# Agonist-Independent Phosphorylation of Purified Cardiac Muscarinic Cholinergic Receptors by Protein Kinase C†

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ABSTRACT: The results of several studies have suggested that muscarinic cholinergic receptors (mAChR) may be regulated by multiple pathways involving phosphorylation of the receptors. Previous studies have demonstrated that chick heart mAChR are phosphorylated by the β-adrenergic receptor kinase (β-AR kinase) in an agonist-dependent manner, and it has been suggested that this process may be linked to receptor desensitization. In this work, we present evidence that protein kinase C can phosphorylate the purified, reconstituted chick heart mAChR and can modify the interaction of the receptors with GTP binding proteins (G-proteins) that couple the receptors to effectors. Phosphorylation of the mAChR with protein kinase C occurred to an extent of ~5 mol of P/mol of receptor. Neither the rate nor the extent of the protein kinase C mediated phosphorylation of mAChR was agonist-dependent. Under the conditions tested, the initial rate of phosphorylation of the mAChR by protein kinase C was significantly more rapid than that obtained with the β-AR kinase. At equilibrium, phosphorylation of mAChR by protein kinase C and β-AR kinase was partially additive. The functional effects of protein kinase C mediated phosphorylation of the mAChR were assessed by comparing the abilities of purified G-proteins ($G_i$ and $G_o$) to reconstitute high-affinity agonist binding to phosphorylated and nonphosphorylated receptors. A significantly larger percentage of the receptors phosphorylated with protein kinase C exhibited G-protein-dependent high-affinity agonist binding, suggeting that phosphorylation of the receptors by protein kinase C modulates receptor function in a positive manner. As mAChR may activate protein kinase C through hydrolysis of polyphosphoinositides, these results suggest that protein kinase C may also participate in the regulation of mAChR function by phosphorylation.

Muscarinic acetylcholine receptors (mAChR) mediate several responses such as inhibition of adenylyl cyclase (Murad et al., 1962; Jacobs et al., 1979), hydrolysis of polyphosphoinositides (Brown & Brown, 1984; Brown et al., 1985), changes in ion permeability (Giles & Noble, 1976; Breitwieser & Szabo, 1985; Pfaffinger et al., 1985; Codina et al., 1987), and increases in intracellular cGMP (George et al., 1970; McKinney & Richelson, 1984). These responses are mediated through the interaction of the receptors with different guanine nucleotide binding proteins (G-proteins) (Nathanson, 1987). Muscarinic receptors possess topological and amino acid similarities with other G-protein-coupled receptors such as the β-adrenergic receptors, α-adrenergic receptors, and rhodopsin (Kubo et al., 1986; Peralta et al., 1987; Bonner et al., 1987; Applebury & Hargrave, 1987; Dohlman et al., 1987).

A property of this family of receptors is that many of the G-protein-coupled receptors appear to be regulated by protein phosphorylation (Sibley et al., 1987; Huganir & Greengard, 1987). The structurally related α- and β-adrenergic receptors (Leeb-Lundberg et al., 1985; Strasser et al., 1986; Benovic et al., 1986, 1987a) and rhodopsin (Applebury & Hargrave, 1987) undergo phosphorylation by one or more protein kinases, and phosphorylation appears to regulate receptor function. Novel protein kinases, referred to as rhodopsin kinase and the β-AR kinase (Schichi & Sommers, 1978; Benovic et al., 1986), have been identified and found to phosphorylate their respective substrates in a relatively specific and agonist-dependent manner.

With regard to mAChR, it has been shown that stimulation of avian and porcine cardiac muscarinic receptors by an agonist in situ results in a marked increase in the phosphorylation of the receptors on serine and threonine residues (Kwatra & Hosey, 1986; Kwatra et al., 1989a). This process has been suggested to be associated with the process of desensitization (Kwatra et al., 1987). In vitro studies have shown that β-AR kinase phosphorylates mAChR in an agonist-dependent manner (Kwatra et al., 1989b). These latter results suggested that the mechanism of phosphorylation, and perhaps desensitization, of adrenergic and muscarinic receptors might follow similar pathways. However, several studies have demonstrated that other, less specific mechanisms of phosphorylation may also be involved in the regulation of mAChR (Burgoyne, 1980, 1981, 1983; Ho & Wang, 1985; Liles et al., 1986). In particular, several lines of evidence have implicated protein kinase C in the regulation of mAChR function (Vincentini et al., 1985; Orellana et al., 1985; Lai & El-Fakahany, 1987; Liles et al., 1986; Conklin et al., 1988). Phorbol esters that activate protein kinase C induce down-regulation of mAChR in neuroblastoma cells (Feigenbaum & El-Fakahany, 1985; Liles et al., 1986) and inhibit mAChR-mediated increases in the hydrolysis of polyphosphoinositides (Orellana et al., 1985; Conklin et al., 1988) and cGMP (Lai & El-Fakahany, 1987). As these results suggest that protein kinase C may play an important role in the regulation of the mAChR, we have determined whether muscarinic receptors are substrates for phosphorylation by protein kinase C. In this work, we have studied the phosphorylation and regulation of chick heart muscarinic receptors by protein kinase C.

## Experimental Procedures

*Materials.* The materials used for the purification of chick heart mAChR were obtained from the sources described previously (Kwatra & Hosey, 1986). TPCK-treated trypsin,

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carbachol, heparin, and deoxycholic and cholic acids were purchased from Sigma Chemical Co., St. Louis, MO. [$^3$H]-QNB and [$\gamma$-$^{32}$P]ATP were from Amersham, Arlington Heights, IL. $\beta$-AR kinase was purified from bovine cerebral cortex with minor modifications of a previously described procedure (Benovic et al., 1987b) and was generously supplied by Drs. Jeffrey Benovic and Robert Lefkowitz, Duke University. The specific activity of the purified enzyme was ~50–80 nmol of $P_i$ min$^{-1}$ (mg of protein)$^{-1}$ (Benovic et al., 1987b), and the preparation appeared to be free of contaminating protein kinases, as it did not phosphorylate basic substrates such as caseins and histones which are commonly used as substrates for protein kinase C, cyclic nucleotide, and $Ca^{2+}$-dependent protein kinases.

*Purification of mAChR and Protein Kinase C.* Muscarinic receptors were purified from chick ventricles by using the muscarinic receptor affinity column (Haga & Haga, 1983; Kwatra & Hosey, 1986) and were reconstituted into phospholipid vesicles as previously described (Kwatra et al., 1989b). The specific activity of the purified receptors, measured with the muscarinic antagonist [$^3$H]quinuclidinyl benzilate (QNB), was ~1 nmol of [$^3$H]QNB bound/mg of protein (Kwatra & Hosey, 1986). Protein kinase C was purified from chick brain according to the procedure of Woodgett and Hunter (1987). This procedure results in the isolation of a doublet of 78–80 kDa and consists of at least two, and probably minimally the $\alpha$, $\beta$, and $\gamma$, isoforms of protein kinase C that cannot be distinguished by molecular weight on SDS gels (Nishizuka, 1988). The preparation was judged to be free of contaminating proteins by silver staining after SDS gel electrophoresis, was highly dependent on calcium, and was insensitive to cyclic nucleotides and calmodulin.

*Phosphorylation of mAChR by Protein Kinase C and $\beta$-AR Kinase.* For in vitro phosphorylation of the purified mAChR by protein kinase C, the reaction mixtures (50 $\mu$L) consisted of 10–15 $\mu$L of reconstituted receptor (0.2–1.0 pmol), 5 mM $CaCl_2$, 0.1 mM [$\gamma$-$^{32}$P]ATP (1–4 cpm/fmol), 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$, ±1 mM carbachol, and 5 $\mu$L of protein kinase C (0.1 nM). The reactions were started by adding ATP and were incubated at 30 °C for the times indicated. The reactions were stopped by adding 25 $\mu$L of SDS sample buffer and were then electrophoresed on SDS gels containing 8.0% polyacrylamide (Laemmli, 1970). Phosphorylation of the receptors was visualized by autoradiography and quantified by scintillation counting of the receptor bands excised from the dried gels. Stoichiometries of phosphorylation were calculated from the amount of receptor loaded onto each gel lane and the specific activity of the ATP. The same procedure was used for the phosphorylation of the mAChR by $\beta$-AR kinase, except $CaCl_2$ was omitted and the protein kinase C was replaced by 5 $\mu$L of $\beta$-AR kinase (50 ng). Phosphoamino acid and phosphopeptide mapping determinations were performed as previously described (Kwatra et al., 1987; O'Callahan et al., 1988).

*Purification of G-Proteins.* The purification of G-proteins from calf brain was carried out according to the method described by Sternweis and Robishaw (1984). This procedure resulted in the purification of a mixture of heterotrimeric $G_i$ and $G_o$ proteins, in a proportion of ~80% $G_o$ and 20% $G_i$ (Sternweis & Robishaw, 1984). We will refer to this preparation as $G_i/G_o$.

*Reconstitution of mAChR with G-Proteins.* The reconstitution of the phosphorylated and nonphosphorylated mAChR with G-proteins was performed as previously described by Haga et al. (1988) using a proportion of 1 pmol of receptor per 200 pmol of the $G_i/G_o$ mixture. Prior to reconstitution with $G_i/G_o$, the phosphorylated and the nonphosphorylated mAChR (which were also subjected to the conditions of the phosphorylation reaction, but without protein kinase C) were chromatographed on a small Sephadex G-50 column (2 mL), in the presence of 20 mM Hepes, pH 8, 0.1 mM EDTA, 0.1 M NaCl, and 5 mM dithiothreitol in order to remove the ATP and other reagents of the phosphorylation reactions.

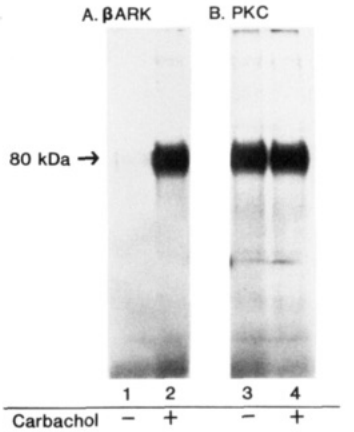


FIGURE 1: Autoradiogram depicting the phosphorylation of chick heart muscarinic receptors by $\beta$-AR kinase and protein kinase C. Phosphorylation of mAChR by $\beta$-AR kinase and protein kinase C was performed for 45 min at 30 °C in reaction mixtures containing reconstituted muscarinic receptors (~0.36 pmol) in the presence or absence of carbachol and either $\beta$-AR kinase (panel A) or protein kinase C (panel B).

*Binding Assays and Data Analysis.* Ligand binding to the reconstituted mAChR was carried out with the antagonist [$^3$H]QNB and varying concentrations of the agonist carbachol according to Haga and Haga (1986). The reactions (1 mL) contained 50–60 fmol of reconstituted receptors ± G-proteins, 25 mM potassium phosphate, pH 7, 0.8 mM EDTA, 3 mM $MgCl_2$, 230 mM NaCl, 0.06% bovine serum albumin, 4 mM Hepes, 0.6–0.7 nM [$^3$H]QNB, and ±0.1 mM Gpp(NH)p as indicated. Reactions were carried out for 75 min at 30 °C and terminated by filtration over Whatman GF/F filters. The data obtained from three experiments performed in duplicate were analyzed with the curve-fitting program LIGAND (Munson & Rodbard, 1980).

## Results and Discussion

In order to investigate the possibility that mAChR could serve as substrates for protein kinase C, the receptors were reconstituted and incubated with protein kinase C as described under Experimental Procedures. For comparison, some parallel studies were performed in the presence of $\beta$-AR kinase. As shown in Figure 1 (lanes 3 and 4), protein kinase C effectively phosphorylated the purified receptors. However, in contrast to the agonist-dependent phosphorylation that was obtained with $\beta$-AR kinase (Figure 1, lanes 1 and 2), a marked phosphorylation of the mAChR with protein kinase C occurred in the absence of the agonist carbachol (lane 3) and was not further increased in the presence of 1 mM carbachol (lane 4). These results demonstrated that chick heart mAChR could serve as substrates in vitro for protein kinase C; the observed phosphorylation required that the purified receptors be reconstituted out of detergent solution and into phospholipid vesicles, as no phosphorylation by protein kinase C could be observed with the purified, nonreconstituted receptors. The results suggested that the mechanism of phosphorylation might be different for protein kinase C and $\beta$-AR kinase, since the

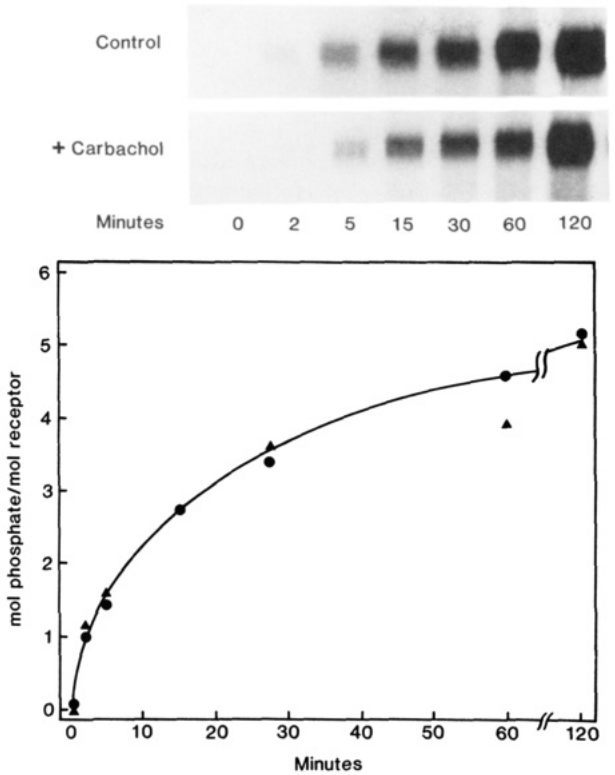


FIGURE 2: Time course of phosphorylation of chick heart muscarinic receptors by protein kinase C in the presence or absence of agonist. The upper panel shows autoradiograms depicting the time course of phosphorylation of mAChR by protein kinase C in the presence or absence of carbachol (control). The lower panel shows the rate and extent of phosphorylation of mAChR by protein kinase C in the presence (▲) or absence (●) of carbachol as determined from counting the excised gel pieces containing the receptors shown in the upper panel. This experiment has been repeated 3 times with similar results. The difference observed at 60 min (carbachol vs control) was due to loading slightly less protein in the sample containing carbachol.

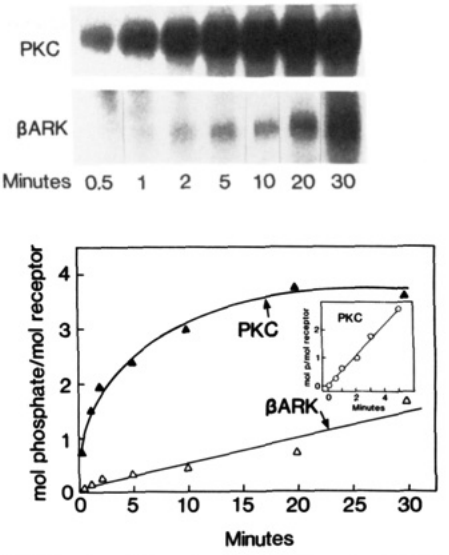


FIGURE 3: Initial rates of the phosphorylation of mAChR by β-AR kinase and protein kinase C. The upper panels shows autoradiograms of the resulting gels of the reactions in which phosphorylation was performed with either protein kinase C or β-AR kinase at the times indicated. All reactions contained carbachol. The lower panel presents the extent of the phosphorylation in each case shown above, which was determined by counting the excised gel pieces containing the receptors.

phosphorylation of mAChR by β-AR kinase required the presence of an agonist, whereas for protein kinase C the mechanism of phosphorylation seemed to be agonist-independent.

In order to examine more accurately whether or not phosphorylation of mAChR by protein kinase C could be affected by agonist occupancy of the receptors, the time courses and extents of phosphorylation of the mAChR by protein kinase C were determined in the presence and absence of carbachol. Figure 2, upper panel, shows the autoradiogram from SDS gels containing receptors phosphorylated by protein kinase C for different times in the presence or absence of carbachol, while the lower panel shows the rates and extents of the phosphorylation that were calculated by counting the radioactivity in the gels pieces containing the phosphorylated receptors. As can be seen, neither the rate nor the extent of phosphorylation of the mAChR by protein kinase C was influenced by the agonist. An identical curve was obtained in both the presence and the absence of carbachol. This finding clearly indicated that the phosphorylation of mAChR by protein kinase C was not affected by agonist occupancy of the receptors. The results also revealed that the stoichiometry of the phosphorylation of mAChR by protein kinase C was ~5 mol of phosphate/mol of receptors, slightly higher than the extent previously observed for the phosphorylation of mAChR by β-AR kinase (Kwatra et al., 1989b).

The initial rate of phosphorylation of the mAChR by protein kinase C was examined and compared to that obtained with β-AR kinase. Figure 3 shows the autoradiograms obtained at the times indicated (upper panel) and the graphical representations of the rates of the reactions for each kinase (lower panel). The phosphorylation of mAChR by protein kinase C was rapid and almost complete in 15 min, reaching an extent of ~4.5 mol of phosphate/mol of receptors. The reaction with protein kinase C was only linear in the first 5 min of incubation, as shown in the inset figure, and this rapid reaction was in marked contrast to that observed with β-AR kinase. The reaction with β-AR kinase was considerably slower and remained linear in the first 30 min of the reaction, reaching an extent of ~1.5 mol of phosphate/mol of receptors. These results suggest that the reconstituted muscarinic receptors may be better substrates for protein kinase C than for β-AR kinase; however, several factors need to be taken into consideration in reviewing these results. First, it needs to be noted that these results were obtained by using a concentration of protein kinase C that was considerably lower (~100-fold) than that of β-AR kinase, however, this difference was somewhat offset by the fact that the specific activity of protein kinase C (defined with histone as a substrate) (Woodgett & Hunter, 1987) was much higher than that of β-AR kinase (defined with β-AR as substrate) (Benovic et al., 1987b). Second, it is widely accepted that kinetic activities of protein kinase C vary considerably with the substrate used (Nishizuka, 1988). Finally, it is possible that the conditions of phosphorylation reactions catalyzed by β-AR kinase are not yet optimal.

Since protein kinase C phosphorylates the mAChR in an agonist-independent manner and β-AR kinase phosphorylates the receptors in an agonist-dependent manner, it was of interest to determine if their effects were additive. In an attempt to address this issue, we studied the rate and extent of phosphorylation of the receptors with one or both kinases present in the reaction mixture (all reactions contained agonist). As shown in Figure 4 (left panel), the presence of both kinases simultaneously in the reaction mixture resulted in an additive incorporation of $^{32}P$ into the receptors, as compared to the results obtained for each kinase alone. The stoichiometry of the additive reaction obtained in two experiments was ~6.5 mol of phosphate/mol of receptors as compared to ~2.5 mol

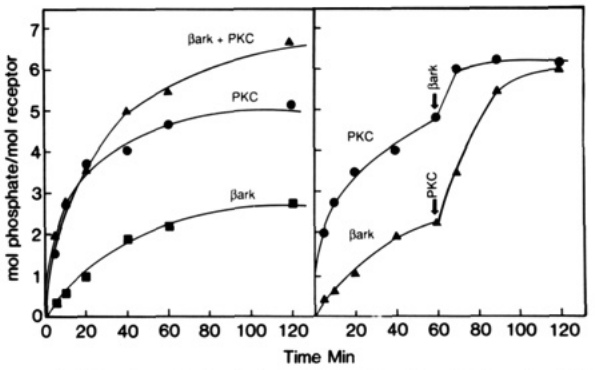


FIGURE 4: Kinetic analysis of the additive phosphorylation of mAChR by protein kinase C and β-AR kinase. In the left panel, mAChR was incubated at 37 °C with protein kinase C, β-AR kinase, or both kinases simultaneously for the time indicated. In the right panel, mAChR were incubated first with either one of the kinases for 60 min before adding the other kinase for an additional 60 min. The samples were electrophoresed on SDS gels, and after autoradiography, the bands were excised, and the $^{32}P$ incorporation was quantitated by Cerenkov counting.

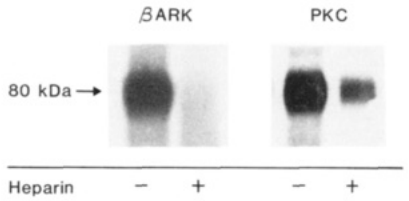


FIGURE 5: Phosphorylation of chick heart muscarinic receptors by β-AR kinase and protein kinase C in the presence or absence of heparin. Autoradiograms of the resulting gels of the reactions in which phosphorylation was performed with either β-AR kinase or protein kinase C in the presence or absence of heparin (1 μM) as indicated. All reactions contained carbachol.

of P/mol of receptor and ~5 mol of P/mol of receptor obtained with β-AR kinase and protein kinase C alone, respectively. Thus, the phosphorylation catalyzed by the two kinases appeared to be additive, but may be slightly less than totally additive.

We also tested whether the phosphorylation by either protein kinase would alter the ability of the reconstituted receptors to serve as substrates for the other kinase. Experiments were performed in which the receptors were phosphorylated for 60 min by one kinase and then for another 60 min by the other kinase. Figure 4 (right panel) shows the rate and extent of the incorporation of $^{32}P$ into the receptors obtained in each experiment. The levels of phosphorylation attained were the same as the level observed when both kinases were added simultaneously. These results suggested that phosphorylation by protein kinase C does not preclude subsequent phosphorylation by β-AR kinase and vice versa, and confirmed the partial additivity of the reaction observed when both kinases were added simultaneously.

It has been demonstrated by Benovic et al. (1989) that heparin totally inhibits β-AR kinase catalyzed phosphorylation of β-adrenergic receptors ($K_i$ = 0.15 μM). In order to more fully characterize the mAChR phosphorylation catalyzed by β-AR kinase and protein kinase C, we also studied the effect of heparin in both processes of phosphorylation. As shown in Figure 5, the presence of heparin in the reaction mixtures resulted in a total inhibition of the mAChR phosphorylation catalyzed by β-AR kinase (lanes 1 and 2). In contrast, only partial inhibition (~65–75%) of the mAChR phosphorylation catalyzed by protein kinase C was observed in the presence of heparin (lanes 3 and 4). The inhibition by heparin appeared to be due to interaction with the substrate, as protein kinase C mediated phosphorylation of histone was found to be unaffected by heparin (data not shown). Therefore, the difference in the inhibition of mAChR phosphorylation by heparin also suggests a certain difference between the amino acid residues modified by each kinase.

The phosphoamino acid composition of the receptors phosphorylated by protein kinase C was determined; ~60% of the $^{32}P$ was recovered as phosphoserine and ~40% as phosphothreonine (data obtained from two experiments). These results are similar to what has been found in previous studies of agonist-induced phosphorylation of chick heart mAChR in situ (Kwatra et al., 1987), and in vitro studies with β-AR kinase (Kwatra et al., 1989b). Two-dimensional phosphopeptide mapping of the receptors after phosphorylation by either protein kinase C or β-AR kinase was performed (Figure 6) and revealed that protein kinase C and β-AR kinase phosphorylated distinct, as well as similar, peptides. Three peptides mainly phosphorylated by protein kinase C (panel A, 1, 2, 3) were also phosphorylated by β-AR kinase (panel B). Peptides 1 and 3 seemed to be phosphorylated to a greater

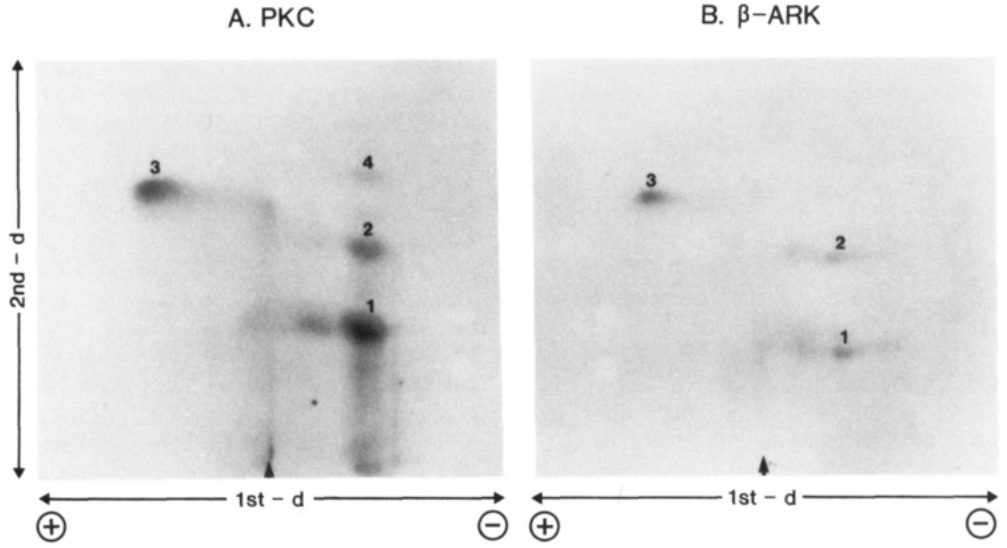


FIGURE 6: Two-dimensional phosphopeptide maps obtained after phosphorylation of mAChR by protein kinase C and β-AR kinase. Purified and reconstituted mAChR were phosphorylated for 120 min by either protein kinase C or β-AR kinase, electrophoresed on SDS gels, and processed as previously described (Kwatra et al., 1987; O'Callahan et al., 1988). The small arrow in each panel indicates the origin.

Table I: Binding Parameters Obtained from Computer Analysis of Observed Data Shown in Figure 7[a]

| | $K_H$ (μM) | $R_H$ (%) | $K_L$ (μM) | $R_L$ (%) |
|---|---|---|---|---|
| | Nonphosphorylated Receptors | | | |
| receptors alone | ND[c] | ND | 52 ± 4.6 | 100 |
| R + $G_i/G_o$ | 0.102 ± 0.05 | 22 ± 2.2[b] | 71 ± 6 | 78 ± 2.2 |
| R + $G_i/G_o$ + Gpp(NH)p | ND | ND | 35 ± 2.5 | 100 |
| | Phosphorylated Receptors | | | |
| receptors alone | ND | ND | 34 ± 3.4 | 100 |
| R + $G_i/G_o$ | 0.09 ± 0.04 | 36 ± 3.8[b] | 83 ± 8.3 | 64 ± 3.4 |
| R + $G_i/G_o$ + Gpp(NH)p | ND | ND | 54 ± 5.2 | 100 |

[a] $K_H$ and $K_L$ refer to $K_i$ values for high- and low-affinity states, respectively, as determined by curve fitting with the LIGAND program. $R_H$ and $R_L$ refer to the percentage of receptors exhibiting the respective affinity. Values are mean ± standard error. [b] $p < 0.05$ as determined by Student's $t$ test. [c] ND, not detectable.

degree by protein kinase C, indicating that they may contain more than one modified residue. In terms of distinct sites, peptide 4 was phosphorylated by protein kinase C (panel B) but was not phosphorylated by β-AR kinase. As the results of the experiments obtained when both kinases were present in the reaction mixture suggest that phosphorylation was partially additive (Figure 4), it is possible that the tryptic peptides that appear to be phosphorylated by both kinases contain multiple sites for phosphorylation. Currently, efforts are under way to sequence the sites phosphorylated by both protein kinases in order to clarify this issue.

The results presented so far suggest that chick heart mAChR can serve as excellent substrates in vitro for protein kinase C. These observations, coupled with previous studies of effects of activators of protein kinase C on mAChR function in various cell types, suggest a role for protein kinase C mediated receptor phosphorylation in the regulation of mAChR. In order to obtain evidence for this proposal, we determined whether phosphorylation by protein kinase C altered the ability of the receptors to interact with the G-proteins that couple the mAChR to effectors. Receptors were subjected to phosphorylation conditions in the presence or absence of protein kinase C; the phosphorylation reagents were removed, the G-proteins were added or not, and the receptors were subsequently assayed for agonist binding properties in antagonist/agonist competition assays. In the absence of added G-proteins, the phosphorylated and nonphosphorylated receptors exhibited low affinity for carbachol (Figure 7 and Table I). The dose response relationships were best fit by a one-state model, and the dissociation constants were not different for the phosphorylated and nonphosphorylated receptors. However, in the presence of added $G_i/G_o$, a significant effect of phosphorylation was observed. As expected (Florio & Sternweis, 1985; Kurose et al., 1986; Haga et al., 1985, 1989), the G-proteins restored high-affinity agonist binding, but, unexpectedly, this occurred to a greater degree with the receptors phosphorylated by protein kinase C than with the nonphosphorylated receptors (Figure 7, Table I). In both cases, the competition curves were best fit by a two-state model (Figure 7, Table I). The dissociation constants, $K_H$, for carbachol binding to the phosphorylated and nonphosphorylated receptors were similar; however, the percentage of receptors exhibiting high-affinity binding was significantly greater for the phosphorylated than the nonphosphorylated receptors (36 ± 3% vs 22 ± 2%, $p < 0.05$). The $G_o/G_i$-dependent high-affinity binding was fully reversed by the addition of Gpp(NH)p, demonstrating that nonphosphorylated and phosphorylated receptors behaved similarly when the guanine nucleotide reversed the receptor/G-protein interaction (Figure 7, Table I). The increased percentage of high-affinity receptors observed after phosphorylation could reflect an increased affinity of the phosphorylated receptors for the $G_i/G_o$, or an effect on another parameter that influences receptor/G-protein interactions.

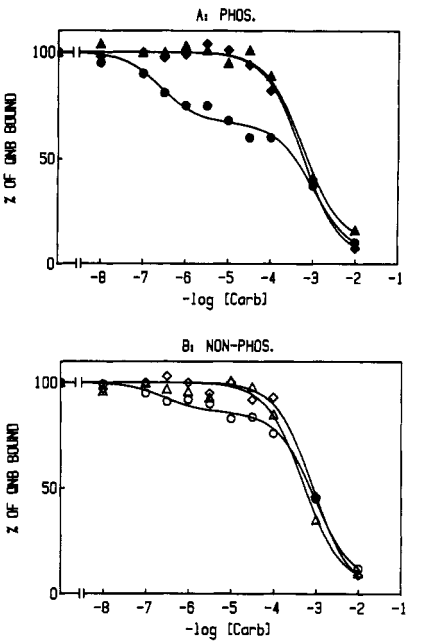


FIGURE 7: Competitive inhibition by carbachol of [$^3$H]QNB binding to protein kinase C phosphorylated (A) and nonphosphorylated (B) mAChR reconstituted in the presence and absence of $G_o/G_i$. Ligand binding assays were performed as described under Experimental Procedures. Receptors with no G-proteins added (♦); receptors reconstituted with $G_i/G_o$ in a 1:200 ratio in the absence (●) and presence (▲) of 0.1 mM Gpp(NH)p. Curve fitting was performed with the LIGAND program, and the resulting parameters are given in Table I. The results shown are the mean curves obtained from three sets of experiments performed in duplicate.

The above results suggest that protein kinase C can positively modify the interaction of mAChR with $G_i/G_o$. These results were somewhat surprising in that most reports concerning the regulation of mAChR function by activators of protein kinase C have demonstrated inhibition of receptor function, or down-regulation of surface mAChR by protein kinase C (Liles et al., 1985; Conklin et al., 1989; Orellana et al., 1985; Vincentini et al., 1985). On the other hand, phorbol esters have been reported to enhance mAChR-mediated arachidonic acid release in A9 L cells (Conklin et al., 1988). Two obvious questions that need to be addressed in future studies are whether protein kinase C phosphorylates mAChR in heart cells and if positive regulation of mAChR by protein kinase C occurs in these cells. In an earlier study, we were unable to detect effects of phorbol esters on the phosphorylation of mAChR in slices of newborn chick heart (Kwatra et al., 1987), under conditions in which we were able to observe a phorbol ester induced translocation of protein kinase C from the cytosol to the membrane (Kwatra and Hosey, unpublished observations). However, it is possible that in intact cells phosphorylation sites are masked by interactions of the receptors with G-proteins, as has been suggested by the in vitro studies of Haga and Haga (1989), and that agonists may be necessary to promote cycling of receptor/G-protein complexes in order for protein kinase C phosphorylation to occur. Another consideration is that the protein kinase C isozyme content in heart is known to be different than that of brain (Nishizuka, 1988) from which the purified protein kinase C preparation used in these studies was derived. Future efforts will determine which protein kinase C isozymes can phosphorylate mAChR.

The finding of positive regulation of mAChR by protein kinase C also will require further studies to elucidate which subtypes of mAChR exhibit this property and if the interaction with more than one G-protein is affected by these events. In the present experiments, we have not attempted to resolve the $G_i$ from $G_o$ to determine if the interaction of the receptors with one or the other G-protein is preferentially modified by receptor phosphorylation. Previous studies by Haga and colleagues (Haga et al., 1989) have shown that there are no significant differences in the ability of either $G_i$ or $G_o$ to induce high-affinity binding to cerebral mAChR; however, it is conceivable that phosphorylation of the receptors might differentially affect coupling to one or the other.

At least five subtypes of mAChR have been identified by cDNA cloning studies (Peralta et al., 1987; Bonner et al., 1987; Liao et al., 1989), and they are known to differentially couple to various G-proteins and different effector systems (Nathanson, 1987). Both inhibitory and stimulatory effects of protein kinase C activation have been reported for the expressed m1 and m3 subtypes (Conklin et al., 1988), while other studies using neuronal cells expressing endogenous m3 and m4 subtypes have only reported inhibitory effects (Liles et al., 1986; Orellana, 1985; El-Fakahany et al., 1988). Evidence from cDNA cloning studies suggest that chick heart contains m4 (Tietje et al., 1990) receptors [nomenclature of Bonner et al. (1987)]. However, mammalian heart appears to contain only m2 receptors (Peralta et al., 1987a). We previously have demonstrated that chick and porcine heart mAChR both undergo agonist-induced phosphorylation in intact cells (Kwatra et al., 1986, 1987, 1989a), although the pharmacological and structural properties of the receptors suggested they were distinct proteins (Kwatra et al., 1989a). It appears that not all cardiac mAChR may be substrates for protein kinase C, since a previous study reported that the m2 mAChR purified from porcine atria were not substrates for protein kinase C (Rosenbaum et al., 1987). Differences in the primary structures in porcine m2 and chick heart m4 mAChR may account for the differences observed with the porcine and chick cardiac mAChR. In either case, the possibility exists either that mAChR subtypes and/or mAChR/effector coupling may be differentially regulated by phosphorylation.

In summary, the present results demonstrate that mAChR purified from chick heart and reconstituted into phospholipid vesicles are excellent substrates in vitro for protein kinase C. Phosphorylation of these receptors by this enzyme occurs in an agonist-independent manner, in contrast to the agonist-dependent phosphorylation that is catalyzed by $\beta$-AR kinase. These results suggest that the recognition of substrates is markedly different for the two enzymes. Furthermore, the agonist dependence and independence have important implications for the functional significance of the phosphorylation catalyzed by each enzyme, since regulation of the mAChR by protein kinase C could conceivably occur in the absence of mAChR activation and/or in the presence of activation of heterologous receptors. An unexpected finding of the present study was that the phosphorylation of the mAChR by protein kinase C resulted in an increased ability of the receptors to recognize agonists with high affinity when the receptors were reconstituted with a purified preparation of $G_i/G_o$. This positive regulation of receptor function will be explored in future studies designed to identify which subtypes of mAChR are affected by protein kinase C in positive and/or negative manners.

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# A Second-Site Mutation at Phenylalanine-137 That Increases Catalytic Efficiency in the Mutant Aspartate-27 → Serine *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: The adaptability of *Escherichia coli* dihydrofolate reductase (DHFR) is being explored by identifying second-site mutations that can partially suppress the deleterious effect associated with removal of the active-site proton donor aspartic acid-27. The Asp27 → serine mutant DHFR (D27S) was previously characterized and the catalytic activity found to be greatly decreased at pH 7.0 [Howell et al. (1986) *Science 231*, 1123-1128]. Using resistance to trimethoprim (a DHFR inhibitor) in a genetic selection procedure, we have isolated a double-mutant DHFR gene containing Asp27 → Ser and Phe137 → Ser mutations (D27S+F137S). The presence of the F137S mutation increases $k_{cat}$ approximately 3-fold and decreases $K_{m(DHF)}$ approximately 2-fold over D27S DHFR values. The overall effect on $k_{cat}/K_{m(DHF)}$ is a 7-fold increase. The D27S+F137S double-mutant DHFR is still 500-fold less active than wild-type DHFR at pH 7. Surprisingly, Phe137 is approximately 15 Å from residue 27 in the active site and is part of a $\beta$-bulge. We propose the F137S mutation likely causes its catalytic effect by slightly altering the conformation of D27S DHFR. This supposition is supported by the observation that the F137S mutation does not have the same kinetic effect when introduced into the wild-type and D27S DHFRs, by the altered distribution of two conformers of free enzyme [see Dunn et al. (1990)] and by a preliminary difference Fourier map comparing the D27S and D27S+F137S DHFR crystal structures.

Dihydrofolate reductase (DHFR;[1] EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). This activity is necessary in maintaining intracellular pools of THF cofactors that are essential in biosynthetic reactions involving the transfer of one-carbon units. Inhibitors of DHFR are clinically important and include the antitumor drug methotrexate (MTX), the antibacterial trimethoprim (TMP), and the antimalarial pyrimethamine.

DHFR has been extensively studied by kinetic methods (Stone & Morrison, 1988a,b; Fierke et al., 1987a; Penner & Frieden, 1987). Recently Fierke et al. (1987b) have suggested

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