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Relationship between agonist binding, phosphorylation and immunoprecipitation of the m_3-muscarinic receptor, and second messenger responses

[1]A.B. Tobin, G.B. Willars, N.T. Burford & S.R. Nahorski

Leicester University, Dept. Of Cell Physiology and Pharmacology, P.O. Box 138, University Road, Leicester, LE1 9HN

1 Phosphoinositidase C-linked m_3-muscarinic receptors expressed in Chinese hamster ovary cells (CHO-m_3 cells) are phosphorylated on serine following agonist stimulation.

2 m_3-Muscarinic receptor phosphorylation is concentration-dependent requiring a carbachol concentration of 13.2 μM for half maximal stimulation.

3 The phosphorylation concentration-response curve lies to the left of the curve for carbachol binding to muscarinic receptors ($K_D = 100$ μM) in membranes from CHO-m_3 cells. In contrast, receptor phosphorylation closely correlates with receptor-mediated phosphoinositidase C activation (EC_{50} for inositol 1,4,5 trisphosphate accumulation during the peak and plateau phases were 7.14 μM and 5.92 μM respectively) but not with rapid agonist-mediated calcium elevation ($EC_{50} = 0.32$ μM) measured in fura-2-AM loaded cells.

4 These data suggest a dissociation of receptor phosphorylation from agonist occupation. Such an apparent 'receptor reserve' for m_3-muscarinic receptor phosphorylation may be indicative of a mechanism that is dependent on a small amplification of the receptor signal, though probably dissociated from the calcium signal.

Keywords: M_3-muscarinic receptor; phosphorylation; calcium mobilisation; inositol 1,4,5 trisphosphate; receptor binding

Introduction

The muscarinic receptor gene family consists of five members, three of which, m_1, m_3 and m_5, are preferentially coupled to phosphoinositidase C probably via the Gq family of guanine nucleotide binding proteins (Caulfield, 1993). We have demonstrated that the human m_3-muscarinic receptor subtype expressed in Chinese hamster ovary (CHO-m_3) cells is phosphorylated on serine following agonist stimulation (Tobin & Nahorski, 1993). The phosphorylation event is rapid, occurring within seconds of agonist addition, and is sustained for at least 30 min. This time course suggests that phosphorylation may mediated rapid changes in receptor activity. Recent studies have demonstrated that phosphoinositide hydrolysis mediated by m_3-muscarinic receptors expressed in CHO-m_3 cells and natively expressed in the human neuroblastoma cell SH-SY5Y undergo a partial desensitization within the first 15-20 s of agonist stimulation (Fisher *et al.*, 1994; Wojcikiewicz *et al.*, 1994). Furthermore, agonist pre-stimulation of CHO-m_3 cells has been shown to attenuate muscarinic receptor mediated inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) and calcium responses (Tobin *et al.*, 1992). The time course for the onset of m_3-muscarinic receptor desensitization correlates with that of receptor phosphorylation suggesting that the two processes may be linked (Wojcikiewicz *et al.*, 1993).

We have previously established that the kinase responsible for m_3-muscarinic receptor phosphorylation is distinct from second messenger activated protein kinases; adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA), guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase, calcium/calmodulin-dependent protein kinase and protein kinase C (PKC) (Tobin & Nahorski, 1993). These studies eliminate a simple feedback mechanism where receptor-generated second messengers, in particular diacylglycerol, activate previously characterized protein kinases that are then able to phosphorylate m_3-muscarinic receptors. Furthermore, *in vitro* studies have also eliminated a role for β-adrenoceptor kinases (β-ARK) (Tobin *et al.*, 1993). This finding is particularly important in light of evidence that β-ARK can phosphorylate a number of G-protein linked receptors, other than β-adrenoceptors, in an agonist-dependent manner (Benovic *et al.*, 1987; Kwatra *et al.*, 1989), including receptors linked to phosphoinositidase C (Kwatra *et al.*, 1993).

An important feature of β-ARK phosphorylation of β-adrenoceptors is the high concentration of agonist required to promote kinase activity (Lohse, 1993). This is in contrast to PKA-mediated phosphorylation of the β-adrenoceptor which is second messenger driven (cyclic AMP) and therefore occurs at lower β-adrenoceptor occupancy due to amplification within the signalling pathway (Lohse *et al.*, 1990). Here we examine this relationship for m_3-muscarinic receptors by comparing receptor occupancy, phosphorylation, agonist regulated $Ins(1,4,5)P_3$ generation and intracellular calcium elevation.

Methods

Cell culture

CHO cell cultures transfected with human m_3-muscarinic receptor cDNA (a kind gift from Dr N.J. Buckley, Wellcome Laboratory for Molecular Pharmacology, Dept. Pharmacology, University College, London) were routinely maintained in αMEM supplemented with penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}), fungizone (2.5 μg ml^{-1}) and newborn calf serum (10% v/v).

Measurement of D-Ins(1,4,5)P_3 mass

Stimulation of CHO-m_3 cells and subsequent analysis of intracellular $Ins(1,4,5)P_3$ levels were carried out essentially as described previously (Tobin *et al.*, 1992). Intact CHO-m_3 cells grown in 24 well dishes were washed in 250 μl Krebs/HEPES buffer (composition mM: HEPES 10, NaCl 118, KCl 4.3, $MgSO_4.7H_2O$ 1.17, KH_2PO_4 1.17, $CaCl_2.2H_2O$ 1.30, $NaHCO_3$

[1] Author for correspondence.

25.0, glucose 11.7, (pH 7.4)) and allowed to stabilize for 10 min at 37°C. The buffer was then replaced with 100 μl Krebs/HEPES buffer containing carbachol for the indicated times. The reaction was stopped with an equal volume of 1 M trichloroacetic acid and Ins(1,4,5)P_3 concentration determined by a radioreceptor assay previously described (Challiss *et al.*, 1988).

Immunoprecipitation of phosphorylated m_3-muscarinic receptors

Labelling of ATP pools in intact CHO-m_3 cells and immunoprecipitation of m_3-muscarinic receptors has been described previously (Tobin & Nahorski, 1993) Briefly, CHO-m_3 cells were harvested using PBS/0.5 mM EDTA and washed twice in phosphate free Krebs/HEPES buffer and resuspended to $1-3\times10^6$ cells ml^{-1}. [^{32}P]-orthophosphate (50 μCi ml^{-1}, final) was added to the cell suspension which was then dispensed into 1 ml aliquots before being left to incubate for 60 min at 37°C. Carbachol was then added directly to the cell suspensions and the incubation continued for 15 min. Stimulations were terminated by rapid centrifugation, aspiration of medium and application of 1 ml of ice cold solubilization buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% NP-40 (Nonidet P40), 0.1% SDS (sodium dodecyl sulphate), 0.5% deoxycholate (pH 7.4). Samples were left on ice for 30 min and then cleared by microcentrifugation. A 3 μl aliquot of purified antiserum 332 (rabbit polyclonal raised against a region of the third intracellular loop of the m_3-muscarinic receptor see Tobin & Nahorski, 1993) was added for 60–90 min. Immunecomplexes were isolated on protein-A-sepharose beads and resolved by 8% SDS-PAGE (SDS-polyacrylamide electrophoresis). The gels were dried and subjected to autoradiography and phosphorylation of m_3-muscarinic receptors assessed with a BioRad model GS 670 densitometer.

Carbachol inhibition of [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding

Carbachol inhibition of [^{3}H]-NMS binding was performed in washed membrane preparations of CHO-m_3 cells in the presence of guanine nucleotides to assess agonist affinity at the free (dissociated from G-proteins) muscarinic receptor (Kenakin, 1993).

Cells were harvested and membranes were prepared in 10 mM HEPES, 10 mM EDTA, pH 7.4 by homogenisation with a Polytron tissue disrupter. The 40,000 *g* membrane pellet was resuspended in binding buffer (10 mM HEPES, 1 mM $MgCl_2$, 100 mM NaCl, pH 7.4). Membranes were freshly prepared prior to each experiment. Reaction tubes containing a range of carbachol concentrations (10^{-7} M–10^{-2} M), ~0.4 nM [^{3}H]-NMS, 100 μM GTP and CHO-m_3 membranes (50 μg protein) in a final volume of 1 ml were prepared. Reactions were started by addition of the CHO-m_3 membranes. Non-specific binding was defined in the presence of 10 μM atropine. Reactions were incubated for 1 h at 37°C and terminated by rapid vacuum filtration through Whatman GF/B filters followed by 2×4 ml washes with cold binding buffer. Filters were removed to scintillation vials containing 5 ml of scintillant and radioactivity was detected >12 h later by liquid scintillation.

Under these conditions, carbachol binding to a single affinity binding site was detected and analysed by use of GraphPad (GraphPad Software Inc. San Diego, CA. U.S.A.).

Measurement of intracellular calcium ($[Ca^2]_i$)

Confluent cultures of cells were harvested in 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA, pH 7.4, washed with Krebs/HEPES buffer and resuspended in 7 ml of Krebs/HEPES buffer. A 1 ml aliquot was removed for determination of cellular autofluorescence in a final volume of 3 ml. To the remaining cells fura-2-AM was added at 5 μM and the cells left with gentle stirring for 30 min at room temperature. Supernatant containing extracellular fura-2-AM was removed following centrifugation of 1 ml aliquots and the cells resuspended in 3 ml of buffer at 37°C. With emission at

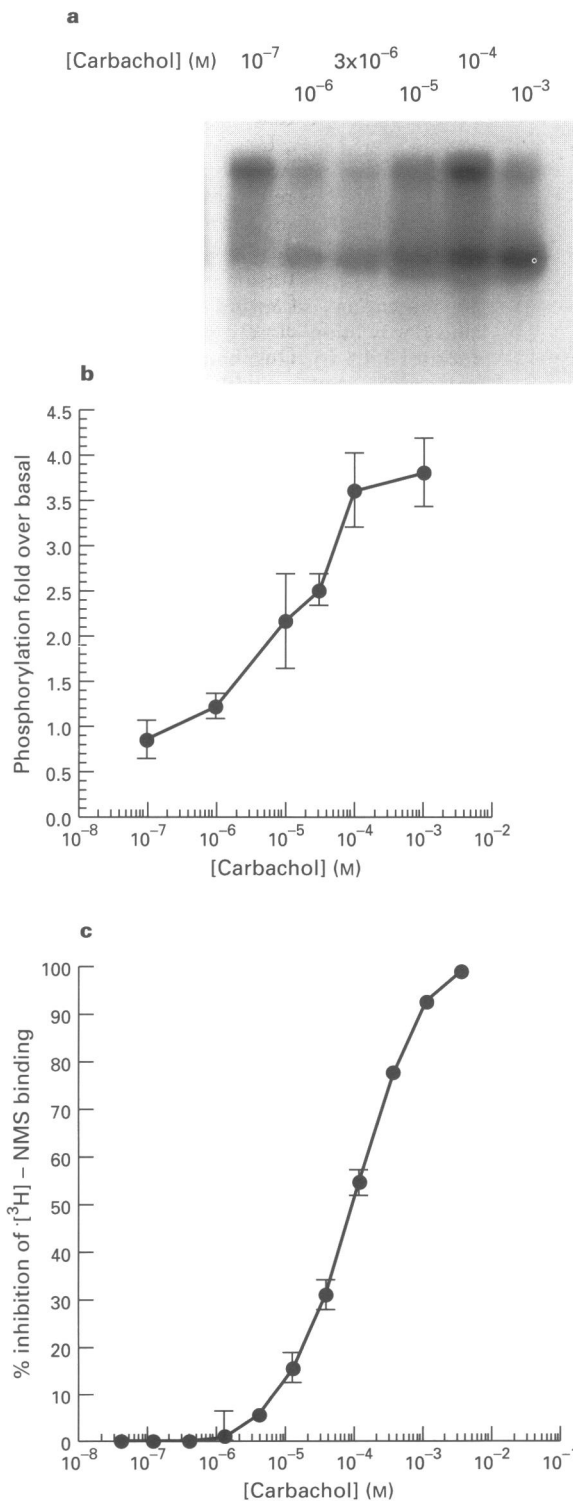

Figure 1 Dose-response curve for m_3-muscarinic receptor phosphorylation and carbachol binding to the m_3-muscarinic receptor. (a) Representative gel of the dose response of carbachol mediated m_3-muscarinic receptor phosphorylation in CHO-m_3 cells. (b) Dose-response curve for m_3-muscarinic receptor phosphorylation. (c) The binding curve for carbachol binding to m_3-muscarinic receptors expressed on CHO-m_3 cells expressed as % inhibition of [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding (see text). The phosphorylation data are mean ± s.e.mean of three experiments.

509 nm and 340/380 nm excitation ratio was recorded every 3.8 s at 37°C as an index of $[Ca^{2+}]_i$. A single concentration of carbachol, in a random sequence, was added to each 3 ml of cells as a 50 μl aliquot. The 340/380 ratio was converted to $[Ca^{2+}]_i$ as previously reported (Grynkiewicx *et al.*, 1985) using 0.1% Triton X-100 in the presence of 1.3 mM Ca^{2+} to determine R_{max} followed by the addition of 6.7 mM EGTA to determine R_{min}.

Curve fitting and statistical analysis

Dose-response curves were fitted by Graph-Pad (Graph-Pad, Software Inc. San Diego, CA, U.S.A.) using a standard four-parameter logistic equation with uniform weighting to all points. Mean EC_{50} values were determined from replicate values derived from individual experiments and are quoted as mean ± standard error of the mean (s.e.mean) of the $\log_{10}$ values. Where appropriate, statistical comparisons have been made by one-way analysis of variance on the $\log_{10}$ data and where $P<0.05$, statistical differences between individual group means were determined by Duncan's multiple range test at $P<0.05$ and $P<0.01$.

Results

Phosphorylation and agonist occupation of the m_3-muscarinic receptor

We have demonstrated previously that m_3-muscarinic receptors expressed in CHO cells transfected with human m_3-muscarinic receptor cDNA (CHO-m_3, $B_{max}=980\pm124$ f-mol mg^{-1} protein ($n=3$)) are phosphorylated under basal conditions and that application of a high dose of a muscarinic agonist dramatically increases the level of phosphorylation (Tobin & Nahorski, 1993). The time course of m_3-muscarinic receptor phosphorylation is rapid reaching a maximum within a minute of agonist application and is sustained for at least 30 min (Tobin & Nahorski, 1993). Consistent with these earlier findings we show here that a maximal concentration of the agonist carbachol (1 mM) increases the level of phosphorylation of the m_3-muscarinic receptor in CHO-m_3 cells by ~4 fold over basal (Figure 1a,b). This has previously been shown to represent ~2 mol of phosphate mol^{-1} of muscarinic receptor (Tobin & Nahorski, 1993). Figure 1b shows the dose-response curve for carbachol-mediated receptor phosphorylation, assessed after 15 min receptor stimulation, which reveals an EC_{50} of 13.2 μM (-4.9 ± 0.20 $\log_{10}$ M ± s.e.mean, $n=3$).

Carbachol displacement of radio-labelled antagonist ([^{3}H]-NMS) binding from membrane preparations of CHO-m_3 cells in the presence of guanine nucleotides was best fitted by a curve representing a single affinity binding site for carbachol with a K_D of 100 μM (-4.00 ± 0.09 $\log_{10}$ M ± s.e.mean, ($n=4$)) and a slope factor of 0.9 ± 0.05 (Figure 1c). These data clearly demonstrate that the occupation curve for the free receptor lies to the right of the dose-response curve for agonist mediated m_3-muscarinic receptor phosphorylation.

Mass Ins(1,4,5)P_3 and intracellular free calcium measurements

Consistent with previous studies (Tobin *et al.*, 1992) the stimulation of intact CHO-m_3 cells with a maximal dose of carbachol (1 mM) resulted in a biphasic production of Ins(1,4,5)P_3 (Figure 2a). Ins(1,4,5)P_3 accumulation peaked after 10 s of agonist application to ~10 fold above basal (basal = 41.8 ± 7.06 pmol mg^{-1} protein, $n=3$), and then fell to a sustained plateau phase ~5 fold over basal after 1 min. The sustained phase of Ins(1,4,5)P_3 generation was maintained for at least 30 min. It is, however, not clear why there is no secondary elevation in Ins (1,4,5)P_3 at ~15 min as reported previously (Tobin *et al.* ,1992).

Dose-response curves for Ins(1,4,5)P_3 were constructed for the responses occurring after 10 and 60 s carbachol stimulation. The EC_{50} s for the early and later phases of Ins(1,4,5)P_3 production were 7.14 μM (-5.26 ± 0.25 $\log_{10}$ M ± s.e.mean, $n=3$) and 5.92 (-5.43 ± 0.32 $\log_{10}$ M ± s.e.mean, $n=3$) respectively (Figure 2b). There was no significant difference between the EC_{50} values for agonist-mediated receptor phosphorylation and the peak/plateau phases of Ins(1,4,5)P_3 production.

Stimulation of CHO-m_3 cells led to a rapid elevation of intracellular calcium that could be detected by a peak of fura-2 fluorescence at 15–25 s. The EC_{50} value of 0.32 μM ($-6.55\pm0.0.14$ $\log_{10}$ M ± s.e.mean, $n=3$) for the rapid peak elevation of $[Ca^{2+}]_i$ (Figure 3) was significantly ($P<0.01$)

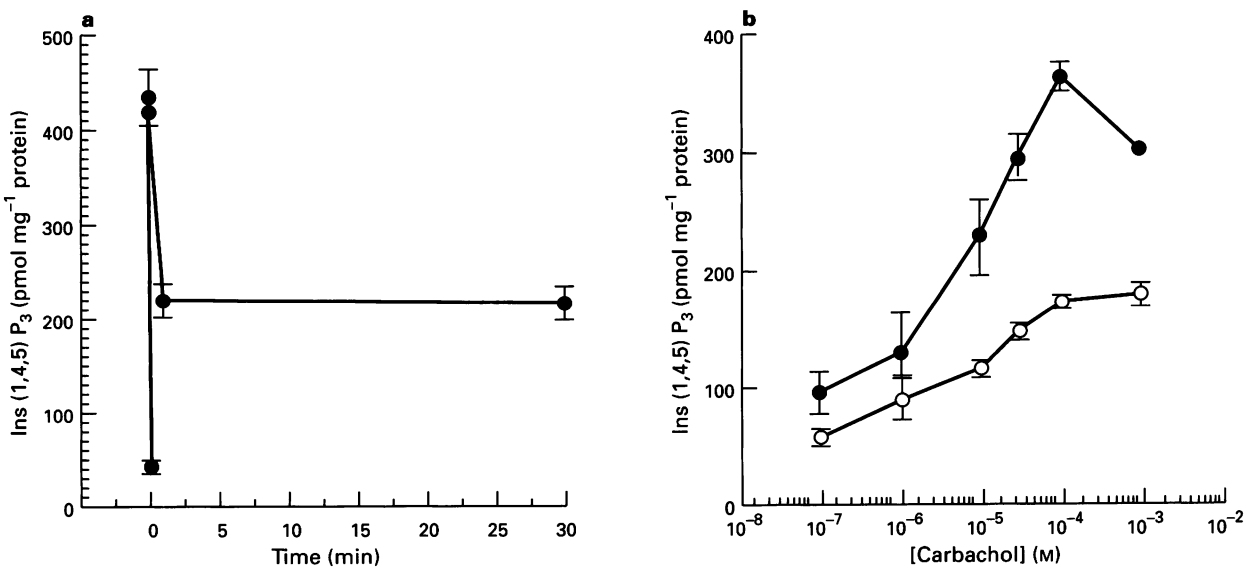

Figure 2 m_3-Muscarinic receptor-mediated generation of Ins(1,4,5)P_3 in CHO-m_3 cells. (a) Time course for Ins(1,4,5)P_3 production induced by 1 mM carbachol in CHO-m_3 cells. (b) The dose-response curves for the generation of Ins(1,4,5)P_3 at 10 s (●) and 60 s (○) stimulation. Results are the mean ± s.e.mean of three experiments.

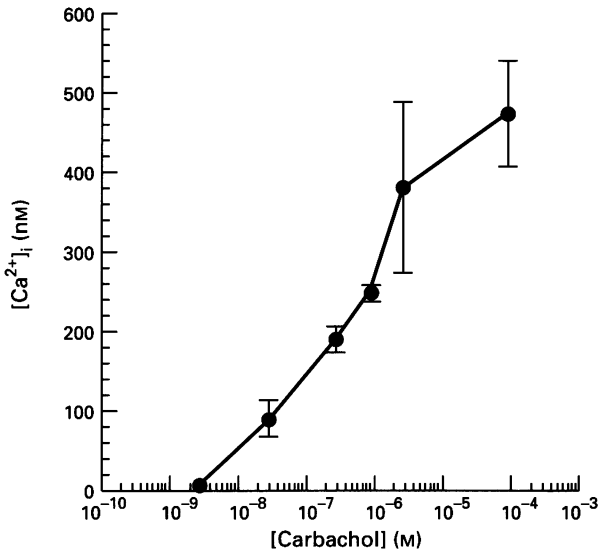

Figure 3 Dose-response curve for carbachol-mediated increase in free intracellular calcium in CHO-m_3 cells. Maximal increases in free intracellular calcium were measured in populations of CHO-m_3 cells loaded with fura-2-AM. The basal calcium concentration was 116 ± 7.0 nM ($n = 18$). Results are the mean ± s.e.mean of three experiments.

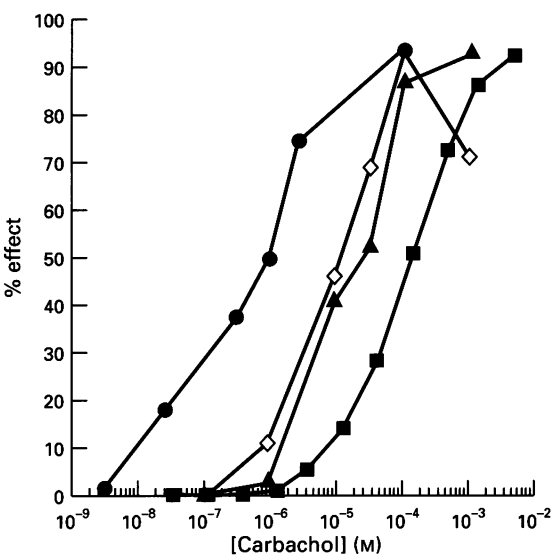

Figure 4 Comparison of carbachol occupancy of the m_3-muscarinic receptor with calcium mobilisation, Ins(1,4,5)P_3 and phosporylation responses. The data for the increase in free intracellular calcium (●), the peak of Ins(1,4,5)P_3 response (◇), the phosphorylation response (▲) and carbachol binding to the m_3-muscarinic receptor (■) were normalized to 100% effect.

lower than the EC_{50} values of both the peak and sustained phases of Ins(1,4,5)P_3 generation and receptor phosphorylation.

Comparison of the potency of carbachol-mediated phosphorylation, Ins(1,4,5)P_3 generation and calcium mobilisation with agonist binding affinity

Figure 4 shows comparison of the dose-response curves for agonist-mediated intracellular calcium elevation, peak Ins(1,4,5,)P_3 production, receptor phosphorylation and agonist occupancy. The data were normalised to 100% effect and show that the dose-response for intracellular calcium elevation lies to the left of the dose-response curve for phosphorylation and Ins(1,4,5)P_3 generation, whereas the agonist occupancy curve lies to the right of the phosphorylation dose-response curve and those for agonist-mediated calcium mobilisation and Ins(1,4,5)P_3 generation.

Discussion

Recent studies from our laboratory have demonstrated that phosphoinositidase C-linked m_3-musacrinic receptors are rapidly phosphorylated on serine following agonist stimulation (Tobin & Nahorski, 1993). Studies with inhibitors have revealed that the kinase involved is likely to be a novel receptor kinase distinct from the known second messenger-regulated kinases and indeed from β-ARK (Tobin & Nahorski, 1993; Tobin *et al.*, 1993). In order to gain further insight into the nature and regulation of this receptor kinase, we have examined the potency of agonist-induced phosphorylation in relation to receptor occupancy. Our data clearly reveal that the dose-response curve for carbachol-mediated phosphorylation lies almost 8 fold to the left of the occupation curve for the free m_3-muscarinic receptor. This suggests that it is unlikely that phosphorylation is directly related to receptor occupancy but that it may be driven by an amplified signal resulting from agonist binding.

Evidence that the agonist occupation curve measured in this study is likely to reflect the agonist binding to the active confirmation of the receptor comes from studies of the cyclic-AMP response mediated by m_3-muscarinic receptors in CHO-m_3 cells. These studies have demonstrated that the EC_{50} value for carbachol-mediated cyclic AMP accumulation is 170 μM (Burford *et al.*, 1995). Since the K_D for carbachol binding to the active form of the receptor can not be less than the EC_{50} of a receptor response these data suggest that the K_D for carbachol binding to the m_3-muscarinic receptor must be at least 170 μM which correlates with the K_D for carbachol binding described in this study (i.e. 100 μM).

The demonstration of a receptor reserve for m_3-muscarinic receptor phosphorylation is particularly important in light of the known activation mechanisms of rhodopsin kinase and β-ARK, the only specific G-protein linked receptor kinases extensively studied to date. Both rhodopsin kinase and β-ARK are activated on association with the agonist-occupied form of their respective receptor substrates (Benovic *et al.*, 1990; Palczewski *et al.*, 1991; Chen *et al.*, 1993). Thus β-ARK and rhodopsin kinase rely on a change in receptor conformation induced by agonist binding to promote an association of the receptor with the kinase and subsequent stimulation of kinase activity (Palczywski & Benovic, 1991). This conformational selectivity ensures that rhodopsin kinase and β-ARK only phosphorylate the agonist occupied form of rhodopsin and β-adrenoceptors, respectively. In the case of β-ARK the lack of a second messenger activator is reflected in the fact that β-ARK phosphorylation of β-adrenoceptors and the subsequent desensitization requires high agonist concentrations (Lohse *et al.*, 1990; Lohse, 1993). Indeed the EC_{50} for isoproterenol mediated β-ARK phosphorylation of the β-adrenoceptor is ~300 nM (see Lohse, 1993) which correlates with isoproterenol receptor occupation, estimated to have a K_D of 100–200 nM (Lohse, 1993; Lohse *et al.*, 1990). This is in contrast with PKA mediated phosphorylation and desensitization of β-adrenoceptors which occurs at low agonist concentrations, correlating with the generation of cyclic AMP which is maximal at concentrations of agonist that occupy only 10% of the β-adrenoceptors (Lohse *et al.*, 1990).

In this study m_3-muscarinic receptor phosphorylation shows a closer correlation with the generation of the second messenger Ins(1,4,5)P_3 than with agonist occupation of the receptor. The EC_{50} for receptor phosphorylation (13.2 μM) and Ins(1,4,5)P_3 production (peak response = 7.14 μM and sustained response = 5.92 μM) were not significantly different. Generation of Ins(1,4,5)P_3 in response to m_3-muscarinic receptor stimulation is due to receptor-mediated dissociation of

the heterotrimeric G-proteins probably of the G_q-family, to yield activated GTP-bound α_q-subunits (Caulfield, 1993). Free α_q-subunits are then able to activate isotypes of PLC_β which catalyse the hydrolysis of phosphoinositide 4,5 bisphosphate to generate $Ins(1,4,5)P_3$ and diacylglycerol (Rhee & Choi, 1992). The receptor reserve observed for the m_3-muscarinic receptor $Ins(1,4,5)P_3$ response is probably attributable to the ability of G-protein linked receptors to activate many G-protein molecules per agonist-occupied receptor. The apparent non-linear relationship between agonist occupation and m_3-muscarinic receptor mediated $Ins(1,4,5)P_3$ accumulation may be complicated by metabolic factors, other than synthesis, that determine a new steady state of the second messenger. However, it may also indicate an amplification at the level of G-proteins, particularly in view of the apparent receptor reserve reported for [^{35}S]-GTPγS (5'-O-(3-thiotriphosphate)) binding to G-proteins in CHO-m_3 cells (Lazareno *et al.*, 1993).

The fact that the phosphorylation response shows a similar dose-response relationship to that of the generation of $Ins(1,4,5)P_3$ further indicates that the kinase is activated downstream of receptor occupancy. Although it is not possible from the present data to establish the mechanism of m_3-muscarinic receptor kinase activation, based on the close correlation with the generation of $Ins(1,4,5)P_3$ one might speculate the involvement of G-protein. The role of βγ-subunits in the translocation of β-ARK to the plasma membrane (Pitcher *et al.*, 1992) certainly sets a precedent for G-protein involvement in G-protein linked receptor phosphorylation. However, βγ-subunit association with β-ARK is thought to offer only a modest 2–4 fold increase in β-ARK activity (Kim *et al.*, 1993). The primary function of β-ARK translocation to the plasma membrane appears not to be kinase activation but rather to bring the kinase in contact with the agonist occupied β-adrenoceptor (Pitcher *et al.*, 1992). Further experiments on purified preparations of the putative muscarinic receptor kinase are presently in progress to investigate the possible involvement of G-proteins in m_3-muscarinic receptor phosphorylation.

Previous studies have demonstrated that m_3-muscarinic receptor phosphorylation could not be initiated by increasing intracellular calcium concentrations using a calcium ionophore, nor could receptor phosphorylation be stimulated by releasing intracellular calcium stores using thapsigargin (Tobin & Nahorski, 1993). These findings indicate that m_3-muscarinic receptor phosphorylation is not promoted by an increase in free intracellular calcium concentration alone and subsequent activation of a calcium-dependent protein kinase. This conclusion is supported by the data presented in the present study where the potency of the agonist-induced increased in free intracellular calcium is ~40 fold greater (EC_{50} = 0.32 μM) than that of agonist-mediated receptor phosphorylation (EC_{50} = 13.2 μM). If calcium was directly involved in activation of the receptor kinase or was an upstream messenger in an activation cascade then carbachol-induced increase in free intracellular calcium would be expected to be equal to or less potent than phosphorylation of the receptor. Since the opposite is true then calcium is unlikely to have any direct role in stimulation receptor kinase activity.

Involvement of the diacylglycerol/PKC arm of the phosphoinositide signal transduction pathway in agonist mediated m_3-muscarinic receptor phosphorylation has been eliminated previously in studies where agonist-mediated receptor phosphorylation was unaffected by the potent PKC inhibitor, RO-318220, at concentrations that suppressed phorbol ester-mediated phosphorylation (Tobin & Nahorski, 1993).

In summary, this study reveals that m_3-muscarinic receptor expressed in CHO cells undergo phosphorylation that is dependent on agonist concentration. The potency of carbachol is greater than its binding affinity to the receptor but closely correlates with the potency of the agonist mediating $Ins(1,4,5)P_3$ accumulation. These data suggest that, unlike β-ARK, events slightly amplified down stream of the receptor mediate muscarinic receptor kinase activation. On the other hand our data do not indicate phosphorylation is driven by a second messenger-regulated kinase as sensitive as PKA at the β-adrenoceptor (see Lohse, 1993). The precise mechanism of phosphorylation awaits ongoing studies with the purified muscarinic receptor kinase and mutants of the m_3-muscarinic receptor lacking potential sites of phosphorylation.

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