0026-895X/89/050553-06$02.00/0

MOLECULAR PHARMACOLOGY, 35:553-558

ACCELERATED COMMUNICATION

The Porcine Heart M2 Muscarinic Receptor: Agonist-Induced Phosphorylation and Comparison of Properties with the Chick Heart Receptor

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Received November 7, 1988; Accepted February 6, 1989

SUMMARY

Recently we showed that the chick heart muscarinic acetylcholine receptor is a phosphoprotein in intact cells and that treatment with agonists results in a striking increase in receptor phosphorylation [J. Biol. Chem. **261**:12429–12432 (1986)]. Furthermore, we showed that the agonist-induced increase in the phosphorylation of chick heart muscarinic receptors correlates with receptor desensitization [*J. Biol. Chem.* **262**:16314–16321 (1987)]. We have now extended studies of receptor phosphorylation to mammalian cardiac muscarinic receptors, in order to test the concept that phosphorylation is of general importance in the regulation of muscarinic receptor function. We have determined that, in intact porcine atria, M2 muscarinic receptors are phosphoproteins and that treatment with the agonist carbachol markedly increases receptor phosphorylation, to 4–6 mol of phosphate/mol of protein. Phosphorylation occurs on serine and threonine residues. Activation of either protein kinase C or cAMP-dependent protein kinase did not mimic the effect of agonists on receptor phosphorylation. These results are very similar to those seen with the chick heart muscarinic receptors. To determine whether the porcine and the chick cardiac muscarinic receptors represent similar or different proteins, we undertook detailed pharmacological studies and, in addition, prepared peptide maps of purified muscarinic receptors from chick heart and porcine atria. Our data show that there are marked differences in the pharmacological properties of the chick and the porcine cardiac muscarinic receptors. The peptide maps of the porcine and chick heart muscarinic receptors are also different, suggesting that muscarinic receptors in chick and porcine cardiac cells differ in their primary structure. Taken together, the data show that porcine and chick cardiac muscarinic receptors possess pharmacological and structural differences, but both receptors undergo agonist-mediated phosphorylation in intact cardiac cells. These data support the possibility that receptor phosphorylation may be of general importance in the regulation of muscarinic receptors.

mAChR are activated by the neurotransmitter acetylcholine and are present in many excitable cells. In the heart, activation of mAChR results in attenuation of the force and rate of contraction. Cardiac mAChR are coupled to a variety of effector systems: the inhibition of adenylate cyclase (1, 2), increased hydrolysis of membrane polyphosphoinositides (3, 4), activation of potassium channels (5–7), and stimulation of cGMP synthesis (8).

It is well established that there are subtypes of mAChR. Initially, two subtypes, termed M1 and M2, were proposed based on the differential sensitivity of receptors to the antagonist pirenzepine (9). M1 receptors were defined as those possessing a high affinity for pirenzepine and were found to be enriched in the cerebral cortex. M2 receptors, on the other hand, were defined as those receptors that exhibited a lower affinity for pirenzepine and were found to be mainly localized in peripheral organs and in some areas of the brain. Most mammalian cardiac mAChR appear to be of the M2 subtype. Recently, a new muscarinic antagonist, AFDX-116, has been described (10). This compound is a selective antagonist of cardiac mAChR. Studies with this and other drugs suggested that the M2 receptors may be heterogeneous and that more than two subtypes of mAChR may exist (11, 12). Indeed, molecular cloning studies have identified five distinct mAChR, denoted as M1, M2, M3, M4, and M5 (13–19; using classification of Ref. 16). The receptors corresponding to the cloned and expressed M1 and M2 subtypes display a pharmacological profile that is similar to the pharmacologically defined M1 and M2 subtypes, respectively, whereas the M3, M4, and M5 sub-

This work was supported by National Institutes of Health Grant HL31601. M.M.K. was supported by a Senior Research Fellowship of the American Heart Association of Metropolitan Chicago, and M.M.H. was supported by an Established Investigatorship from the American Heart Association during the tenure of this work.

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptors; QNB, quinuclidinyl benzilate; PMA, phorbol 12-myristate 13-acetate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

types have intermediate properties (13, 15–19). The possibility that different receptor subtypes couple to different effector systems has been substantiated by the recent demonstration that the expressed M1, M4, and M5 subtypes preferentially couple to hydrolysis of polyphosphoinositides, whereas the M2 and M3 subtypes preferentially couple to attenuation of adenylate cyclase (15, 17, 18, 20–22).

In previous studies we showed that the mAChR present in chick heart is a phosphoprotein in intact cells and that its phosphorylation is markedly increased by treatment with the agonist carbachol (23). Furthermore, we have shown that the agonist-induced increase in the phosphorylation of chick heart mAChR appears to correlate with receptor desensitization (24). In order to test the possibility that mAChR phosphorylation is of general importance, it is necessary to determine whether each receptor subtype undergoes agonist-mediated phosphorylation. Because it was suggested earlier that the chick heart mAChR may not be an M2 subtype (4), as a first approach we have determined whether mammalian cardiac mAChR, which are the prototypical M2 receptors, undergo agonist-induced phosphorylation in intact tissue. These studies were performed with porcine atrial M2 mAChR. The primary amino acid sequence of this receptor has been deduced from cDNA cloning studies (14, 19). We have found that this mAChR, like the chick heart mAChR, existed as a phosphoprotein in intact cells and that its phosphorylation was strikingly increased by treatment of the atrial preparations with the agonist carbachol. To determine whether the chick and the porcine heart mAChR were similar or different proteins, peptide maps were generated and detailed pharmacological studies were performed. The results showed that the porcine and the chick heart mAChR are structurally and pharmacologically distinct and that both are regulated *in vivo* by phosphorylation.

Experimental Procedures

Materials. Newborn chicks were obtained from Corn Belt Hatcheries (Forrest, IL). Fresh atria from young Minnesota miniature swine were kindly supplied by Dr. Y. B. Kim (Department of Microbiology, Chicago Medical School). Inorganic ^{32}P, $Na^{125}I$, and [^{3}H]QNB were purchased from Amersham (Arlington Heights, IL). Oxotremorine was purchased from K & K Laboratories, Inc. (Plainview, NY). McN-A343 was from Research Biochemicals, Inc. (Wayland, MA). AHR-602 was kindly supplied by Dr. L. Sancilio (A. H. Robins Co., Richmond, VA). Pirenzepine and AFDX-116 were generously supplied by Boehringer Ingelheim (Ridgefield, CT). *Staphylococcus aureus* V8 protease, chymotrypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin, subtilisin, PMA, carbamylcholine chloride (carbachol), and isoproterenol were purchased from Sigma Chemical Company (St. Louis, MO). Forskolin was from Calbiochem (San Diego, CA). Wheat germ agglutinin-Sepharose 6MB was prepared according to protocol provided by Pharmacia Biotechnology, Inc. (Piscataway, NJ). The mAChR affinity ligand 3-(2′-aminobenzhydryloxy)tropane was synthesized and coupled to epoxy-activated Sepharose (Pharmacia Biotechnology) as described by Haga and Haga (25, 26).

***In situ* phosphorylation studies.** Fresh porcine atria were trimmed of fat and vessels and sliced using a Stadie-Riggs tissue slicer. The slices were incubated in oxygenated Tyrode's solution, which contained 0.4 to 0.6 Ci/ml of ^{32}P, at 37° for 75–90 min. After this incubation period, the slices were washed twice with the Tyrode's solution and were divided into different groups for drug treatments. After the drug treatment, the tissue was further processed and the mAChR were purified as described earlier (23, 24). Equal amounts of purified receptors, as determined by [^{3}H]QNB binding, from control and drug-treated groups were electrophoresed on 8.5% sodium dodecyl sulfate-polyacrylamide gels. The phosphorylated receptor protein was visualized by silver staining and autoradiography. The stoichiometry of phosphorylation was determined as previously described (23, 24). Phosphoamino acid analysis was performed as in Ref. 27.

Iodination of the chick and the porcine mAChR and peptide mapping. Chick and porcine cardiac mAChR were purified (23) and iodinated using $Na^{125}I$ and chloramine T (26). These ^{125}I-labeled receptors were used to generate peptide maps according to the method of Cleveland *et al.* (28) utilizing sodium dodecyl sulfate gels containing a gradient of 7–17% polyacrylamide.

Membrane isolation and receptor binding assays. Chick heart ventricles or porcine atria were homogenized (Polytron PT-20 probe, setting 5, with two sec bursts) in 5 volumes of buffer A (20 mM potassium phosphate, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.25 mM sucrose, pH 7.0) that contained the following protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin, 1 μM pepstatin, and 2 μg/ml leupeptin. In the case of porcine atria, the homogenate was filtered through cheesecloth before centrifugation. The homogenates were centrifuged at 30,000 × *g* for 30 min, the pellets were resuspended in buffer B (buffer A without sucrose), and crude membranes were prepared (29). These membranes were used in ligand-binding assays, which were performed with [^{3}H]QNB and various competing ligands (29) in a reaction (2 ml) containing 10 mM $NaKPO_4$, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 85–100 pM [^{3}H]QNB, with or without 0.1 mM Gpp(NH)p where indicated. The chick and porcine membranes were present at protein concentrations of 125–150 μg and 200–300 μg; respectively. All drug solutions, except AFDX-116, were prepared in 10 mM $NaKPO_4$ buffer, pH 7.4. A 10 mM stock solution of AFDX-116 was prepared in 0.05 N HCl, which was diluted with 10 mM $NaKPO_4$, pH 7.4, to obtain the desired final concentrations.

Results

Phosphorylation of porcine atrial mAChR in intact tissue. In order to determine whether the porcine cardiac mAChR undergo agonist-mediated phosphorylation in cardiac cells, porcine atrial mAChR were purified by affinity chromatography from control or carbachol-treated (0.1 mM) tissue that had been bathed in a ^{32}P-containing physiological salt solution. As was the case for our previous studies with the chick heart, analysis of the receptor eluted from the affinity column with atropine demonstrated the presence of a single major phosphopeptide at the position of the receptor peptide of ~80 kDa (Fig. 1). The results of these studies showed that the M2 mAChR is a phosphoprotein in intact porcine atrial tissue. More importantly, the phosphorylation of the receptor was markedly increased in the intact cells by exposure of the tissue to the agonist carbachol (Fig. 1). (No other phosphoproteins eluted from the affinity gel for both control and carbachol-treated preparations.) Based on the ^{32}P content of the excised gel bands containing the receptor protein, the agonist treatment led to a 5–6-fold increase in the phosphorylation of the receptor. Calculation of the stoichiometry of phosphorylation showed that agonist treatment resulted in the incorporation of 4.2–5.8 mol of phosphate/mol of protein (two experiments). The present results with the porcine cardiac mAChR are very similar to our previous results with the chick heart mAChR (23, 24). Thus, both the chick and the porcine cardiac mAChR are phosphoproteins in intact tissue and both of these receptors display a striking increase in their phosphorylation to 4–6 mol of phosphate/mol of receptor, upon agonist treatment *in situ*.

The identify of phosphorylated amino acid(s) in the porcine atrial mAChR was determined. It was found that the agonist-

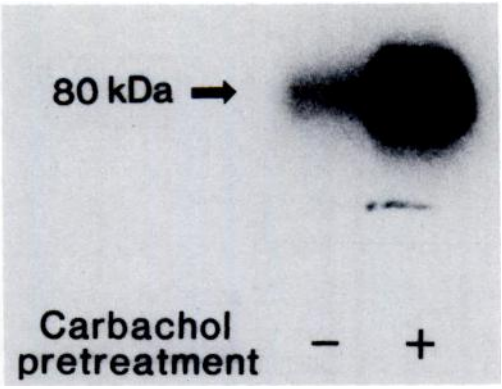

Fig. 1. Autoradiogram of purified porcine atrial muscarinic receptor showing the effect of carbachol on its phosphorylation in intact tissue. ^{32}P-labeled porcine atrial slices were treated with or without 0.1 mM carbachol for 10 min. After agonist treatment, membranes were prepared and the receptor was purified as described in the text. Equal amounts of receptors, as measured by [^{3}H]QNB binding, were applied to each lane of an 8.5% sodium dodecyl sulfate-polyacrylamide gel. Shown is the resulting autoradiogram depicting the 80-kDa phosphorylated receptors. Direct counting of the gel pieces containing the receptor showed that the control sample contained 60 cpm whereas the carbachol-treated sample contained 300 cpm. The stoichiometry of phosphorylation for the carbachol-stimulated phosphorylation was calculated to be 4–6 mol of phosphate/mol of receptor. Similar agonist-induced increases in receptor phosphorylation were observed in five separate experiments.

induced phosphorylation occurred on serine and threonine residues (data not shown). These amino acids were also found to be phosphorylated in the case of agonist-induced phosphorylation of the chick heart mAChR (24).

Nature of the protein kinase(s) involved in the agonist-induced phosphorylation of porcine atrial mAChR. Activation of cardiac mAChR results in attenuation of adenylate cyclase and increased hydrolysis of membrane phosphoinositides. Because the latter reaction would lead to formation of diacylglycerols, which are activators of protein kinase C, one consequence of stimulation of mAChR may be the activation of protein kinase C. To determine whether the agonist-induced phosphorylation of porcine atrial M2 mAChR was mediated via protein kinase C, the effect of PMA, a tumor-promoting phorbol ester and a potent activator of protein kinase C, on the phosphorylation of mAChR was studied. It was found that the treatment of porcine atrial slices with 100 nM PMA for 10 min had no effect on the phosphorylation of mAChR (data not shown). These results indicated that protein kinase C was probably not involved in the observed agonist-induced phosphorylation of porcine atrial mAChR.

Recently, it was shown that the porcine atrial mAChR could be phosphorylated *in vitro* by cAMP-dependent protein kinase (30). To determine whether the porcine atrial mAChR was a substrate for the kinase in *intact cells*, the intracellular levels of cAMP were increased by treating atrial tissue with the β-adrenergic agonist isoproterenol (10 μM) and the direct adenylate cyclase activator forskolin (50 μM) for 10 min. This treatment had no effect on the phosphorylation of mAChR (data not shown). That this treatment resulted in the activation of cAMP-dependent protein kinase was confirmed by determining that the treatment led to an increase in the phosphorylation of a known substrate of this enzyme, troponin I (data not shown). Thus, activation of cAMP-dependent protein kinase did not result in the phosphorylation of mAChR in intact porcine atrial tissue.

Are porcine atrial and chick ventricular mAChR similar or different proteins? Although the chick and the porcine cardiac mAChR behave in a similar fashion with respect to their phosphorylation in intact tissue, these two receptors differ in their sensitivity to the muscarinic antagonist pirenzepine. The chick heart mAChR has a high affinity for pirenzepine (4), a behavior typical of an M1 receptor, whereas the porcine atrial mAChR has a lower affinity for pirenzepine and is a prototypical M2 subtype (14, 19). These differences are likely not due to the chick receptor originating from ventricular tissue, and the porcine receptor from atrial tissue, because no significant differences have been detected in atrial and ventricular receptors isolated from the same species (31, 32). Given the differences in the sensitivity of the chick and the porcine cardiac mAChR to pirenzepine, it was of interest to further compare the properties of these two receptors, in order to determine whether they represent similar or different proteins. To address this question, we undertook a more extensive pharmacological evaluation of these receptors and, in addition, we compared the peptide maps of the purified receptors.

Pharmacology of the chick and the porcine cardiac mAChR. Competition curves were generated using the antagonist [^{3}H]QNB and various muscarinic agonists and antagonists with the chick ventricular and porcine atrial membranes (Fig. 2; Table 1). The prototypical muscarinic antagonist atropine had similar affinities for both receptors, whereas, as ex-

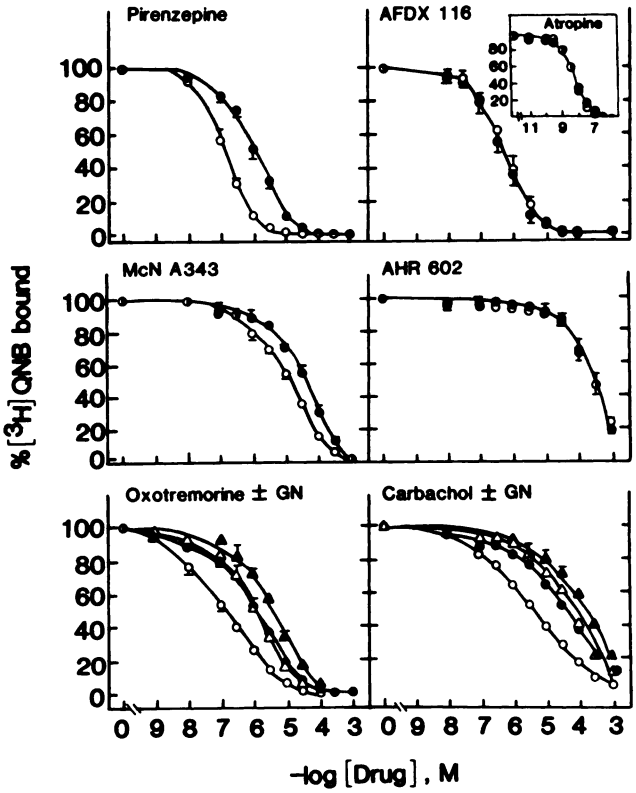

Fig. 2. Competition of agonists and antagonists at muscarinic receptors in chick ventricular and porcine atrial membranes. Assays were performed using [^{3}H]QNB as the radioligand, as described in the text. *Data points* shown are the means of three to five experiments performed in duplicate. *Bars* indicate the standard error. The effect of Gpp(NH)p (*GN*) on the affinity of carbachol and oxotremorine for the chick (*open triangles*) and the porcine (*closed triangles*) mAChR were also determined. *Open symbols*, chick heart membranes; *closed symbols*, porcine atrial membranes. Analysis of the data is contained in Table 1.

TABLE 1

Pharmacological profile of chick and porcine cardiac muscarinic receptors as assessed by [^{3}H]QNB/ligand competition studies

Values are mean ± standard error.

	Chick		Porcine	
	IC_{50}	K_i	IC_{50}	K_i
		nM		
Atropine	4.63 ± 0.6	1.04 ± 0.09	4.56 ± 0.4	1.56 ± 0.01
Pirenzepine	140 ± 29	30.2 ± 4.3	1,100 ± 260	357 ± 60[a]
AFDX-116	430 ± 140	96 ± 18	360 ± 102	184 ± 63
McN A343	13,700 ± 380	3,200 ± 800	39,100 ± 540	14,000 ± 2,000[a]
AHR 602	240,000 ± 50,000	45,000 ± 11,000	220,000 ± 50,000	69,000 ± 12,000
Oxotremorine	198 ± 38	K_1 = 19 ± 16 (65 ± 19%)[b] K_2 = 806 ± 390 (35 ± 16%)	1,190 ± 220[c]	K_1 = 43 ± 23 (40 ± 19%) K_2 = 1730 ± 590 (60 ± 19%)
Oxotremorine + Gpp(NH)p	1,280 ± 210	450 ± 50	3,950 ± 660	1,890 ± 206[d]
Carbachol	4,240 ± 1,180	K_1 = 1,700 ± 430 (52 ± 8%) K_2 = 13,600 ± 2900 (48 ± 4%)	33,000 ± 5,100[c]	K_1 = 4690 ± 850 (57 ± 3%) K_2 = 192,000 ± 21,000 (43 ± 3%)
Carbachol + Gpp(NH)p	57,000 ± 16,400	K_1 = 950 ± 580 (27 ± 6%) K_2 = 34,900 ± 6,300 (73 ± 6%)	176,000 ± 55,000[a]	K_1 = 4,460 ± 400 (28 ± 8%) K_2 = 166,000 ± 29,000 (72 ± 8%)

[a] Significantly different from chick, $p < 0.01$.
[b] K_1 and K_2 refer to K_i values of different affinity states as determined by curve fitting with the LIGAND program. Numbers in parentheses refer to percentage of receptors exhibiting the respective affinity.
[c] Significantly different from chick, $p < 0.001$.
[d] Significantly different from chick, $p < 0.005$.
[e] Significantly different from chick, $p < 0.05$.

pected, the selective antagonist pirenzepine had a higher affinity for the chick than for the porcine receptor (Fig. 2; Table 1). These results are in agreement with the results of Brown *et al.* (4), who originally suggested that the chick heart receptor was more like an M1 than M2 receptor. The cardioselective antagonist AFDX-116 (10), on the other hand, exhibited similar affinities for the chick and porcine cardiac mAChR (Fig. 2; Table 1). The behavior of two different partial agonists, McN A343 and AHR 602, which have been proposed to be M1 selective (see Refs. 33 and 34), toward these two receptors was also studied. AHR 602 had similar affinities for the chick and the porcine cardiac receptors, but the agonist McN A343 displayed a higher affinity for the chick receptor (Fig. 2; Table 1).

The affinities of the classical agonists carbachol and oxotremorine for these two receptors were also determined. The IC_{50} values for both agonists were significantly lower (higher apparent affinity) for the chick than for the porcine cardiac mAChR (Fig. 2; Table 1). Agonists are known to bind to multiple affinity states of mAChR, therefore, the competition curves for carbachol and oxotremorine were analyzed with the curve-fitting program LIGAND (35). The results of this analysis (Table 1) indicated that oxotremorine and carbachol bound to two affinity states of the chick and porcine receptors. In each case the calculated affinities were approximately 2–4-fold more favorable for the chick than for the porcine receptors. In addition, for oxotremorine, the percentage of receptors exhibiting the higher affinity was larger in the chick than the porcine preparation (Table 1). Taken together, these results would explain the higher apparent affinity of the chick receptors for agonists, as seen by the competition curves.

The expression of the different receptor affinity states can be modulated by guanine nucleotides, which through binding to GTP-binding proteins convert high affinity states into lower affinity states for agonists (see Refs. 29, 31, 32, and 36). To determine whether the observed differences in the binding of agonists by the chick and the porcine mAChR were due to a differential interaction of the receptors with their GTP-binding proteins, we studied the effect of Gpp(NH)p, a hydrolysis-resistant analog of GTP, on the affinities of carbachol and oxotremorine for the chick and the porcine cardiac mAChR (Fig. 2; Table I). The results showed that Gpp(NH)p, as expected (29), increased the proportion of receptors in the lower affinity states, but this treatment did not abolish the differences in the affinities of the agonists for the chick and the porcine receptors. In the case of oxotremorine, only one affinity state was observed after the Gpp(NH)p treatment, and the K_i values for the chick receptor were 4-fold lower than those observed for the porcine receptors (Table 1). Thus, even after conversion of receptors into a single affinity state, significant differences in the affinity of the chick and porcine cardiac receptors for agonists can be discerned.

Peptide maps of purified chick and porcine cardiac mAChR. The basis of the observed pharmacological differences between the chick and the porcine receptor is not known. To discern whether there are any differences in the primary structure of these two receptors, we prepared peptide maps (28) of the purified iodinated chick and porcine cardiac mAChR. To generate these maps, three different proteases, *S. aureus* V8 protease, subtilisin, and chymotrypsin, were used. In addition, each protease was used at three different concentrations. The results showed that the peptide maps of the chick and the porcine mAChR obtained with each of these proteases were different (Fig. 3). These results indicated that the chick and the porcine cardiac mAChR contain differences in their primary structures.

Discussion

In the present study we have shown that a mammalian (porcine) cardiac mAChR, like the chick heart mAChR (23, 24), was found to be a phosphoprotein in intact cells and that treatment with the agonist carbachol led to a striking increase in the phosphorylation of the receptor, to the level of 4–6 mol of phosphate/mol of receptor. The present results extend our knowledge of mAChR phosphorylation in two ways. First, the results show that agonist-induced phosphorylation of mAChR occurs in mammalian cardiac cells and is, thus, not limited to avian receptors. Second, because the results with the porcine cardiac mAChR were obtained in atrial tissue whereas the

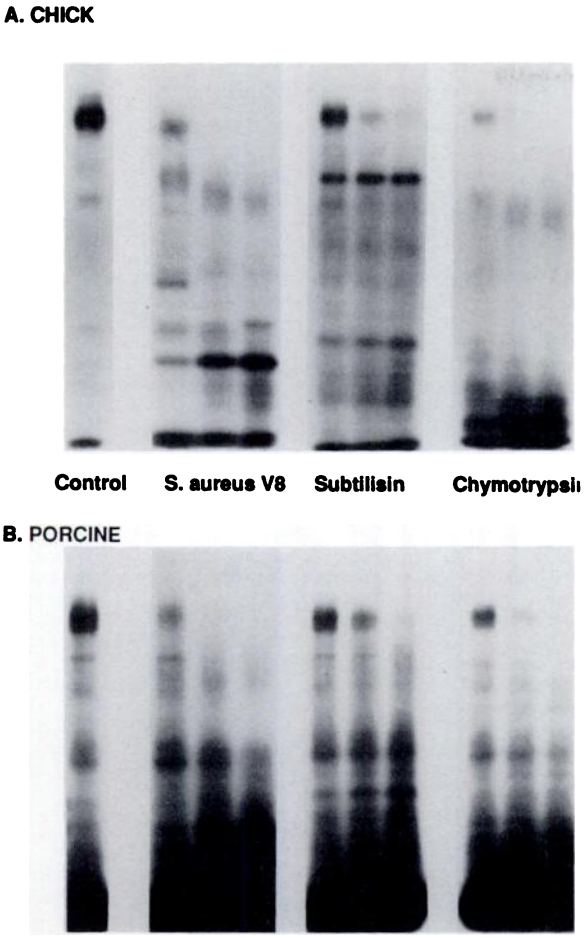

Fig. 3. Peptide maps of the purified chick (A) and porcine (B) cardiac mAChR. Purified and iodinated mAChR were subjected to limited proteolysis (22) with the proteases indicated. Each protease was used at three different concentrations and these are, from *left* to *right*; *S. aureus* V8 protease, 0.01, 0.1, and 1 μg; subtilisin, 0.01, 0.1, and 1 ng; chymotrypsin, 5, 25, and 50 μg. The ^{125}I-labeled receptors were added to the protease solutions and the mixtures were loaded onto 7–17% sodium dodecyl sulfate-polyacrylamide gels and electrophoresed. Shown is an autoradiogram depicting the peptide fragments generated.

previous results (23, 24) were obtained with mAChR derived from ventricular tissue, the data demonstrate that mAChR phosphorylation occurs in both atrial and ventricular tissue. This is of interest because mAChR in atria and ventricles are coupled to different physiological responses. In atria, mAChR primarily regulate K^+ currents (5–7) and, in doing so, induce negative chronotropic effects. In ventricles, the mAChR primarily regulate voltage-dependent Ca^{2+} currents through a mechanism believed to involve the attenuation of adenylate cyclase and dephosphorylation of Ca channels (37). Although previous studies have been unable to determine that mAChR in atria and ventricles are different proteins (31, 32), the present results demonstrate that both atrial and ventricular mAChR are subject to agonist-induced phosphorylation *in vivo*.

Limited experiments aimed at discerning the identity of the kinase responsible for the phosphorylation of mAChR indicated that in intact tissue the porcine atrial mAChR is not a substrate for protein kinase C or cAMP-dependent kinase. One would expect the latter result, because the activation of cardiac mAChR results in decreased levels of cAMP and would not be expected to cause activation of cAMP-dependent protein kinase (1, 2). Hence, although the porcine atrial mAChR was shown to be highly phosphorylated by cAMP-dependent protein kinase *in vitro* (30), this does not appear to be the case in the intact cell. Accordingly, phosphorylation by cAMP-dependent protein kinase is not implicated in the mechanism of agonist-induced phosphorylation of this receptor in intact tissue. The involvement of protein kinase C in the regulation of mAChR has been suggested by a number of workers (38, 39). However, activation of protein kinase C with the tumor-promoting phorbol ester PMA had no effect on the phosphorylation of porcine atrial mAChR in intact tissue. In support of this conclusion is a recent observation (30) that the purified porcine atrial mAChR was not found to be a substrate for protein kinase C *in vitro*. Thus, the kinase responsible for phosphorylation of the porcine atrial mAChR in the cardiac cell remains to be identified. In the case of the chick heart mAChR, we showed previously that this receptor did not appear to be a substrate for either cAMP- or cGMP-dependent protein kinases, protein kinase C, or a Ca/calmodulin-dependent protein kinase *in situ* (24), and we had suggested that a receptor-specific kinase, as has been shown to be the case with the β-adrenergic receptor (40), may catalyze the phosphorylation of the receptor. Because our results on the phosphorylation of the porcine atrial mAChR are similar to those seen with the chick heart mAChR, it is possible that a receptor-specific kinase may also be responsible for the agonist-induced *in situ* phosphorylation of the porcine atrial mAChR.

Because it is now recognized that there are multiple subtypes of mAChR, in studying the phosphorylation of the receptors it is important to identify the receptor subtype. Earlier studies suggested that the chick cardiac mAChR may not be of the M2 subtype (4), although most other cardiac mAChR are classified as M2 receptors (see Refs. 9, 14, 19, 33, and 34). Therefore, it was of interest to determine whether the porcine and the chick cardiac mAChR were similar or different proteins. Detailed pharmacological evaluation of the chick and porcine cardiac mAChR revealed substantial differences in the interaction of these two receptors with various agonists, partial agonists, and selective antagonists. The peptide maps derived from the chick and the porcine cardiac mAChR were also different. Studies of the molecular cloning of cDNAs for the human, porcine, and rat M2 mAChR have shown that there is a high degree of homology (98%) across these species (14–16, 18, 19). This observation implies that the mAChR were highly conserved during evolution. However, the marked differences observed in the pharmacology and peptide maps of the chick and the porcine cardiac mAChR suggest either that these are different receptor subtypes or that they may both be M2 receptors but with a lower degree of homology than is found for mammalian M2 receptors.

In summary, we have shown that a mammalian M2 mAChR was found to be a phosphoprotein in intact cardiac atrial cells and that its phosphorylation was strikingly increased by treatment of the tissue with the agonist carbachol. In the intact cell, the porcine atrial mAChR does not appear to be not a substrate for either protein kinase C or for the cAMP-dependent protein kinase. These results are similar to our previous observations on the chick heart mAChR (23, 24). The porcine and the chick heart mAChR behave differently in pharmacological assays and appear to be structurally different, based on peptide mapping. The results suggest that, although these two receptors appear

to be different proteins, both appear to be regulated in cardiac cells by agonist-induced phosphorylation. These results are consistent with the possibility that phosphorylation of mAChR may be of general importance in the regulation of mAChR function.

Acknowledgments

We thank Dr. E. Leung (University of Illinois) for performing the LIGAND analyses of the data shown in Table 1.

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