Phosphorylation of the Porcine Atrial Muscarinic Acetylcholine Receptor by Cyclic AMP Dependent Protein Kinase†

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ABSTRACT: cAMP-dependent protein kinase, protein kinase C, cGMP-dependent protein kinase, smooth muscle myosin light-chain kinase, and phosphorylase kinase were examined with respect to their ability to phosphorylate porcine atrial muscarinic receptors (mAcChRs). Experiments were performed both in detergent solution and in a reconstituted system containing the mAcChR alone or in the presence of the purified porcine atrial inhibitor guanine nucleotide binding protein (G_i). Only cAMP-dependent protein kinase was capable of phosphorylating the receptor under any of the experimental conditions examined. Phosphorylation of the mAcChR in the detergent-solubilized state resulted in a loss of ligand binding sites that was reversible upon treatment with calcineurin in the presence of calcium and calmodulin. Upon reconstitution, the apparent stoichiometry of phosphorylation was increased by about 15-fold. Carbachol-stimulated covalent incorporation of phosphate was found only in the reconstituted system in the presence of G_i, suggesting that the large agonist-stimulated increase in phosphorylation observed in vivo [Kwatra, M. M., & Hosey, M. M. (1986) *J. Biol. Chem. 261*, 12429–12432] may in part result from a unique receptor conformation that occurs upon association with this protein. Ligand binding studies indicated that phosphorylation of the mAcChR in the detergent-solubilized or reconstituted state did not affect its interaction with carbachol or L-quinuclidinyl benzilate in vitro. Carbachol-induced stimulation of the GTPase activity of G_i in the reconstituted system was also unaffected by phosphorylation.

Atrial muscarinic acetylcholine receptors (mAcChRs)[1] mediate the regulation of the heartbeat by the parasympathetic nervous system. The mechanisms by which acetylcholine binding to the receptor is transduced into physiological responses include the activation of inward-rectifying potassium channels, inhibition of adenylyl cyclase, stimulation of phosphoinositide turnover, and an increase in cGMP formation [reviewed in Sokolovsky et al. (1983) and McKinney and Richelson (1984)]. Muscarinic activation of potassium channels (Pfaffinger et al., 1985; Breitwieser & Szabo, 1985; Yatani et al., 1987; Logothetis et al., 1987; Codina et al., 1987) and inhibition of adenylyl cyclase (Jakobs et al., 1979; Rodbell, 1980) are known to be mediated through the inhibitory guanine nucleotide binding protein G_i, while activation of phosphoinositide turnover may be mediated in part by G_i or by an as yet unidentified guanine nucleotide binding protein (Masters et al., 1985; Hepler & Harden, 1986).

Phosphorylation of membrane-bound neurotransmitter receptors and ion channels has been demonstrated for the nicotinic acetylcholine receptor (Gordon et al., 1977; Saitoh & Changeux, 1981; Huganir & Greengard, 1983), adrenergic receptors (Stadel et al., 1983; Benovic et al., 1985; Bouvier et al., 1987), the potential-dependent sodium channel (Costa et al., 1982), and voltage-sensitive calcium channels (Curtis & Catterall, 1985). Phosphorylation of mAcChRs in the presence of calcium and calmodulin has been suggested as a potential regulatory mechanism in that muscarinic binding sites were lost from rat brain synaptosomes (Burgoyne, 1980, 1981) and the guanine nucleotide mediated conversion of high-affinity to low-affinity agonist sites was partially inhibited under phosphorylating conditions (Burgoyne, 1983). It was further shown that the calcium–calmodulin-dependent phosphorylation of brain receptors could be reversed by calcineurin, a calcium–calmodulin-dependent phosphatase (Ho & Wang, 1985), and that phosphorylation of purified brain mAcChRs by cAMP-dependent protein kinase was also reversed by calcineurin (Ho et al., 1986). Attenuation of the muscarinic activation of phosphoinositide metabolism (Orellana et al., 1985) and the rapid internalization of mAcChRs in neuroblastoma cells (Liles et al., 1986) after treatment with phorbol esters suggest that protein kinase C may also play a role in the regulation of muscarinic responses. Most importantly, phosphorylation of the mAcChR in chick heart preparations has been demonstrated in vivo (Kwatra & Hosey, 1986) where it was shown that agonist treatment caused a 10–12-fold increase in the apparent stoichiometry of phosphate incorporation.

The experiments described below were initiated in order to gain information regarding possible phosphorylation of the purified porcine atrial mAcChR, either in detergent solution or after reconstitution with lipids in the presence of purified porcine atrial G_i. Purified cAMP-dependent protein kinase,

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; L-QNB, the L isomer of quinuclidinyl benzilate; G_i, the inhibitory guanine nucleotide binding protein; PC, soybean L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidylserine; SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; kDa, kilodalton(s); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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smooth muscle myosin light-chain kinase, phosphorylase kinase, cGMP-dependent protein kinase, and protein kinase C were examined for their abilities to catalyze the in vitro phosphorylation of the receptor. Of these enzymes, only the first proved effective. This report deals with the effects of varying experimental conditions on the enzyme-catalyzed phosphorylation and with the properties of the phosphorylated receptor.

Materials and Methods

L-[^{3}H]QNB (30–40 Ci/mmol; 1 Ci = 37 GBq), [^{35}S]-GTPγS (1000 Ci/mmol), [γ-^{32}P]GTP, and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from New England Nuclear. Cholesterol, PC, PS, CHAPS, ATP, cholic acid, cGMP, digitonin, bovine intestinal alkaline phosphatase, carbachol, acetylcholine, and L-hyoscyamine were from Sigma. Bio-Gel P-2, SDS, acrylamide, and *N,N'*-methylenebis(acrylamide) were from Bio-Rad Laboratories. mAcChR (10–15 nmol of L-[^{3}H]QNB/mg of protein) was purified from porcine atria (Peterson et al., 1984), and G_i (1.4–8 nmol of [^{35}S]GTPγS/mg of protein) was purified by sequential chromatography on DEAE-Sephacel, Ultragel ACA-34, octyl-Sepharose, and hydroxylapatite followed by sucrose gradient centrifugation in cholate buffers as a byproduct of the mAcChR purification procedure (details will be published elsewhere). Calcineurin was purified by the procedure of Klee et al. (1983), cGMP-dependent protein kinase according to Glass and Krebs (1979), phosphorylase kinase as described by Malencik and Fischer (1982), protein kinase C by the method of Kitano et al. (1986), myosin light-chain kinase as described by Malencik et al. (1982), and troponin I by the procedure of Kerrick et al. (1980). The catalytic subunit of cAMP-dependent protein kinase was purified from beef heart (Peters et al., 1977), while porcine brain calmodulin was prepared according to Schreiber et al. (1981) with a final purification as described by Charbonneau and Cormier (1979).

Reconstitution of mAcChR and mAcChR–G_i. mAcChRs were reconstituted into PC/PS/cholesterol (1:1:0.1 w/w) as described in the previous paper (Tota et al., 1987).

Reconstituted mAcChR was quantitated in terms of L-[^{3}H]QNB binding sites using the DEAE-filter disk assay (Peterson & Schimerlik, 1984) and G_i by [^{35}S]GTPγS binding according to Northup et al. (1982) modified by the substitution of 10 mM HEPES, pH 7.4, for Tris and deletion of detergent. GTPase activity was measured as described by Sunyer et al. (1984) as modified in the previous paper (Tota et al., 1987). Protein analysis was done by the colloidal gold method (Stoshek, 1987). Typical recoveries after reconstitution were 30–40% for both mAcChR and G_i.

Phosphorylation of mAcChR. Purified mAcChR (5–9 pmol) in 0.03% w/v digitonin, 0.006% w/v cholate, or reconstituted mAcChR was incubated with 0.39 μM cAMP-dependent protein kinase catalytic subunit for times varying up to 60 min at 25 °C in a buffer system consisting of 55 mM MOPS, 0.1 M DTT, 0.11 M KCl, 2.2 mM $MgCl_2$, 0.5 mM EGTA, and 19 μM [γ-^{32}P]ATP (2.0–3.5 Ci/mmol), pH 7.4, in 70-μL final volume. In general, reaction conditions were chosen so that phosphorylation reactions were completed in 10 min. For experiments with the reconstituted system, mAcChR and G_i were 2 and 10 pmol, respectively, in 70-μL final volume. Phosphorylation experiments utilizing all other protein kinases were done at identical concentrations of receptor, G_i, protein kinase, [γ-^{32}P]ATP, and DTT with the following additions and/or deletions: cGMP-dependent protein kinase, plus 10 μM cGMP; myosin light-chain kinase, plus 0.78 μM calmodulin, 100 μM Ca^{2+}, and no EGTA; phosphorylase kinase, plus 100 μM Ca^{2+} and 50 mM phosphoglycerol, pH 8.3; and protein kinase C, as described by Kikkawa et al. (1983) for phosphorylation of histones. Muscarinic ligands were preincubated with the mAcChR for 30 min prior to addition of [γ-^{32}P]ATP to start the reaction.

After a given time interval, reactions were terminated by the addition of Laemmli sample buffer and electrophoresed on 8–18% linear acrylamide gradient gels using the discontinuous buffer system Laemmli (1970). Silver staining was done according to Wray et al. (1981). In one experiment, phosphorylated proteins were resolved on 10% acrylamide–SDS/8 M urea gels according to Perrie et al. (1973). Incorporation of ^{32}P into the mAcCHR (moles of ^{32}P per mole of L-[^{3}H]QNB binding sites) was determined by scanning gels with the Ambis β scanning system using troponin I maximally phosphorylated by the catalytic subunit of cyclic AMP dependent protein kinase (2 mol of [^{32}P]P_i/mol of troponin I; Malencik & Fischer, 1982) as a standard. Standard curves prepared by scanning radioactivity in the gel as a function of the amount of ^{32}P-labeled troponin I were linear up to about 3 μg of protein (about 2000 cpm/cm^2). ^{32}P incorporation into mAcChRs was quantitated by using the linear portion of the standard curve.

Dephosphorylation of ^{32}P-Labeled mAcChRs. Purified mAcChR (30–50 pmol) was phosphorylated as described above and loaded onto a Bio-Gel P-2 column to remove free [γ-^{32}P]ATP. Dephosphorylation experiments with calcineurin were carried out by incubating the desalted, phosphorylated receptor with calcineurin (15 or 150 nM), calmodulin (200 nM), $CaCl_2$ (2 mM), and $MnCl_2$ (5 mM) for 2 h at 25 °C and terminating the reaction with Laemmli sample buffer. Experiments with alkaline phosphatase were identical with the exception of alkaline phosphatase (0.33 μM), $MgCl_2$ (10 mM), and glycine buffer, pH 8.8. Additional incubation times and concentrations are as noted in the text.

Ligand Binding Studies. The dissociation constant and total binding sites for L-[^{3}H]QNB binding to the mAcChR under both phosphorylating and nonphosphorylating conditions were determined by Scatchard (1949) analysis. Equilibrium titrations of both phosphorylated and nonphosphorylated mAcChR were performed with the agonist carbachol and analyzed according to a two-site model using Marquardt's algorithm as described by Duggleby (1984):

$$\bar{Y} = \frac{[Q]}{K}\left(\frac{F_1}{1 + [I]/K_1 + [Q]/K} + \frac{F_2}{1 + [I]/K_2 + [Q]/K}\right) \quad (1)$$

In eq 1, $\bar{Y}$ equals the fractional saturation of mAcChR by L-[^{3}H]QNB, and [Q] and [I] are the free L-[^{3}H]QNB and carbachol concentrations, respectively. F_1 and F_2 are the fractions of L-[^{3}H]QNB sites having high affinity and low affinity for carbachol with dissociation constants K_1 and K_2, respectively, and K is the overall dissociation constant for L-[^{3}H]QNB. Data were normalized according to eq 2 where $\bar{Y}_0$ was the fractional saturation in the absence of inhibitor.

$$\% \text{ specifically bound} = (\bar{Y}/\bar{Y}_0) \times 100 \quad (2)$$

Results

As shown in Figure 1, the purified porcine atrial mAcChR in detergent solution was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Similar incorporation of ^{32}P (0.7 mol of ^{32}P/mol of L-[^{3}H]QNB binding sites) was found in the absence of muscarinic ligands (lane 2) or in the presence of the agonist carbachol (lane 3), while

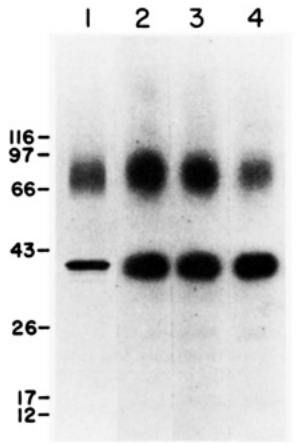

FIGURE 1: Phosphorylation of purified cardiac mAcChR by cAMP-dependent protein kinase. Purified receptor in 0.03% (w/v) digitonin/0.006% (w/v) cholate was incubated with kinase for 60 min at 25 °C and electrophoresed on 8–18% linear acrylamide gradient gels as described under Materials and Methods. Lane 1 shows a silver-stained gel pattern of purified mAcChR (0.20 μM) and cAMP-dependent protein kinase (0.39 μM) prior to autoradiography. Lanes 2–4 are autoradiograms of mAcChR plus kinase alone (2), plus 10 mM carbachol (3), or plus 100 μM L-hyoscyamine (4). Molecular weight markers were β-galactosidase (M_r 116 116), phosphorylase *b* (M_r 97 111), bovine serum albumin (M_r 66 296), ovalbumin (M_r 42 807), chymotrypsinogen A (M_r 25 666), and cytochrome *c* (M_r 11 761).

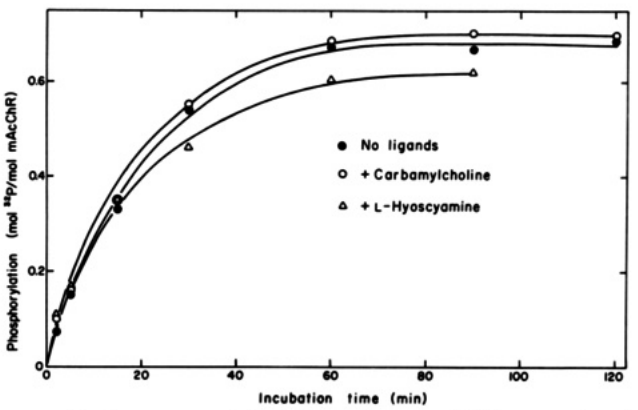

FIGURE 2: Time course of cAMP-dependent protein kinase catalyzed phosphorylation of purified mAcChR in digitonin/cholate. Purified mAcChR was phosphorylated at 25 °C for the indicated periods of time in the absence of ligands (●), the presence of 10 mM carbachol (○), or the presence of 100 μM L-hyoscyamine (△). The reactions were terminated with the addition of Laemmli sample buffer, electrophoresed, scanned, and autoradiographed as described under Materials and Methods.

preincubation with the antagonist L-hyoscyamine (lane 4) resulted in a slightly lower amount of ^{32}P incorporation (0.6 mol of ^{32}P/mol of L-[^{3}H]QNB sites). Attempts to phosphorylate the detergent-solubilized mAcChR with cGMP-dependent protein kinase, phosphorylase kinase, myosin light-chain kinase, and protein kinase C showed no incorporation of ^{32}P. Control experiments indicated that all of the above enzymes were active under the experimental assay conditions.

The time course of mAcChR phosphorylation by cAMP-dependent protein kinase is shown in Figure 2. Maximal phosphorylation was achieved after about 60 min under those conditions regardless of whether muscarinic ligands were present. Although the initial rate of phosphorylation was almost identical in the presence or absence of ligands, the final level of ^{32}P incorporation was about 15% lower in the presence of the antagonist L-hyoscyamine than in the absence of ligands or in the presence of the agonist carbachol.

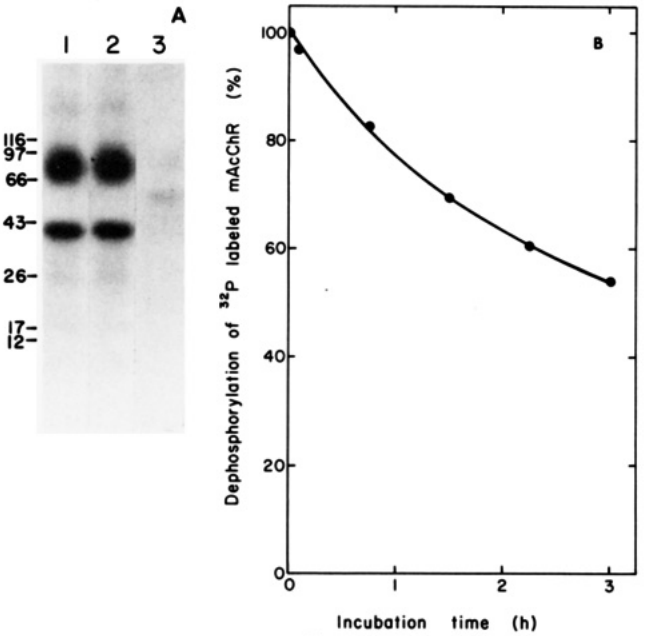

FIGURE 3: Dephosphorylation of ^{32}P-labeled mAcChR by calcineurin in digitonin/cholate. (A) Receptor was phosphorylated and desalted on a Bio-Gel P-2 column as described under Materials and Methods. Lane 1 contains receptor (0.15 μM); lane 2, receptor plus alkaline phosphatase (0.33 μM); lane 3, receptor plus calcineurin (0.15 μM). (B) Time course of calcineurin-catalyzed dephosphorylation of ^{32}P-labeled mAcChR. Receptor was phosphorylated and desalted as above and incubated with 15 nM calcineurin. Reactions were terminated and electrophoresed as described in Figure 1.

The effects of phosphorylation of the detergent-solubilized mAcChR on the binding of L-[^{3}H]QNB are shown in Figure 1A of the supplementary material (see paragraph at end of paper regarding supplementary material). In parallel experiments, the mAcChR was incubated with the same reaction mixture either plus or minus 20 μM ATP. Analysis of the binding data indicated that the dissociation constant for L-[^{3}H]QNB was essentially the same for the preparation exposed to phosphorylating conditions (1.39 ± 0.10 nM) compared to the control in the absence of ATP (1.46 ± 0.08 nM); however, the total number of L-[^{3}H]QNB binding sites was reduced by about 50%. Carbachol titration of specifically bound L-[^{3}H]QNB for control and phosphorylated preparations of detergent-solubilized mAcChR showed essentially no effect on agonist binding (Figure 1B,C of supplementary material). Control experiments also indicated that 20 μM ATP did not influence receptor stability or L-[^{3}H]QNB binding.

Dephosphorylation of ^{32}P-labeled mAcChR was attempted by using either alkaline phosphatase or the calcium-calmodulin-dependent phosphatase calcineurin. Treatment of phosphorylated mAcChR with alkaline phosphatase did not result in loss of ^{32}P covalently incorporated into the mAcChR (Figure 3A, lane 1 versus lane 2). Incubation of the phosphorylated mAcChR with 150 nM calcineurin in the presence of Ca^{2+} and calmodulin, as described under Materials and Methods, completely dephosphorylated the mAcChR (Figure 3A, lane 1 versus lane 3) and resulted in complete recovery of the L-[^{3}H]QNB binding sites lost upon phosphorylation of the receptor by the catalytic subunit of cAMP-dependent protein kinase. A sample time course for mAcChR dephosphorylation using 15 nM calcineurin is shown in Figure 3B. Treatment of the purified mAcChR with calcineurin prior to phosphorylation did not result in a significant difference in ^{32}P incorporation, indicating that the potential phosphorylation sites for cAMP-dependent protein kinase were available in the purified mAcChR.

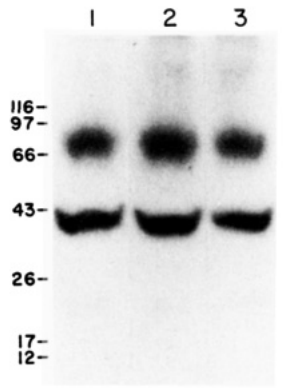

FIGURE 4: Phosphorylation of reconstituted mAcChR by cAMP-dependent protein kinase. Purified mAcChR was reconstituted into lipid vesicles as described under Materials and Methods. Lanes 1–3 are autoradiograms of reconstituted mAcChR (0.02 μM) plus kinase (0.39 μM) (1), plus 10 mM carbachol (2), or plus 100 μM L-hyoscyamine (3). Reactions were terminated and analyzed as described under Materials and Methods. The abnormal shape of the kinase band may be due to the presence of lipids.

mAcChR was reconstituted into lipids alone or in the presence of a 5-fold molar excess of purified porcine atrial G_i. For both preparations, the catalytic subunit of cAMP-dependent protein kinase was the only enzyme effective in phosphorylating the mAcChR. Figure 4 shows the result of cAMP-dependent protein kinase catalyzed phosphorylation of reconstituted mAcChR alone in the presence and absence of ligands. Similar incorporation of ^{32}P into the reconstituted mAcChR was found in the absence of ligands and in the presence of the agonist carbachol, while about 25% lower incorporation was found for the L-hyoscyamine-treated sample. The striking difference between phosphorylation of detergent-solubilized and reconstituted receptor was that the apparent stoichiometry of ^{32}P incorporation increased by about 15-fold to 10 mol of ^{32}P/mol of L-[^{3}H]QNB binding sites in the reconstituted system. To determine whether the detergent system used for the solubilized mAcChR inhibited phosphorylation, the concentration of digitonin was varied from 0.01% to 0.1% w/v, maintaining a 5:1 ratio of digitonin to cholate. ^{32}P incorporation into the solubilized mAcChR was independent of detergent concentration over the range examined, remaining at about 0.7 mol of [^{32}P]P_i/mol of L-[^{3}H]QNB binding sites as described above. The ligand binding properties of the reconstituted, phosphorylated mAcChR appear identical with those of the nonphosphorylated protein with respect to both L-[^{3}H]QNB binding (Figure 2A, supplementary material) and carbachol binding as measured by titration of specifically bound L-[^{3}H]QNB (Figure 2B,C, supplementary material).

After reconstitution of the mAcChR with purified G_i, cAMP-dependent protein kinase catalyzed ^{32}P incorporation into the mAcChR in the absence of ligands or the presence of the antagonist L-hyoscyamine was the same as found for reconstituted mAcChR alone (about 10 mol of [^{32}P]P_i/mol of L-[^{3}H]QNB sites). In the presence of the agonist carbachol, the rate of ^{32}P incorporation was unaffected, but the extent of incorporation increased 2-fold, to 20 mol of ^{32}P/mol of L-[^{3}H]QNB binding sites. Addition of 1 μM GTPγS to uncouple the mAcChR from G_i reduced the level of ^{32}P incorporation in the presence of carbachol to about 10 mol/mol of L-[^{3}H]QNB binding sites. GTPγS had no effect on mAcChR phosphorylation in the presence of L-hyoscyamine. The ratios of moles of ^{32}P incorporated per mole of L-[^{3}H]QNB binding sites for the detergent-solubilized mAcChR as well as the reconstituted preparations are summarized in Table I. The ligand binding properties of the phosphorylated mAcChR plus

Table I: Phosphorylation Stoichiometries[a] for Atrial Muscarinic Receptors

treatment	purified[b]	reconstituted	reconstituted with G_i
no ligand	0.7 ± 0.02	10.0 ± 0.21	10.4 ± 0.23
carbachol, 2 mM	0.7 ± 0.03	10.5 ± 0.32	20.7 ± 0.81
L-hyoscyamine, 100 μM	0.6 ± 0.02	7.5 ± 0.27	10.8 ± 0.15

[a] Moles of ^{32}P per mole of L-[^{3}H]QNB sites, 60-min incubation time, average of three determinations. [b] 0.08% w/v digitonin/0.016% w/v cholate.

Table II: Ligand Binding Studies of Phosphorylated and Nonphosphorylated Atrial Muscarinic Receptors[a]

	nonphosphorylated	phosphorylated
	(1) Detergent Solubilized	
K (M)	$(1.46 \pm 0.08) \times 10^{-9}$	$(1.50 \pm 0.10) \times 10^{-9}$
F_1	0.28 ± 0.01	0.21 ± 0.02
K_1 (M)	$(2.56 \pm 0.65) \times 10^{-6}$	$(3.75 \pm 1.12) \times 10^{-6}$
F_2	0.72 ± 0.01	0.79 ± 0.02
K_2 (M)	$(9.6 \pm 0.4) \times 10^{-4}$	$(1.51 \pm 0.12) \times 10^{-3}$
	(2) Reconstituted Muscarinic Receptor	
K (M)	$(4.04 \pm 0.58) \times 10^{-10}$	$(4.21 \pm 0.98) \times 10^{-10}$
F_1	0.13 ± 0.02	0.16 ± 0.03
K_1 (M)	$(1.03 \pm 0.60) \times 10^{-6}$	$(1.62 \pm 1.08) \times 10^{-6}$
F_2	0.87 ± 0.02	0.84 ± 0.03
K_2 (M)	$(4.6 \pm 0.3) \times 10^{-4}$	$(5.0 \pm 0.6) \times 10^{-4}$
	(3) Reconstituted Muscarinic Receptor plus G_i	
K (M)	$(4.25 \pm 0.68) \times 10^{-10}$	$(5.15 \pm 0.26) \times 10^{-10}$
F_1	0.54 × 0.03	0.51 ± 0.01
K_1 (M)	$(6.5 \pm 1.7) \times 10^{-7}$	$(7.0 \pm 0.9) \times 10^{-7}$
F_2	0.46 ± 0.03	0.49 ± 0.01
K_2 (M)	$(1.8 \pm 0.5) \times 10^{-4}$	$(1.5 \pm 0.1) \times 10^{-4}$

[a] Ligand binding studies were done as described under Materials and Methods and in the respective figure captions. K is the dissociation constant for L-[^{3}H]QNB; F_1 and F_2 are the fractions of high- and low-affinity carbachol binding sites having dissociation constants for carbachol of K_1 and K_2, respectively.

G_i were identical with controls in the absence of ATP (supplementary material, Figure 3A,B). The results of the ligand bindng experiments for the purified and reconstituted preparations are summarized in Table II. Phosphorylation of the mAcChR also had no effect on the maximal stimulation of the GTPase activity of G_i (measured at 2 mM carbachol and 500 nM [γ-^{32}P]GTP).

In order to determine whether cAMP-dependent protein kinase also phosphorylated G_i, attempts were made to resolve the polypeptide components of the reconstituted system using 8–18% gradient SDS–polyacrylamide gels. Under these conditions, resolution between the catalytic subunit of cAMP-dependent protein kinase (39 kDa), which undergoes autophosphorylation, and α_i (41 kDa) and β_i (35 kDa) was poor. However, adequate resolution of these three polypeptides was obtained by using 10% acrylamide–SDS/8 M urea gels (Perrie et al., 1973) which were then standard, scanned with the Ambis β scanning system, and subjected to autoradiography. The results clearly showed no label incorporation into the α, β, or γ subunits of G_i, indicating that only the mAcChR was a substrate for this enzyme.

Discussion

The results presented above demonstrate that the purified porcine atrial mAcChR in detergent solution and reconstituted into lipids in the presence and absence of G_i is a substrate for cAMP-dependent protein kinase. In the detergent-solubilized preparation, the mAcChR was phosphorylated in a time-dependent manner (Figure 2) to about 0.7 mol of ^{32}P/mol of L-[^{3}H]QNB sites (Table I). Under these conditions, about 50% of the L-[^{3}H]QNB binding sites were lost, and the ligand

binding properties of the remaining binding sites appeared to be unaffected by phosphorylation (supplementary material Figure 1, Table II). The rate of phosphorylation and the final stoichiometry of phosphorylation were the same in the absence of ligands or in the presence of carbachol. Including the antagonist L-hyoscyamine reduced the stoichiometry only slightly (about 15%). In agreement with studies on mAcChR-containing synaptic membranes (Burgoyne, 1980, 1981), the in vitro studies in detergent solution indicate that it is possible to lose ligand binding activity upon phosphorylation of the protein without internalization and/or degradation occurring.

Treatment of the phosphorylated atrial mAcChR with calcineurin in the presence of calmodulin and Ca^{2+} (Figure 3A,B) resulted in dephosphorylation of the mAcChR and a reversal of the loss of L-[^{3}H]QNB binding sites, in agreement with previous results using synaptic membranes from rat brain (Ho & Wang, 1985; Ho et al., 1986). The regeneration of L-[^{3}H]QNB sites appeared to be specific for calcineurin, in that treatment with alkaline phosphatase had no effect (Figure 3A). Since it was very unlikely that phosphorylation physically occluded the ligand binding site, these results suggest that the covalent modification of the detergent-solubilized mAcChR stabilized a conformation of the protein that could not bind ligands. Dephosphorylation then permitted a return to the active conformation.

Phosphorylation of the mAcChR reconstituted into lipids resulted in a large increase in the apparent stoichiometry of phosphorylation to about 10 mol of P_i/mol of L-[^{3}H]QNB sites. A higher stoichiometry of ^{32}P incorporation upon reconstitution has also been found for the β-adrenergic receptor (Benovic et al., 1985). The reconstituted system behaved similarly to the detergent-solubilized preparation in that the ligand binding properties were unaffected by phosphorylation (Table II) and the apparent stoichiometry of phosphorylation was similar for the unliganded mAcChR and mAcChR plus carbachol. The antagonist L-hyoscyamine reduced incorporation of ^{32}P only slightly (30%). In this case, however, there was no loss of L-[^{3}H]QNB sites after treatment with cAMP-dependent protein kinase as was observed for the detergent-solubilized preparation. This result suggested either that incorporation into lipids prevented phosphorylation at the unique site or sites that resulted in the loss of the ligand binding activity, or that reconstitution stabilized mAcChR structure such that the protein could still bind ligands after it was phosphorylated.

Sequence studies on physiologically significant substrates for cAMP-dependent protein kinase showed that the phosphorylated serine and threonine residues occur in conjunction with arginine or lysine residues located from two to five positions away in the N-terminal direction (Carlson et al., 1979). Common patterns include -Arg-Arg-X-Ser- and -Lys-Arg-X-X-Ser- (Huang et al., 1979). The sequence of porcine atrial mAcChR (Kubo et al., 1986; Peralta et al., 1987) contains regions that could be targets of phosphorylation. They include Thr-386, Thr-388, Ser-213, Ser-215, Thr-136, Thr-137, Thr-56, Thr-126, Thr-130, Thr-271, Ser-282, Ser-283, Ser-320, Thr-329, Thr-331, Thr-369, and Thr-450.

In studies of the cyclic AMP dependent protein kinase catalyzed phosphorylation of native and denatured lysozyme (Bylund & Krebs, 1975), additional phosphorylation sites for the enzyme were found in the denatured protein that were not phosphorylated in the native conformation. Control experiments indicated that about 30% of the L-[^{3}H]QNB bindng sites were recovered in the reconstituted preparation, while almost all of the total protein was recovered. Thus, denatured mAcChRs with additional exposed phosphorylation sites may be present. The phosphorylation of denatured mAcChRs at these sites would tend to skew the phosphorylation stoichiometry toward higher values.

Although the stoichiometry of phosphorylation may be poorly determined in the reconstituted system due to the presence of denatured mAcChR, there are several significant conclusions. The first was that phosphorylation did not appear to alter the ligand binding properties of the mAcChR with respect to either L-[^{3}H]QNB or carbachol, either for the reconstituted mAcChR alone or for it in the presence of G_i. If this is true for the in vivo system, these results suggest that phosphorylation may serve as a signal for receptor internalization as opposed to modulating mAcChR–ligand interactions. The fact that there was no effect of mAcChR phosphorylation on the agonist-induced stimulation of the GTPase activity of G_i also supports this notion.

Second, the inability of protein kinase C to phosphorylate the mAcChR under any of the above conditions suggests that the effects of this enzyme in accelerating mAcChR internalization in neuroblastoma cells (Liles et al., 1986) may occur at sites other than the receptor itself.

Studies of the effects of protein kinase C on inositol triphosphate formation in 1321N1 astrocytoma cells (Orellana et al., 1987) showed that treatment with 4β-phorbol 12β-myristate 13α-acetate reduced inositol trisphosphate formation induced by GTPγS plus carbachol but did not affect carbachol binding to the mAcChR or the effect of GTP on agonist binding. Those authors suggested that the effect of protein kinase C on inositol phosphate metabolism occurred at a site distal to the receptor, possibly inhibition of G protein interaction with phospholipase C. Preliminary data obtained during the course of these experiments indicated that treatment of the reconstituted mAcChR plus G_i with protein kinase C results in the phosphorylation of the γ subunit of G_i. Whether this modulates coupling of G_i to other signaling systems in vivo has not yet been determined.

Finally, the experiments with the reconstituted system suggest that carbachol-stimulated phosphorylation of the mAcChR occurs only in the presence of G_i. This observation strongly suggests that mAcChR association with G_i either transiently or in a binary complex induces a conformational change in the protein which exposes additional sites for phosphorylation by cAMP-dependent protein kinase.

In summary, these studies show that purified and reconstituted porcine atrial mAcChR preparations are in vitro substrates for cAMP-dependent protein kinase, but not myosin light-chain kinase, phosphorylase kinase, cGMP-dependent protein kinase, or protein kinase C. The ligand binding properties of the mAcChR and its ability to stimulate the GTPase activity of G_i were not affected by phosphorylation. Agonist-stimulated phosphorylation of the mAcChR was found only in the presence of G_i, although determination of accurate stoichiometries of phosphorylation was serverly hampered by the necessity of determining extremely low protein concentrations accurately in the presence of lipid.

Studies such as these provide an opportunity to directly measure events such as receptor phosphorylation in a relatively well-defined system containing homogeneous components. However, there are several potential artifacts arising in part from the high concentrations of kinase used (0.4 μM), differing lipid compositions for the in vitro experiments compared to heart cell membranes, and the absence of other components that may be part of the muscarinic signal-transducing system in vivo. Alternatively, it is not yet known whether a

mAcChR-specific kinase exists similar to that found for the β-adrenergic receptor (Benovic et al., 1986).

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SUPPLEMENTARY MATERIAL AVAILABLE

Figures quantitating the ligand binding properties of the phosphorylated and nonphosphorylated mAcChR in detergent and reconstituted in the presence and absence of G_i (10 pages). Ordering information is given on any current masthead page.

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