Phosphorylation of a phosphoinositidase C-linked muscarinic receptor by a novel kinase distinct from β-adrenergic receptor kinase

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Muscarinic receptor kinase activity previously described in intact CHO cells transfected with human m3-muscarinic receptor cDNA (CHO-m3 cells) [Tobin, A.B and Nahorski, S.R (1993) J. Biol. Chem. 268, 9817–9823] was found to be associated, at least in part, with a crude membrane fraction of CHO-m3 cell lysates. Phosphorylation of the m3-muscarinic receptor was agonist dependent, reaching a maximum after 10 min exposure to carbachol (1 mM) and was completely blocked by atropine (10 μM). m3-Muscarinic receptor phosphorylation was insensitive to Zn^{2+} (0.1 mM) and heparin (1 μg/ml), concentrations that inhibit endogenous β-adrenergic receptor kinase activity present in CHO-m3 cells strongly suggesting that the m3-muscarinic receptor kinase is distinct from β-adrenergic receptor kinase. A role for protein kinase C can also be eliminated on the basis that the potent protein kinase C inhibitor, Ro-318220 (1 μM), had no effect on agonist-mediated m3-muscarinic receptor phosphorylation. Further, the inability of calcium (300 μM), cAMP (0.2 mM) and cGMP (0.2 mM) to elevate the basal phosphorylation state of m3-muscarinic receptors eliminates a role for protein kinases regulated by these second messengers. Finally, agonist mediated phosphorylation appears to be independent of G-protein activation as both GDP-β-S (500 μM) and GTP-γ-S (100 μM) did not influence m3-muscarinic receptor phosphorylation.

Muscarinic acetylcholine receptor; Muscarinic receptor kinase; Phosphoinositidase C; β-Adrenergic receptor kinase; Protein kinase C

1. INTRODUCTION

The muscarinic receptor gene family consists of five subtypes, three of which (m1, m3 and m5) are efficiently coupled to phosphoinositide hydrolysis via a pertussis toxin insensitive G-protein [1]. We have recently demonstrated that the phosphoinositidase C (PIC)-linked human m3-muscarinic receptor expressed in transfected CHO cells (CHO-m3 cells) undergoes stoichiometric phosphorylation (~2 mol phosphate/mol receptor), primarily on serine, following agonist activation [2]. Although the precise identity of the kinase involved was not determined by this earlier study it was possible to conclude that the m3-muscarinic receptor kinase was distinct from protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase and cAMP-dependent protein kinase (PKA) [2].

It is now clear that a number of G-protein linked receptors are phosphorylated in response to agonist occupation. In the case of the cyclase-linked β-adrenoceptor the role played by PKA and β-adrenergic receptor kinase (β-ARK) in uncoupling the β-adrenoceptor from Gsα has been clearly delineated [3]. However, in vitro studies utilising reconstituted systems have demonstrated that β-ARK is also able to phosphorylate the cyclase linked m2-muscarinic [4,5] and $α_2$-adrenergic receptors [6], and the PIC-linked substance P receptor

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[7] suggesting that β-ARK may be acting as a general G-protein receptor kinase with a wide substrate specificity [7].

Similarly, PKC is known to phosphorylate a number of G-protein linked receptors including the m2-muscarinic receptor [8], 5-HT_{1a} receptors [9], CCK receptor [10] and $α_1$-adrenoceptor [11]. Furthermore, phorbol ester activation of PKC either diminishes [12] or completely abolishes [13,14] muscarinic receptor mediated phosphoinositide hydrolysis in cultured cell lines, indicating that PKC is able to influence the activity of at least one component of the muscarinic receptor signal transduction pathway although it is not clear that phosphorylation of the receptor itself is involved.

In the present study we establish that the kinase involved in m3-muscarinic receptor phosphorylation is associated with the particulate fraction and can be distinguished from either β-ARK, PKC or other known second messenger regulated kinases.

2. MATERIALS AND METHODS

2.1. Materials

Cell culture reagents were obtained from Gibco. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. All other reagents were obtained from Sigma. CHO cells transfected with recombinant human m3-muscarinic receptor cDNA (CHO-m3 cells, expression level = 1343 ± 46 fmol/mg protein, [15]) were a kind gift from Dr. N.J. Buckley, National Institute for Medical Research, London, UK. Details of the antisera to the human m3-receptor have been previously published [2].

2.2. Preparation of crude CHO/CHO-m3 membrane fraction

Non-transfected CHO or CHO-m3 cells were harvested and resuspended in kinase buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM NaCl, 1 mM EGTA, 2 mM DTT + protease inhibitors 1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml aprotinin), 4–6 flasks in 25 ml. Cells were disrupted by a 5 s pulse in an ultraturrax homogeniser (max. setting). Following centrifugation at 15,000 × g 10 min at 4°C the particulate membrane fraction was resuspended in kinase buffer to a concentration of 10 mg protein/ml.

2.3. Phosphorylation of m3-muscarinic receptors contained in CHO-m3 membrane fractions

CHO/CHO-m3 membranes (500 μg protein) resuspended in kinase buffer were incubated with 50 μM [γ-^{32}P]ATP (1–4 dpm/fmol) for 10 min at 30°C in the presence of various experimental agents. The final volume was 100 μl. Reactions were stopped by a 30 s centrifugation in a microfuge, removal of the supernatant, and solubilisation of m3-muscarinic receptors with 1 ml solubilisation buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate + protease's inhibitors). In the case of the time course the centrifugation step was omitted and the reaction terminated by direct addition of 1 ml of solubilisation buffer. Solubilised receptors were then immunoprecipitated as described previously [2] and proteins resolved on 8% SDS-PAGE. Following autoradiography gels were either subjected to densitometric analysis using a BioRad model GS 670 densitometer or radioactive bands were cut from the gel extracted in hyamine hydroxide and activity determined by scintillation counting. Stoichiometry of phosphorylation was calculated based on a recovery of m3-muscarinic receptors of ~ 80% following immunoprecipitation (~ 500 fmol receptor/assay, see [2]).

2.4. Assay for protein kinase C activity

Membranes from CHO-m3 cells were prepared either from unstimulated cells or, as a positive control, from CHO-m3 cells pretreated with PMA (100 nM, 10 min) to induce translocation of PKC to the plasma membrane [16]. The myelin basic protein fragment$_{4-14}$ QKRPSQRSKYL (25 μg/assay) was used as a substrate for PKC [17] in an assay with CHO-m3 membranes (200–350 μg protein) in kinase buffer containing 50 μM [γ-^{32}P]ATP (100 dpm/pmol). In reactions utilising membranes from PMA pretreated cells the kinase assay was carried out in the presence of PMA (100 nM). The total reaction volume was 100 μl. The reaction was initiated by addition of ATP and terminated by centrifugation at 13,000 × g for 5 min to sediment membranes. An aliquot of supernatant (75 μl) was placed on a square of Whatman P81 cellulose phosphate filter and washed 3 times with 75 mM H_3PO_4, and once with ethanol before being subjected to liquid scintillation counting.

2.5. Assay for β-ARK activity

The activity of β-ARK was determined essentially as described previously [18,19]. CHO-m3 cells were harvested and resuspended in twice the volume of kinase buffer. Cells were lysed by a 5 s pulse in an ultraturrax homogeniser (max setting) and a cytosolic extract prepared by centrifugation at 300,000 × g for 30 min (4°C). An aliquot of cytosolic extract (5 μg of protein) was mixed with urea-treated bovine rod outer segment membranes, (~ 10 μg of protein prepared as described previously [20]) in kinase buffer containing 100 μM [γ-^{32}P]ATP (~ 270 dpm/pmol). The final volume was 20 μl. The reaction was carried out at 30°C for 10 min and was terminated by addition of 20 μl SDS-PAGE sample buffer. Proteins were separated on a 12% SDS-PAGE gel. Following Coomassie blue staining and autoradiography the band corresponding to rhodopsin was excised, protein extracted using hyamine hydroxide, and the radioactivity determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

A crude low speed (15,000 × g) membrane preparation of CHO-m3 cells was found to contain m3-muscar-

inic receptor kinase activity (Fig. 1A). Incubation of CHO-m3 membranes with [γ-^{32}P]ATP followed by immunoprecipitation of m3-muscarinic receptors revealed that in the absence of agonist the m3-muscarinic receptor was phosphorylated. Inclusion of a maximal dose of the muscarinic agonist carbachol (1 mM) resulted in a dramatic increase in phosphorylation state of the m3-muscarinic receptor (Fig. 1A), which was completely blocked by the muscarinic antagonist atropine (10 μM, Fig. 1B). No m3-muscarinic receptor kinase activity was detected in cytoplasmic extracts from CHO-m3 cells nor from non-transfected CHO cells. In fact, addition of cytosolic extracts to CHO-m3 membranes decreased the level of agonist mediated phosphorylation (data not shown). Due to the likely presence of protein phosphatases in cytosolic preparations the possibility that the m3-muscarinic receptor kinase also exists in a soluble form cannot be eliminated. However, it is clear that at least a proportion of the m3-muscarinic receptor kinase is associated with the particulate fraction. These observations are not restricted to transfected CHO cells since preliminary studies on the human neuroblastoma cell line SH-SY5Y, which express m3-muscarinic receptors at ~ 400 fmol/mg protein [21,22], have also identified a kinase that phosphorylates the agonist occupied m3-muscarinic receptor in a similar manner to that observed in CHO-m3 membranes (data not shown).

Membrane phosphorylation of m3-muscarinic receptors peaks at 10 min agonist exposure (Fig. 2), where the stoichiometry is ~ 0.1mol phosphate/mol receptor ($n = 3$), after which the m3-muscarinic receptor undergoes dephosphorylation. In contrast, phosphorylation of the m3-muscarinic receptor in intact CHO-m3 cells occurred within seconds of agonist occupation and was maintained for at least 30 min, where the maximum
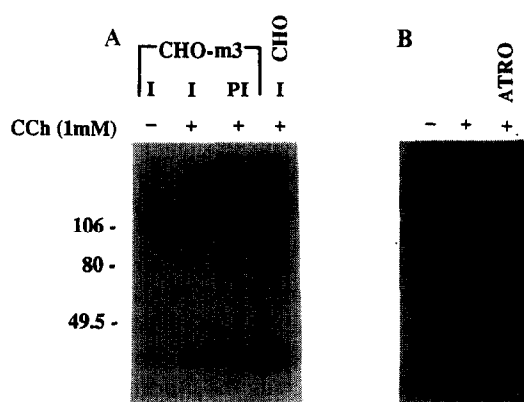

Fig. 1. Phosphorylation of m3-muscarinic receptors in the particulate fraction of CHO-m3 lysate. A: membrane preparations derived from either CHO-m3 cells or non transfected CHO cells were incubated in a buffer containing [γ-^{32}P]ATP ± 1 mM carbachol (CCh) for 10 min at 30°C. Following solubilisation, m3-muscarinic receptors were immunoprecipitated with immune serum (I) or pre-immune serum (PI) antisera B. The effect of atropine (10 μM) on m3-muscarinic receptor phosphorylation. Positions of prestained molecular weight markers are indicated in kDa.
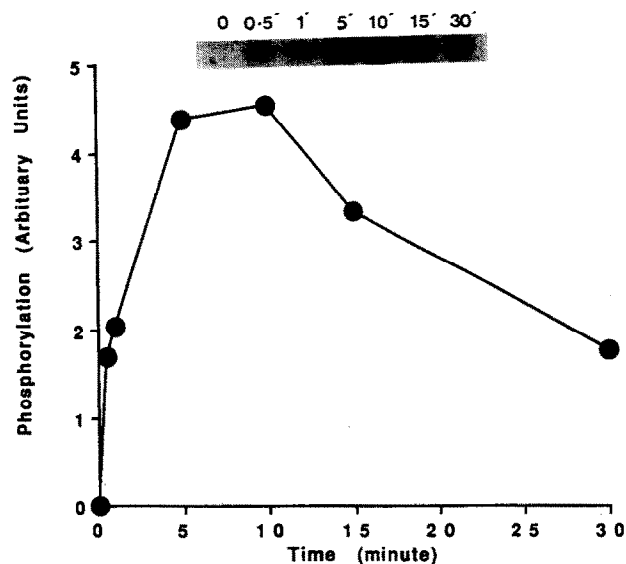
Fig. 2. Time course for m3-muscarinic receptor phosphorylation in CHO-m3 cell membranes. Legend as for Fig. 1. Data is representative of two determinations. Data was analysed by densitometry.

stoichiometry reached was ~ 2mol phosphate/mol receptor. The difference between intact cells and membrane preparations may be attributable to factors missing in the membrane preparation that are necessary for efficient m3-muscarinic receptor phosphorylation, combined with rapidly falling ATP levels (during the first 10 min the concentration of ATP in the kinase assay fell by 80%; $n = 3$) due to the presence of contaminating ATPases. Application of okadaic acid (1 μM) to the membrane preparation enhanced agonist-mediated phosphorylation by ~ 5 fold ($n = 2$, data not shown) suggesting that deregulation of protein phosphatase activity may also contribute to the low stoichiometry of phosphorylation.

Alternatively, a proportion of the m3-muscarinic receptor kinase may be cytoplasmic, therefore, providing an additional component of kinase activity in intact cells that would be missing in membrane preparations. It should be noted that early studies on partially purified β-ARK reported low levels of β-adrenoceptor phosphorylation (0.2 mol phosphate/mol receptor [23]) a situation significantly improved by further purification of the kinase [24]. Similarly, we anticipate that purification of the m3-muscarinic receptor kinase will allow for a precise determination of its intracellular localisation, and rate of in vitro phosphorylation together with an accurate measure of stoichiometry.

The primary consequence of m3-muscarinic receptor activation in membrane preparations (preliminary results indicate that there is a ~ 2-fold increase in Ins(1,4,5)P3 production in CHO-m3 membranes stimulated by 1 mM carbachol under phosphorylating conditions) and intact CHO-m3 cells [15] is the hydrolysis of phosphoinositides resulting in the generation of inositol

polyphosphates and the PKC activator, diacylglycerol. Thus a reasonable candidate for the kinase mediating phosphorylation of the m3-muscarinic receptor might be PKC. This, however, does not appear to be the case since the potent PKC inhibitor, RO-318220 [25], had no effect on agonist-mediated m3-muscarinic receptor phosphorylation (Fig. 3). Furthermore, since membranes were prepared from unstimulated cells it might be expected that levels of PKC activity associated with the membrane fraction would be low. This was found to be the case. In a PKC assay using a peptide pseudo-substrate, membranes prepared for the study of m3-muscarinic receptor phosphorylation had no detectable PKC activity (Fig. 4). However, in control membranes, where CHO-m3 cells had been pretreated with PMA (100 nM, for 10 min) to induce translocation of PKC to the plasma membrane [16], phosphorylation of the pseudo-substrate was readily detectable (Fig. 4). The absence of PKC in membrane preparations was further demonstrated by the ineffectiveness of PMA (100 nM) to induce m3-muscarinic receptor phosphorylation (data not shown). These data support that previously obtained in intact cells where agonist-mediated m3-muscarinic receptor phosphorylation was not affected by Ro-318220 at a concentration that completely suppressed phorbol ester-induced phosphorylation [2]. Hence the data presented here and previously [2] conclude that the agonist-mediated m3-muscarinic receptor kinase is distinct from PKC.

The involvement of other second messenger activated protein kinases, PKA, cGMP-dependent protein kinase and Ca^{2+}/calmodulin dependent protein kinase can be eliminated on the basis of the lack of kinase activity detected following the application of cAMP (0.2 mM), cGMP (0.2 mM) and Ca^{2+} (final concentration = C300 μM), respectively (Figure 3). Indeed, elevated $[Ca^{2+}]$
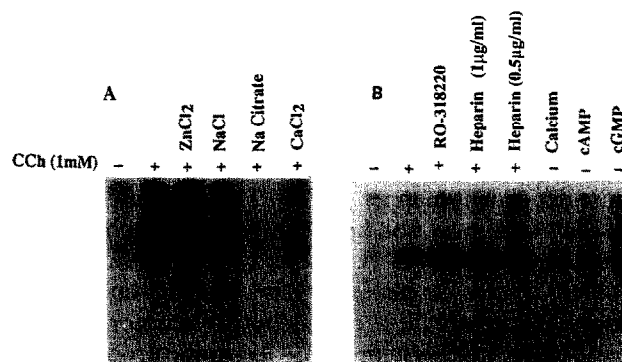

Fig. 3. Characterisation of the m3-muscarinic receptor kinase. A: the effect of $ZnCl_2$ (0.1 mM), NaCl (110 mM), Na-citrate (20 mM) and $CaCl_2$ (300 μM) on carbachol (CCh, 1 mM) mediated m3-muscarinic receptor phosphorylation. B: the effect of Ro-318220 (1 μM) and heparin (1 μg/ml) on agonist mediated m3-muscarinic receptor phosphorylation and the effect of calcium (300 μM), cAMP (0.2 mM) and cGMP (0.2 mM) on basal m3-muscarinic receptor phosphorylation. Data are representative of 2–3 independent determinations with each reagent.
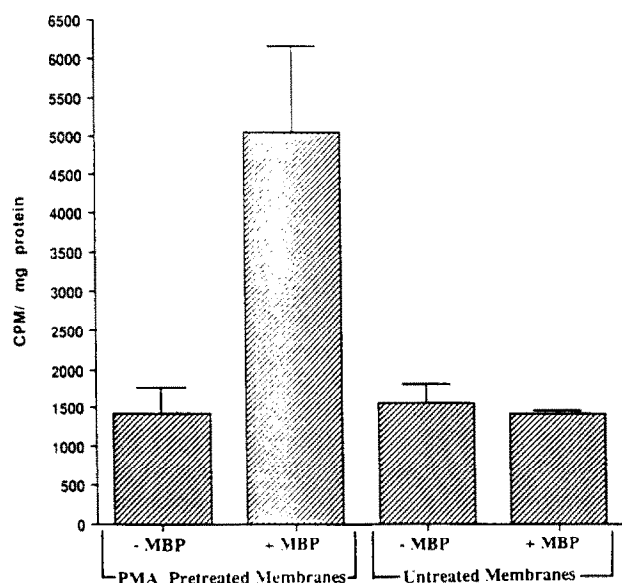
Fig. 4. PKC activity associated with CHO-m3 membrane preparations. CHO-m3 membranes were either prepared from non-treated cells, or as a positive control, from cells pre-treated with PMA (100 nM, 10 min) to induce translocation of PKC to the plasma membrane. PKC activity was then assayed as described in the text using 25 μg/ assay of myelin basic protein fragment$_{4-14}$ (MPB). Results are the mean ± standard error of 3 experiments carried out in triplicate.

decreased the level of agonist driven phosphorylation (Fig. 3). Whether this is a direct effect on the receptor kinase or activation of calcium-sensitive phosphatases has yet to be determined but may be important in the regulation of m3-muscarinic receptor phosphorylation in the intact cell where m3-muscarinic receptor activation results in elevated intracellular calcium concentrations [15].

In vitro studies have established that β-ARK, in addition to acting on β-adrenoceptors, is also able to phosphorylate a number of other cyclase-linked receptors including m2-muscarinic receptors [4,5] and α_2-adrenoceptors [6]. Further, an m2-muscarinic receptor kinase, recently purified from the porcine cerebrum, possesses properties nearly identical to β-ARK suggesting that one or more of the members of the β-ARK family is able to phosphorylate m2-muscarinic receptors in an agonist-dependent manner [26,27]. The substrate specificity of β-ARK may not, however, be limited to receptors coupled to cyclase. A recent study demonstrating that β-ARK is able to phosphorylate the PIC-linked substance P receptor in a reconstituted receptor/Gq-protein/β-ARK lipid system [7] has led to the proposal that β-ARK may be a general G-protein receptor kinase [7].

It is clear, however, from the present study that β-ARK is unlikely to be the kinase involved in agonist-mediated phosphorylation of m3-muscarinic receptors in CHO-m3 membranes. Despite showing sensitivity to high concentrations of sodium chloride (110 mM) and sodium citrate (20 mM; Fig. 3), properties shared with β-ARK [24], m3-muscarinic receptor phosphorylation

is unaffected by 50 μM Zn^{2+} (data not shown) and 100 μM Zn^{2+} (Fig. 3), concentrations shown previously to reduce purified β-ARK activity to 55% and 6% of controls, respectively [24]. Furthermore, heparin (4000–6000 MW) at 1 μg/ml had no inhibitory effect on m3-muscarinic receptor phosphorylation (Fig. 3). This concentration of heparin has previously been shown to completely inhibit β-ARK activity in permeabilised A431 cells [28] and to reduce the phosphorylation of rhodopsin by purified β-ARK [29] and the phosphorylation of m2-muscarinic receptors by a β-ARK related kinase [27] to 20–25% of controls. m3-Muscarinic receptor phosphorylation in our system is, however, partially inhibited by heparin at 10 μg/ml (data not shown). As an internal control, β-ARK was assayed in CHO-m3 cytosolic extracts using rhodopsin as a substrate. In agreement with a previous report [19] low levels of endogenous β-ARK activity were detected in CHO-m3 cytoplasmic extracts (~9 pmol of phosphate incorporated/mg cytosolic protein/minute ($n = 5$)). In the presence of 1 μg/ml heparin or 50 μM Zn^{2+}, conditions that had no effect on m3-muscarinic receptor phosphorylation, endogenous β-ARK activity was reduced by 82% and 81%, respectively ($n = 4$). Therefore, since the m3-receptor kinase is at least partly particulate and insensitive to inhibition by Zn^{2+} and heparin at concentrations that inhibit endogenous β-ARK activity it would seem likely that this kinase is distinct from β-ARK.

Stimulation of the signal transduction pathway coupled to m3-muscarinic receptors does not appear to be a prerequisite to receptor phosphorylation since GDP-β-S (500 μM) which prevents receptor-mediated G-protein activation [30,31] had no inhibitory effect on m3-receptor phosphorylation ($n = 3$). Furthermore, GTP-γ-S (100 μM) shown previously to enhance m3-muscarinic receptor mediated phosphoinositide hydrolysis and calcium mobilisation in permeabilised SH-SY5Y cells [31] and to augment agonist mediated Ins(1,4,5)P3 production in CHO-m3 membranes (Tobin, Keys and Nahorski, unpublished data), had no effect on the basal state of the m3-muscarinic receptor phosphorylation ($n = 3$) nor any effect on agonist-mediated phosphorylation ($n = 3$). An alternative mechanism for m3-muscarinic receptor phosphorylation is that the kinase is constitutively active and that a conformational change in the receptor induced by agonist occupation 'opens' phosphorylation sites which previously were protected from the receptor kinase. Although activation of the m3-muscarinic receptor kinase via a mechanism dependent of G-proteins cannot be completely ruled out evidence in support for a constitutively active kinase has been obtained from a preliminary study where a fragment of the m3-muscarinic receptor was phosphorylated by CHO-m3 membranes in an agonist- independent manner.

Drawing primarily from work on the β-adrenoceptor/ adenylate cyclase system, receptor phosphorylation is

considered to be a general process mediating a reduction in receptor responsiveness or desensitisation [3]. Analysis of m3-muscarinic receptor mediated total inositol phosphoinositide hydrolysis and Ins(1,4,5)P3 production in the first 60 s of agonist stimulation has revealed that the m3-muscarinic receptor phosphoinositide response undergoes rapid partial desensitisation [15,32]. Furthermore, a short 5 min pre-exposure of CHO-m3 cells to carbachol reduces the phosphoinositide and calcium mobilisation responses to a subsequent application of agonist [15]. These desensitisation events correlate temporally with phosphorylation of m3-muscarinic receptors in intact cells [2] and suggest that the two processes could be connected. Understanding the nature of the kinase involved in m3-muscarinic receptor phosphorylation provides valuable information necessary to determine the functional significance of m3-muscarinic receptor phosphorylation and any possible role this process may have in receptor desensitisation.

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