic agonist nicotine (at concentrations up to 10 μM) had no effect on ACh release. The inhibitory effect of oxotremorine (5 μM) was completely abolished by 50 nM atropine, a muscarinic antagonist (Fig. 1). Lower concentrations of atropine

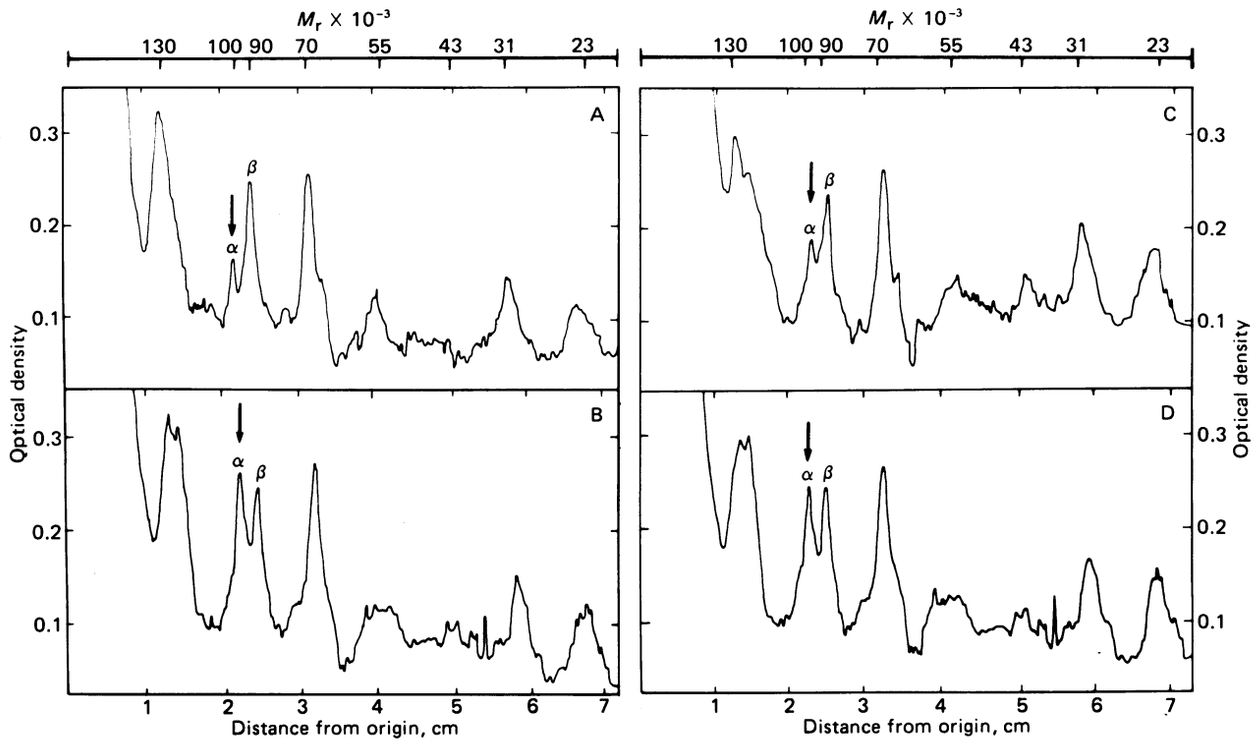

FIG. 3. Densitogram of an autoradiograph depicting the effect of muscarinic ligands on Ca^{2+}-dependent K^+-depolarization-induced phosphorylation of proteins in purified *Torpedo* synaptosomes. The synaptosomes were preincubated with $^{32}P_i$ for 30 min in the absence of Ca^{2+}. Aliquots were then incubated for 1 min as follows: (*A*) control; (*B*) in the presence of 1 mM Ca^{2+} and 130 mM K^+; (*C*) in the presence of 1 mM Ca^{2+}, 130 mM K^+, and 2 μM oxotremorine; (*D*) in the presence of 1 mM Ca^{2+}, 130 mM K^+, 2 μM oxotremorine, and 50 nM atropine. The reactions were terminated and the gels were processed for autoradiography.

(20 nM) partially reversed the inhibitory effect of oxotremorine (2 μM). The muscarinic antagonist *N*-methyl-4-piperidyl benzilate (10 nM) (13) also reversed the oxotremorine (2 μM) inhibition of AcCho release. Atropine by itself (at concentrations up to 0.2 μM) had no effect on AcCho levels of either control or stimulated synaptosomes.

It should be noted that the AcCho release assay used in this study is based on measurement of decrease in total synaptosomal AcCho content (9). Hence, it is essential to establish that, under the experimental conditions used, there was no synthesis of AcCho. Experiments with the acetylcholinesterase inhibitor phospholine iodide, in which both intrasynaptosomal and external AcCho are measured (9), revealed that atropine and oxotremorine did not induce the synthesis of AcCho, thus ensuring that the effects of these muscarinic ligands are indeed on AcCho release.

Effect of Oxotremorine on Synaptosomal $^{45}Ca^{2+}$ Uptake. Presynaptic AcCho release is triggered by increased levels of free Ca^{2+} in the nerve-ending (for review, see ref. 17). Thus, the possibility that muscarinic agonists prevent Ca^{2+} entry into the presynaptic cytosol and thereby inhibit AcCho release was examined by investigating the effect of oxotremorine on K^+-depolarization-induced synaptosomal $^{45}Ca^{2+}$ uptake. The uptake followed a time course similar to that of AcCho liberation (Fig. 2). The total synaptosomal Ca content prior to depolarization (0.12 nmol of Ca/mg of protein, as determined by atomic absorption spectrometry) was much lower than that of $^{45}Ca^{2+}$ after K^+-depolarization (2.5 nmol of Ca^{2+}/mg of protein), implying that K^+-depolarization induced net influx of Ca^{2+} and not a mere exchange. Oxotremorine (2 μM) had no detectable effect on the K^+-depolarization-induced $^{45}Ca^{2+}$ uptake, and neither did atropine (50 nM). Thus, it seems that the mechanism of the muscarinic inhibition of AcCho liberation is not via blockade of the Ca^{2+} channels. This conclusion is further supported by the finding that oxotremorine also blocked AcCho release induced by Ca^{2+} plus ionophore A23187.

Effect of Muscarinic Activation on Protein Phosphorylation. Incubation of intact *Torpedo* synaptosomes with $^{32}P_i$ for 30 min resulted in the incorporation of $^{32}P_i$ into several protein bands (Fig. 3*A*). Incubation of the synaptosomes for 1 min in a high-K^+ (130 mM) buffer, which contained 1 mM Ca^{2+}, resulted in more than a 3-fold increase in $^{32}P_i$ incorporation into a single protein band (for simplicity, designated band α) with an apparent subunit molecular weight of 100,000 (Fig. 3*B*). The other $^{32}P_i$-containing protein bands were unaffected by this treatment. The time course of $^{32}P_i$ incorporation into band α (Fig. 4) was similar to that of $^{45}Ca^{2+}$ uptake (Fig. 2) and of AcCho release (Fig. 1); all were maximal 45–60 sec after K^+-depolarization in the presence of Ca^{2+}. In the absence of Ca^{2+}, K^+-depolarization had no effect on $^{32}P_i$ incorporation into band α, whereas Ca^{2+} by itself increased the $^{32}P_i$ content only slightly (data not shown).

The effects of muscarinic ligands on $^{32}P_i$ incorporation into band α after stimulation with K^+ plus Ca^{2+} is shown in Fig. 3. Oxotremorine (2 μM) abolished the increase in $^{32}P_i$ incorporation into band α (Fig. 3*C*). Lower concentrations of oxotremorine (0.5 μM), which only partially inhibited AcCho liberation, also partially inhibited the phosphorylation of band α. Atropine by itself (at concentrations up to 0.2 μM) had no effect on the $^{32}P_i$ autoradiograph. However, when atropine (50 nM) was introduced together with oxotremorine (2 μM), it abolished the inhibitory effect of oxotremorine on the phosphorylation of band (Fig. 3*D*). The kinetics of $^{32}P_i$ incorporation into band α were similar in the presence of both oxotremorine (2 μM) and atropine (50 nM) and in their absence (Fig. 4). Lower concentrations of atropine (20 nM) partially reversed the inhibitory

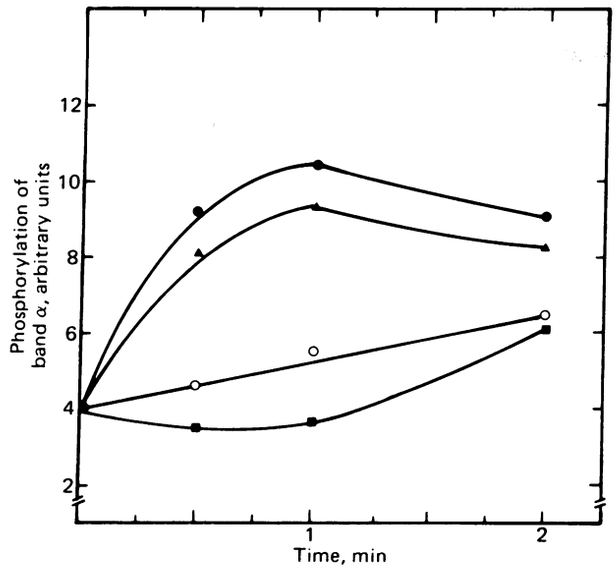

FIG. 4. Time course of the effect of muscarinic ligands on the phosphorylation of band α. ○, Modified KRB in the presence of 1 mM Ca^{2+}; ●, 130 mM K^+-modified KRB containing 1 mM Ca^{2+}; ■, 130 mM K^+-modified KRB containing 1 mM Ca^{2+} and 2 μM oxotremorine; ▲, 130 mM K^+-modified KRB containing 1 mM Ca^{2+}, 2 μM oxotremorine, and 50 mM atropine. The arbitrary unit of phosphorylation corresponds to the ratio of the area of band α to that of band β (which is not affected by the above treatments). Both areas were calculated by connecting the troughs adjacent to the peaks and measuring the area above them. The results presented are means of three experiments; SEM was <15%.

effect of oxotremorine (2 μM). Similarly, at higher concentrations of oxotremorine (4 μM), treatment with 50 nM atropine resulted in partial phosphorylation of band α (about 50% $^{32}P_i$ incorporation relative to that observed in the absence of muscarinic ligands).

DISCUSSION

The results presented in this communication demonstrate that in the electric organ of *Torpedo* there are presynaptic muscarinic receptors that regulate AcCho release and modulate the extent of phosphorylation of a specific protein (band α) that is phosphorylated after the permeation of Ca^{2+} into the presynaptic nerve ending. Activation of the muscarinic receptors by the agonist oxotremorine results in blockade of both AcCho liberation and $^{32}P_i$ incorporation into band α. The effects of oxotremorine are reversed by the muscarinic antagonist atropine.

The effective concentrations of oxotremorine used here (0.5–2.0 μM) are higher than its previously published binding constant to the receptor [I_{50} = 0.1–0.2 μM (13)]. This difference is due to the ionic strength of the *Torpedo* modified KRB which is higher than that of the mammalian physiological buffer (13). We have recently found (unpublished data) that in the *Torpedo* modified KRB the affinity constants of oxotremorine and atropine to the receptor are, respectively, I_{50} = 1–2 μM and $K_d = 6 \pm 2 \times 10^{-9}$ M. Similar effects of ionic strength on the binding of muscarinic ligands to mouse brain receptors have been reported by Birdsall *et al.* (18).

The present findings differ from those previously obtained with intact brain and peripheral preparations (3–5, 19) in that atropine by itself (at concentration up to 0.2 μM) has no effect on synaptosomal AcCho release whereas in the intact tissue preparations it enhances the liberation of AcCho. In nonhomogenized intact tissues, the inhibitory presynaptic receptors may be partially activated by spontaneously released AcCho. Consequently, atropine will relieve this basal inhibition and consequently lead to enhanced AcCho liberation.

The mechanism by which presynaptic AcCho release is induced by Ca^{2+} consists of two consecutive steps: (*i*) depolarization of the nerve ending results in an increase in the Ca^{2+} permeability of the presynaptic membrane, and (*ii*) Ca^{2+} invades the nerve ending and triggers a chain of events which result in the release of AcCho. Therefore, the muscarinic receptor may inhibit AcCho release by either blocking the Ca^{2+} channel or by interfering with the coupling between internal Ca^{2+} and AcCho release. The present observation that muscarinic ligands do not affect $^{45}Ca^{2+}$ entry into the *Torpedo* synaptosomes suggests that the mechanism of presynaptic muscarinic inhibition of AcCho release is mediated via blockade of the coupling between Ca^{2+} stimulation and AcCho secretion. This proposal is also supported by the finding that oxotremorine inhibits AcCho liberation even when Ca^{2+} is introduced into the nerve ending via the Ca^{2+} ionophore A23187 and not through the instrinsic voltage-dependent Ca^{2+} channel. Nevertheless, the possibility can not be excluded that oxotremorine inhibits AcCho release by blocking permeation of Ca^{2+} into a specific subsynaptosomal compartment.

The findings presented in this report suggest that protein phosphorylation may be involved in the mediation of the coupling between Ca^{2+} stimulation and AcCho liberation. This notion is supported by the parallel effects of muscarinic ligands on both AcCho release and $^{32}P_i$ incorporation into band α.

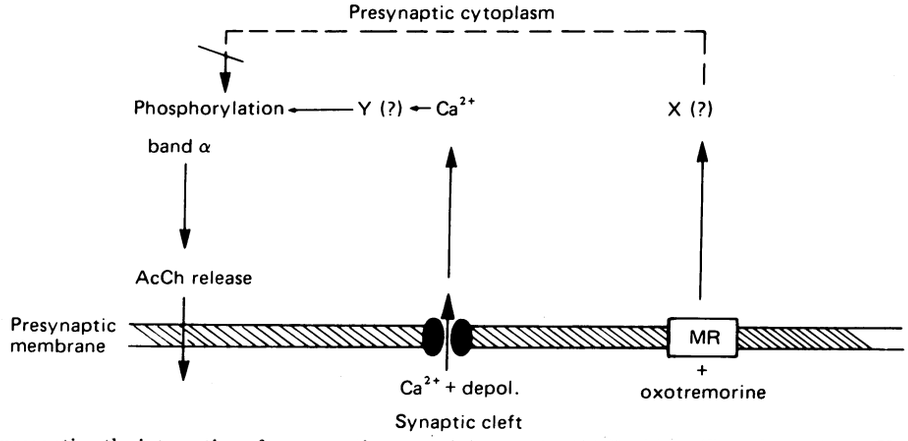

FIG. 5. Scheme representing the interaction of presynaptic muscarinic receptor (MR) with AcCho release. X and Y represent hypothetical intermediates.

The apparent molecular weight of band α (100,000) differs from that of the previously reported mammalian brain proteins that are phosphorylated upon stimulation by Ca^{2+} (10–12). This difference is most likely due to the fact that brain synaptosomes contain a heterogeneous population of nerve endings of which only a small fraction are cholinergic, whereas *Torpedo* synaptosomes are purely cholinergic. The subcellular localization of band α is not known. *Torpedo* synaptosomes contain only low levels of postsynaptic membranes (9); hence, band α is most likely a presynaptic protein. It is of interest to note that purified *Torpedo* synaptic vesicles are enriched in Ca^{2+}, Mg^{2+}-ATPase activity (20) as well as in a protein of an apparent subunit molecular weight of 100,000 (21). Because the phosphorylated subunit of muscle Ca^{2+}-dependent ATPase has been reported to have a molecular weight of about 100,000 (22), it is possible that band α may be a phosphorylated intermediate of a synaptosomal Ca^{2+}-dependent ATPase.

In conclusion, the results presented demonstrate the presence of inhibitory presynaptic muscarinic receptors in *Torpedo* and suggest that these receptors inhibit AcCho liberation by interfering with the phosphorylation of a specific protein and not by blocking the voltage-sensitive presynaptic Ca^{2+} channel (Fig. 5). The mechanisms by which the muscarinic receptor mediates its effects on the phosphorylation of band α and on AcCho liberation are not known. In any systems, muscarinic-mediated effects are accompanied by increases in cellular cyclic GMP levels (for review, see refs. 23 and 24). Hence, it is possible that the hypothetical intermediate X depicted in Fig. 5 is cyclic GMP.

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