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Evidence for cross-talk between M_2 and M_3 muscarinic acetylcholine receptors in the regulation of second messenger and extracellular signal-regulated kinase signalling pathways in Chinese hamster ovary cells

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1 We have examined possible mechanisms of cross-talk between the $G_{q/11}$-linked M_3 muscarinic acetylcholine (mACh) receptor and the $G_{i/o}$-linked M_2 mACh receptor by stable receptor coexpression in Chinese hamster ovary (CHO) cells. A number of second messenger (cyclic AMP, Ins(1,4,5)P_3) and mitogen-activated protein kinase (ERK and JNK) responses stimulated by the mACh receptor agonist methacholine were examined in CHO-m2m3 cells and compared to those stimulated in CHO-m2 and CHO-m3 cell-lines, expressing comparable levels of M_2 or M_3 mACh receptors.

2 Based on comparisons between cell-lines and pertussis toxin (PTx) pretreatment to eliminate receptor-$G_{i/o}$ coupling, evidence was obtained for (i) an M_2 mACh receptor-mediated contribution to the predominantly M_3 mACh receptor-mediated Ins(1,4,5)P_3 response and (ii) a facilitation of the inhibitory effect of M_2 mACh receptor on forskolin-stimulated cyclic AMP accumulation by M_3 mACh receptor coactivation at low agonist concentrations (MCh 10^{-9} – 10^{-6} M).

3 The most profound cross-talk effects were observed with respect to ERK activation. Thus, while MCh stimulated ERK activation in both CHO-m2 and CHO-m3 cells (pEC_{50} values: 5.64 ± 0.09 and 5.57 ± 0.16, respectively), the concentration – effect relation was approx 50-fold left-shifted in CHO-m2m3 cells (pEC_{50}: 7.17 ± 0.07). In addition, the ERK response was greater and more sustained in CHO-m2m3 cells. In contrast, only minor differences were seen in the time-courses and concentration-dependencies of JNK activation in CHO-m3 and CHO-m2m3 cells.

4 Costimulation of endogenous $P2Y_2$ purinoceptors also caused an approx 10-fold left-shift in the MCh-stimulated ERK response in CHO-m2 cells, suggesting that the $G_{q/11}/G_{i/o}$ interaction to affect ERK activation is not specific to muscarinic receptors.

5 PTx pretreatment of cells had unexpected effects on ERK activation by MCh in both CHO-m2m3 and CHO-m3 cells. Thus, in CHO-m3 cells PTx pretreatment caused a marked left-shift in the MCh concentration – effect curve, while in PTx-treated CHO-m2m3 cells the maximal responsiveness was decreased, but the potency of MCh was only slightly affected.

6 The data presented here strongly suggest that cross-talk between M_2 and M_3 mACh receptors occurs at the level of both second messenger and ERK regulation. Further, these data provide novel insights into the involvement of $G_{i/o}$ proteins in both positive and negative modulation of ERK responses evoked by G protein-coupled receptors.

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Abbreviations: CCh, carbachol; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-[γ-thio]triphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; JNK, c-Jun *N*-terminal kinase; mACh, muscarinic acetylcholine; MAPK, mitogen-activated protein kinase; MCh, methacholine; NMS, *N*-methyl-scopolamine; PLC, phosphoinositide-specific phospholipase C; PTx, pertussis toxin; UTP, uridine 5′-triphosphate

Introduction

Acetylcholine, as a neurotransmitter, and as a possible non-neuronal mediator (Wessler *et al*., 1998), exerts many of its actions via interaction with one or more of the five mammalian muscarinic acetylcholine (mACh) receptor subtypes (Bonner *et al*., 1987; Caulfield & Birdsall, 1998). The mACh receptors can be grouped into two subsets,

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based on sequence similarities and receptor-G protein-effector coupling preferences (Wess, 1993; Caulfield & Birdsall, 1998). Thus, M_1, M_3 and M_5 mACh receptors preferentially couple via $G_{q/11}$ proteins to the activation of phospholipase C (PLC), while M_2 and M_4 mACh receptors link to downstream effectors via $G_{i/o}$ protein activation (e.g. to inhibit adenylyl cyclase). Despite such apparent exclusivity between the signalling pathways activated by $M_1/M_3/M_5$ and M_2/M_4 mACh receptors, there are many opportunities for 'cross-talk' (Eglen *et al*., 1994; Challiss & Blank, 1997; Selbie & Hill, 1998). For example, M_2/M_4 mACh receptors can affect signalling mediated by $M_1/M_3/M_5$ mACh receptors through G_i-derived $\beta\gamma$-subunit effects on PLCs (Rhee, 2001) or modulate changes in intracellular Ca^{2+} concentrations through altering K^+-conductance (Kurachi, 1995), or activating nonselective cation channels (Zholos & Bolton, 1997; Wang *et al*., 1999). However, interpretation of potential cross-talk is complicated by the demonstration that $M_1/M_3/M_5$ mACh receptors can also couple to pertussis toxin (PTx) -sensitive G proteins (Offermanns *et al*., 1994; Akam *et al*., 2001) and the reported stimulatory effects of M_1 (and M_3/M_5) mACh receptors on adenylyl cyclase activity probably result from direct receptor coupling to G_s proteins (Burford & Nahorski, 1996).

Compelling evidence has accumulated in recent years linking a wide range of G protein-coupled receptors (GPCRs) to the control of mitogen-activated protein kinases (MAPKs) and, as a consequence, the regulation of cell growth and proliferation (Dhanasekaran *et al*., 1995; Gutkind, 1998; Gudermann *et al*., 2000). In the case of mACh receptors, activation of MAPK pathways has been demonstrated for both the $M_1/M_3/M_5$ and M_2/M_4 subfamilies (Winitz *et al*., 1993; Crespo *et al*., 1994; Mitchell *et al*., 1995), suggesting the potential for mACh receptor cross-talk in the regulation of extracellular signal-regulated kinase (ERK) and c-Jun *N*-terminal kinase (JNK) cascades (Schaeffer & Weber, 1999).

Understanding the mechanisms by which cross-talk between mACh receptors may occur is clearly an important objective, as many cells and tissues express more than one mACh receptor subtype. For example, a wide variety of smooth muscle types express both M_2 and M_3 mACh receptors (see Eglen *et al*., 1994; 1996) and the respective roles of each subtype have been the subject of much investigation and speculation. The role of the M_3 mACh receptor in smooth muscle contraction is well established (Eglen *et al*., 1994); however, the role of the usually more abundant M_2 mACh receptor subpopulation is less clear (Eglen *et al*., 1994; 1996). With respect to the regulation of muscle contraction, pharmacological (Hegde *et al*., 1997; Sawyer & Ehlert, 1999) and genetic/gene knockout (Matsui *et al*., 2000; Stengel *et al*., 2000; 2002) approaches have shown either small direct effects of the M_2 population on contraction or, more commonly, an indirect role through inhibition of the actions of relaxants. To date, very little work has been carried out to analyse the potential for cross-talk between signalling pathways following M_2 and M_3 mACh receptor coactivation.

In the present study we have utilized Chinese hamster ovary (CHO) cells recombinantly expressing M_2 and M_3 mACh receptors, either alone or in combination, to investigate the possible cross-talk between the second messenger-generating and ERK- and JNK-signalling pathways downstream of these receptors.

Methods

Materials

1-[*N*-methyl-^{3}H]-scopolamine (80 – 85 Ci mmol^{-1}), [2,8-^{3}H]-cyclic AMP (30 – 50 Ci mmol^{-1}), [*methyl*-^{3}H]-thymidine (25 Ci mmol^{-1}) and [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) were obtained from Amersham Biosciences U.K. Ltd. (Little Chalfont, U.K.). Guanosine 5′-(γ-thio-[^{35}S])triphosphate (1250 Ci mmol^{-1}) and D-[inositol-1-^{3}H(N)]-1,4,5-trisphosphate (30 Ci mmol^{-1}) were obtained from NEN Life Science Products (Zaventem, Belgium). PTx, methacholine (MCh), carbachol (CCh) and forskolin were from Sigma-Aldrich Co. Ltd. (Poole, U.K.). All other reagents were of analytical grade and were obtained from the sources given by Wylie *et al*. (1999) and Akam *et al*. (2001).

Cell culture and transfection

CHO cell-lines expressing human M_2 (CHO-m2) or M_3 (CHO-m3) mACh receptors were originally obtained from Dr N.J. Buckley (then of the National Institute for Medical Research, Mill Hill, London) and were grown in minimal essential medium (MEMα) supplemented with newborn calf serum (10%), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and amphotericin B (2.5 μg ml^{-1}). To generate M_2/M_3-coexpressing cell-lines, cDNA encoding the human m3-mACh receptor was inserted into a pCEP4 vector (Invitrogen BV, Groningen, The Netherlands). Low passage CHO-m2 cells were transfected with this plasmid by calcium phosphate – DNA coprecipitation (Sambrook *et al*., 1989). After 24 h, cells were harvested and seeded into Petri dishes at various cell densities and grown in the constant presence of 400 – 800 μg ml^{-1} hygromycin B to select for antibiotic-resistant colonies. Colonies were selected after 21 – 28 days and expression of M_3 mACh receptor initially assessed by Western blotting using an anti-m3-mACh receptor-specific antibody (Tobin & Nahorski, 1993). M_3 mACh receptor-positive clones were expanded and radioligand binding and Ins(1,4,5)P_3 mass determinations made to verify expression and function of this receptor, while M_2 mACh receptor function was assessed by inhibition of forskolin-stimulated adenylyl cyclase (see below). Based on these criteria, three cell-lines were archived and the B2 clone selected for the most extensive further characterization.

[^{3}H]-NMS saturation and displacement radioligand binding

CHO cell-lines expressing M_2 and/or M_3 mACh receptors were grown to confluence in 175 cm^2 flasks and harvested using HBS – EDTA (10 mM HEPES, 0.9% NaCl, 0.02% EDTA, pH 7.4). A cell-membrane fraction was then prepared by homogenization and centrifugation, and [^{3}H]-NMS saturation binding performed as described previously (Burford & Nahorski,1996; Akam *et al*., 2001), except that GTP was omitted and membranes were incubated with the radioligand for 90 min at 37°C before separating bound and free fractions by rapid vacuum filtration. Nonspecific binding was defined in the presence of 1 μM atropine. Displacement analysis for tripitramine was performed using approx 0.8 nM [^{3}H]-NMS and a wide range of antagonist concentrations (0.1 nM – 100 μM); again incubations were for 90 min at 37°C.

[^{35}S]-GTPγS binding and G_qα immunoprecipitation

Membranes prepared from CHO-m2, -m3 and -m2m3 cells were incubated with approx 1 nM [^{35}S]-GTPγS in a buffer containing 100 mM NaCl, 1 μM GDP ± agonist for 2 min at 30°C. Residual binding in the presence of 10 μM GTPγS was considered nonspecific (NSB) and subtracted from basal and agonist-stimulated total counts. Incubations were terminated, membranes solubilized and G proteins immunoprecipitated using an 'in-house' G_qα-specific antibody (raised against the C-terminal sequence CLQLNLKEYNLV) exactly as described previously by Akam *et al*. (2001). Scintillation fluid was added to thoroughly washed protein A-sepharose-G_qα-[^{35}S]-GTPγS complexes and radioactivity determined.

Incubation methods and Ins(1,4,5)P_3 and cyclic AMP determination

For experiments in intact cells, the different CHO cell-lines were grown to confluence in 24-well multiwells. Where cells were pretreated with PTx, it was added (100 ng ml^{-1}) for 20 – 24 h before experimentation. For all experiments, monolayers were washed with HEPES-buffered Krebs – Henseleit buffer (KHB: composition in mM: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; $MgSO_4$, 1.2; NaH_2PO_4, 1.2, $CaCl_2$, 1.3; D-glucose, 11; HEPES, 10; pH 7.4 after equilibration with O_2/CO_2 95 : 5). Cells were challenged with the concentrations of agonists, and for the times indicated in the Results section. Incubations were terminated by aspiration and rapid addition of 250 μl ice-cold 0.5 M trichloroacetic acid (TCA) and transferal of the multiwell plate to an icebath. After extraction for 30 min, TCA extracts were collected and neutralized using tri-*n*-octylamine/freon (Challiss *et al*., 1988). Cyclic AMP and Ins(1,4,5)P_3 were determined using the radioreceptor assays of Brown *et al*. (1971) and Challiss *et al*. (1988), respectively.

ERK and JNK activity determinations

CHO-m2, -m3 or -m2m3 cells were grown in six-well plates. Where cells were pretreated with PTx, it was added (100 ng ml^{-1}) for 20 – 24 h before experimentation. Incubations were performed in KHB as described above. Incubations were terminated by aspiration and washing each well with ice-cold phosphate-buffered saline. Cells were processed for ERK and JNK assay exactly as described previously (Wylie *et al*., 1999) with 200 μl (ERK) and/or 400 μl (JNK) aliquots of supernatant being taken for subsequent activity assays. ERK proteins were immunoprecipitated and activity assays performed exactly as described previously (Wylie *et al*., 1999). GST-c-Jun beads were prepared and used to determine JNK activity exactly as previously described (Wylie *et al*., 1999).

Data analysis

All data are presented as means ± s.e. mean for the indicated number of separate experiments performed with the indicated individual experiment replication. Radioligand binding data and agonist/antagonist concentration – effect curves were analysed using a commercially available program (GraphPad Prism version 3.0; GraphPad Software, San Diego, CA, U.S.A.). Where responses to MCh were biphasic (see cyclic AMP data), concentration – response relations were fitted by a sum of two logistic equations:

$$f = I_{max} + F_s\left\{1 - \frac{c^{n_1}}{c^{n_1} + K_1^{n_1}}\right\} + S_{max}\left\{\frac{c^{n_2}}{c^{n_2} + K_2^{n_2}}\right\}$$

where I_{max} is the maximum inhibition of the forskolin-stimulated adenylyl cyclase activity by MCh (F_s) with IC_{50} value K_1 and Hill coefficient n_1; S_{max} is the maximum stimulation of cAMP accumulation by high concentrations of MCh with EC_{50} value and Hill coefficient K_2 and n_2, respectively, and c is the concentration of MCh. Fitting was done by least-squares minimization using the above equation and GraphPad Prism (version 3.0).

Statistical differences between datasets were assessed by one-way analysis of variance followed by Duncan's multiple-range test at $P < 0.05$ using SPSS (version 10, SPSS, Chicago, IL, U.S.A.).

Results

Establishing CHO-m2m3 cell-lines

CHO cells stably expressing M_2 mACh receptors (CHO-m2; B_{max} 858 ± 51 pmol mg^{-1} protein) were transfected with a plasmid encoding the M_3 mACh receptor. Hygromycin-resistant clones were initially assessed by Western blotting using a M_3-specific antibody (Tobin & Nahorski, 1993) and cells expressing M_3 mACh receptor immunoreactivity screened for second messenger responses by assessing the ability of MCh to stimulate Ins(1,4,5)P_3 generation and inhibit forskolin-stimulated cyclic AMP accumulation. Two cell-lines (termed B2 and B7) emerged from this screening and were evaluated for total mACh receptor expression by performing [^{3}H]-NMS binding on suspensions of intact cells. In addition, to subdivide the M_2/M_3 subpopulations in B2 and B7 cell-lines, displacement of specific [^{3}H]-NMS binding from membranes by the highly M_2-selective antagonist tripitramine (Melchiorre *et al*., 1993) was assessed. [^{3}H]-NMS displacement analysis in membranes derived from CHO-m2 and CHO-m3 cells demonstrated tripitramine to be highly discriminating (approx 400 fold) between M_2 (pK_i 9.4) and M_3 (pK_i 6.8) mACh receptors. The results of these binding studies are summarized in Table 1. It can be seen that for both the B2 and B7 cell-lines there is an approx 3 : 1 $M_3 : M_2$ ratio. The CHO-m2m3 B2 cell-line was selected for further study. Key findings of this study were reproduced in the B7 cell-line to provide evidence against clonal variation underlying any of the observed effects.

[^{35}S]-GTPγS binding and Gα-subunit-selective immunoprecipitation to assess receptor – $G_{q/11}$ coupling

Receptor coupling to G_q proteins was initially investigated in membranes prepared from CHO-m2, -m3 or -m2m3 (B2) cell-lines by [^{35}S]-GTPγS binding and subsequent immunoprecipitation using a G_qα-specific antibody (Akam *et al*., 2001). Membranes prepared from CHO-m3, but not -m2 cells showed robust agonist-stimulated increases in [^{35}S]-GTPγS associated with immunoprecipitated G_qα (Figure 1). A comparable increase in [^{35}S]-GTPγS binding to G_qα was observed in CHO-m2m3 membranes and MCh stimulated this response with similar potencies in the two preparations (pEC_{50} values: CHO-m3, 4.49 ± 0.34; CHO-m2m3, 4.29 ± 0.37). PTx pretreat-

Table 1 Assessment of receptor expression levels in CHO-m2, -m3 and -m2m3 cell-lines by [^{3}H]-NMS saturation binding and quantitation of M_2 and M_3 mACh receptor subpopulations in B2 and B7 clones

Cell-line	B_{max} *(pmol mg^{-1} protein)*	*Ratio M_2 : M_3*	*No. expts. (n)*
CHO-m2	0.86±0.05	100 : 0	4
CHO-m3	1.48±0.12	0 : 100	4
CHO-m2m3 (B2)	2.16±0.21	27 : 73	6
CHO-m2m3 (B7)	2.28±0.26	30 : 70	3

B_{max} values are expressed as means±s.e. mean for the indicated number (n) of experiments. M_2 : M_3 ratios were determined by two-site analysis (GraphPad Prism) of tripitramine displacement isotherms for a single concentration of [^{3}H]-NMS (approx 0.8 nM).

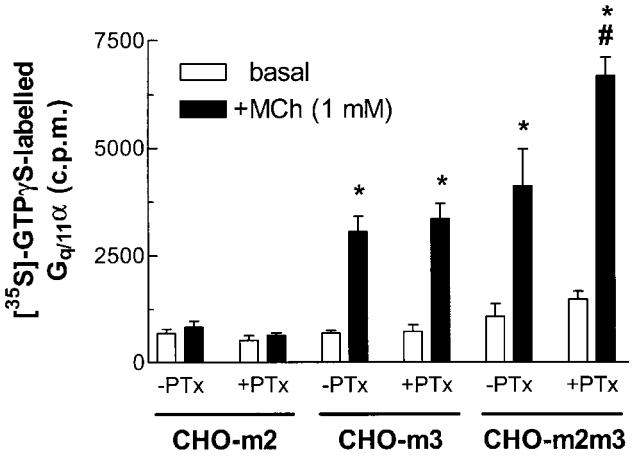

Figure 1 Effects of PTx pretreatment on basal and MCh-stimulated [^{35}S]-GTPγS binding to $G_{q/11}\alpha$ in membranes prepared from CHO-m2, -m3 and -m2m3 cells. CHO cells were preincubated in the presence of PTx (100 ng ml^{-1}; 24 h) or vehicle before preparation of membranes. MCh (1 mM) or buffer additions to membranes were for 2 min at 30°C and, following solubilization, [^{35}S]-GTPγS-$G_{q/11}\alpha$ complexes were immunoprecipitated and radioactivity assessed as described in the Methods section. Data are shown as means±s.e. mean for four separate experiments performed in duplicate. For both CHO-m3 and CHO-m2m3 membranes, a significant ($^*P<0.05$) increase in $G_{q/11}\alpha$-associated [^{35}S]-GTPγS binding was stimulated by MCh irrespective of PTx pretreatment. In addition, following PTx pretreatment a greater agonist-stimulated $G_{q/11}\alpha$-[^{35}S]-GTPγS binding was observed ($^{\#}P<0.05$) in CHO-m2m3, but not CHO-m2 or -m3 membranes.

ment prior to membrane preparation only appeared to affect receptor-G protein coupling in the case of the CHO-m2m3 cell-line. Thus, MCh stimulated an approx 70% greater accumulation of [^{35}S]-GTPγS – $G_q\alpha$ complexes in membranes prepared from PTx pretreated CHO-m2m3 cells (Figure 1), despite the increase in E_{max}, the potency of MCh was similar in PTx-treated M_3-expressing cell-lines (pEC_{50} values: CHO-m3, 4.29±0.31; CHO-m2m3, 4.20±0.14).

Comparison of Ins(1,4,5)P_3 responses in CHO-m2, -m3 and -m2m3 cell-lines

MCh (1 mM) stimulated robust increases in Ins(1,4,5)P_3 accumulation in the CHO-m3 and -m2m3 cell-lines (Figure 2a), but not in CHO-m2 cells where no agonist-stimulated increase in this second messenger was observed. As can be seen in Figure 2, the amplitude of the increase in Ins(1,4,5)P_3 accumulation was greater in the CHO-m2m3 cell-line, with both a greater peak (25±9%) and plateau response being consistently observed. Examination of the concentration-dependency of the initial peak response (at 15 s; Figure 2b) confirmed the greater peak increase in Ins(1,4,5)P_3 in the CHO-m2m3 cell-line and also demonstrated a three-fold decrease in the EC_{50} value in this cell-line (pEC_{50} values: CHO-m3, 5.21±0.10; CHO-m2m3, 5.67±0.11; $P<0.05$). As we have shown previously (Wylie *et al*., 1999) PTx pretreatment of CHO-m3 cells had no effect on either the time-course (data not shown) or concentration-dependency of Ins(1,4,5)P_3 accumulation (Figure 2c). Interestingly, PTx treatment of CHO-m2m3 cells did affect the Ins(1,4,5)P_3 response, generating E_{max} and EC_{50} values in this cell-line that were indistinguishable from those obtained in control CHO-m3 cells (Figure 2d).

Effects of PTx pretreatment on basal and agonist-stimulated cyclic AMP responses in CHO-m2m3 cells

MCh (1 mM) stimulated a robust (eight-fold) increase in cyclic AMP accumulation in CHO-m2m3 cells and PTx pretreatment affected this second messenger response (Figure 3a). Thus, the cyclic AMP response to MCh was approx two-fold greater in PTx pretreated CHO-m2m3 cells, without a change in the concentration-dependency assessed at the 5 min peak (pEC_{50} values: −PTx, 4.24±0.22; +PTx, 4.38±0.19; Figure 3b). The data shown in Figures 2 and 3 provide evidence consistent with a M_2 mACh receptor-G_i modulation of both Ins(1,4,5)P_3 and cyclic AMP responses in the CHO-m2m3 cell-line.

Modulatory effects of mACh receptor activation on forskolin-stimulated adenylyl cyclase activity in CHO-m2, -m3 and -m2m3 cells

The adenylyl cyclase activator forskolin (10 μM) stimulated comparable, large (>100 fold) increases in cyclic AMP accumulations in CHO-m2, -m3 and -m2m3 cell-lines. The effects of increasing concentrations of MCh on forskolin-stimulated cyclic AMP responses in CHO-m2, -m3 and -m2m3 cells are shown in Figure 4. In CHO-m2 cells, MCh concentration-dependently inhibited forskolin-stimulated cyclic AMP accumulation (pIC_{50}: 6.60±0.06) with a maximally effective concentration of agonist causing a >90% inhibition. In both CHO-m3 and -m2m3 cells, a biphasic modulation of forskolin-stimulated adenylyl cyclase activity by MCh was observed (Figure 4). At low agonist concentrations, inhibitory effects were observed that were maximal between 0.1 and 1 μM MCh for both cell-lines, whereas at higher MCh concentrations this effect was superseded by an enhancement of the

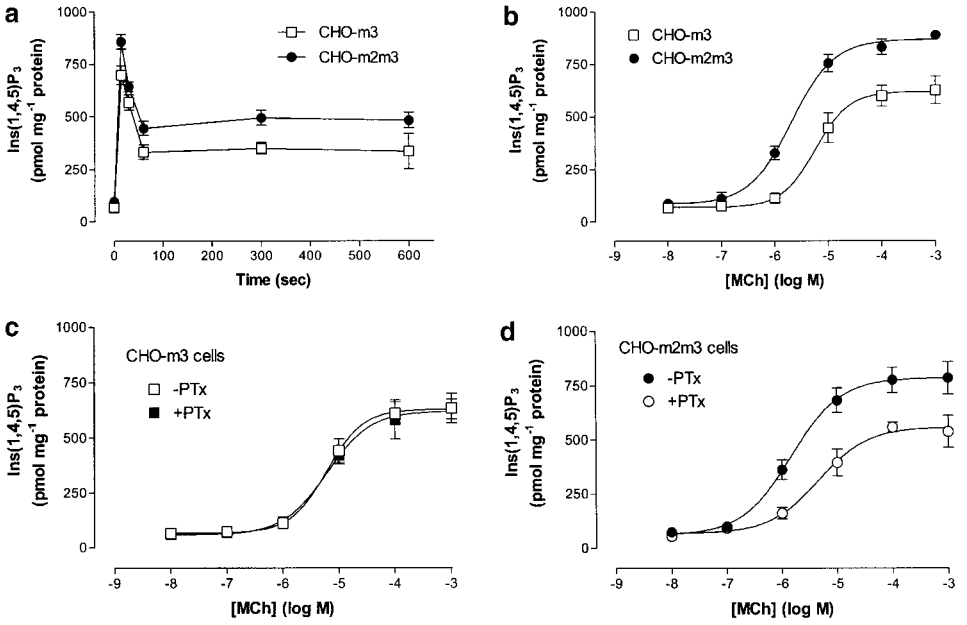

Figure 2 Comparison of time- and concentration-dependent agonist-stimulated Ins(1,4,5)P_3 accumulations in CHO-m3 and -m2m3 cells. Cell monolayers were stimulated with either MCh (1 mM) for the times indicated (a), or for 15 s with different concentrations of MCh (b). CHO-m3 (c) or CHO-m2m3 (d) cells were pretreated with PTx (100 ng ml^{-1}, 24 h) or vehicle before challenge with the indicated concentrations of MCh for 15 s. Incubations were terminated and extracts prepared for Ins(1,4,5)P_3 mass determination as described in the Methods section. Data are shown as means ± s.e. mean for three (panels a and c) or four (panels b and d) separate experiments performed in duplicate.

forskolin response. Although the modulatory effects of MCh were similar in the CHO-m3 and -m2m3 cell-lines, subtle differences could be observed. Thus, analysis of the curves (using the equation given in the Methods) revealed that K_I values (see Data analysis section for K_I definition) for MCh-mediated inhibitions were 22 ± 5 *versus* 38 ± 5 nM in CHO-m2m3 and -m3 cells, respectively, and MCh caused a significantly greater maximum inhibitory effect (66 ± 3% *versus* 42 ± 4%; $P<0.05$) in CHO-m2m3 compared to CHO-m3 cells over the $10^{-9}-10^{-6}$ M concentration range (see Figure 4 inset).

Further evidence that both M_2 and M_3 mACh receptors shape the modulatory effects of MCh on forskolin-stimulated adenylyl cyclase activity in the CHO-m2m3 cell-line is provided in Figure 5. Thus, while PTx treatment of CHO-m3 cells has no significant effect on the MCh modulation of cyclic AMP levels, a marked attenuation of the inhibitory effect seen at low MCh concentrations was observed in the CHO-m2m3 cell-line (Figure 5). These data together strongly suggest that M_2 and M_3 mACh receptors cooperate in the regulation of adenylyl cyclase activity at low agonist concentrations, whereas at high agonist concentration the adenylyl cyclase stimulatory effect mediated by the M_3 mACh receptor predominates.

Time- and concentration-dependent effects of M_2 and M_3 mACh receptor stimulation on ERK and JNK activities

MCh stimulation of CHO-m2 and -m3 cells resulted in rapid, robust (15 – 20 fold-over-basal) increases in ERK activity that peaked at 5 min and had returned to close to basal levels within 30 min (Figure 6a). The stimulatory effect of MCh was concentration-dependent (assessed at 5 min) generating EC_{50} values of 2 – 3 μM for each cell-line (pEC_{50} values: CHO-m2, 5.64 ± 0.09; CHO-m3, 5.57 ± 0.16; Figure 6b). Strikingly, two potentially important differences were observed in the CHO-m2m3 cell-line. Firstly, the magnitude of the ERK response was greater and the increase in activity sustained over a longer period (Figure 6a). Thus, whereas ERK activity had returned to close to basal levels in CHO-m2 and -m3 cells (2.4 ± 0.4 and 2.5 ± 0.6-fold-over-basal, respectively) after 30 min exposure to agonist, it was still substantially elevated in CHO-m2m3 cells (8.2 ± 0.8-fold-over-basal; $P<0.05$ compared to CHO-m2 and -m3 cells, Figure 6a). Secondly, the concentration – effect curve for ERK activation by MCh was >25-fold left-shifted (pEC_{50}: 7.17 ± 0.07; $P<0.05$ compared to CHO-m2 and -m3 cells, Figure 6b). These key differences were also seen in parallel experiments using the CHO-m2m3 B7 clone (data not shown).

With respect to JNK activity, MCh addition to CHO-m2 cells failed to cause a reproducible increase in the activity of this MAPK, whereas stimulation of CHO-m3 and -m2m3 cells produced time- and concentration-dependent increases in JNK activity (Figure 7). MCh stimulated similar maximal five to six-fold increases in JNK activity in both CHO-m3 and -m2m3 cells at 45 min after agonist addition, although basal JNK activity was consistently higher in the coexpressing cell-line (CHO-m3, 1.01 ± 0.38; CHO-m2m3, 2.25 ± 0.05 pmol phosphate incorporated into c-Jun min^{-1} mg^{-1} protein; $P<0.05$). The concentration-dependency of JNK activation by MCh was 2.5-fold left-shifted in the coexpressing cell-line (pEC_{50} values: CHO-m3, 6.11 ± 0.11; CHO-m2m3, 6.55 ± 0.13; $P<0.05$) compared to the >25-fold shift noted for ERK activation in the coexpressing cell-line.

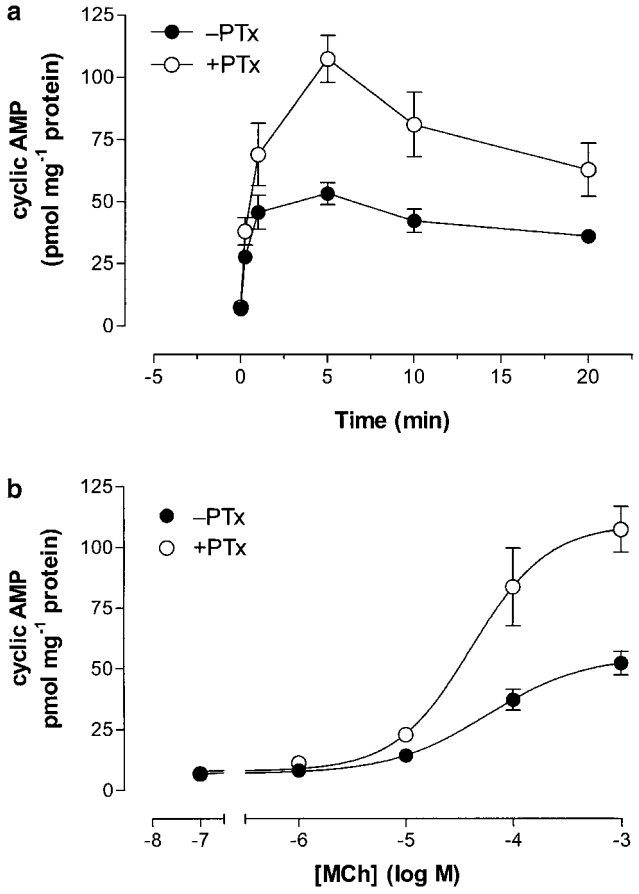

Figure 3 Effects of PTx pretreatment on time- and concentration-dependencies of MCh-stimulated cyclic AMP accumulations in CHO-m2m3 cells. CHO-m2m3 cell monolayers were pretreated with PTx (100 ng ml^{-1}, 24 h) or vehicle before challenge with either 1 mM MCh for the times indicated (a), or with different concentrations of MCh for 5 min (b). Incubations were terminated and extracts prepared for cyclic AMP mass determination as described in the Methods section. Data are shown as means ± s.e. mean for three separate experiments performed in duplicate.

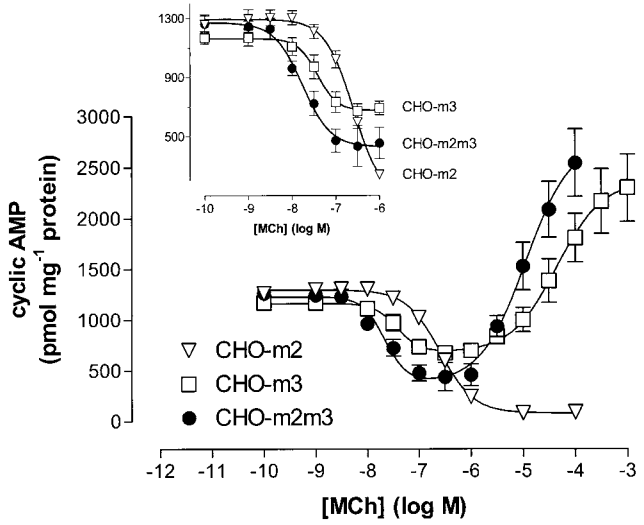

Figure 4 Effects of M_2 and/or M_3 mACh receptor activation on forskolin-stimulated cyclic AMP accumulations in CHO-m2, -m3 and -m2m3 cells. The indicated concentrations of MCh were added to cell monolayers for 10 min before challenge with forskolin (10 μM). After a further period of 10 min incubations were terminated and cyclic AMP mass determined as described in the Methods section. Data are shown as means ± s.e. mean for three (CHO-m2) or four (CHO-m3 and -m2m3) separate experiments performed in duplicate. The inset focuses on the inhibitory effects of 10^{-9} – 10^{-6} M MCh in the three cell-lines.

Coactivation of P2Y and M_2 mACh receptors also facilitates ERK activation

CHO cells possess endogenous $P2Y_2$ purinoceptors that can mediate an ERK response (Dickenson *et al*., 1998). This $G_{q/11}$-coupled receptor caused a modest concentration-dependent increase in ERK activity in the CHO-m2 cell-line (pEC_{50}: 5.40 ± 0.34; E_{max}, 5.0 ± 0.5-fold-over-basal) in response to UTP (Figure 8 inset). Stimulation of CHO-m2 cells with MCh in the presence of UTP (10 μM) produced a concentration – effect curve for ERK activation, which was approx 10-fold left-shifted (pEC_{50} values: −UTP, 5.53 ± 0.24; +UTP, 6.51 ± 0.31; $P<0.05$) without significantly affecting the maximal response (Figure 8). Thus, both the native UTP receptor and recombinant M_3 mACh receptor increase the apparent potency of MCh at the M_2 mACh receptor to cause ERK activation.

Effects of PTx on the concentration-dependent activation of ERK by MCh in CHO-m2, -m3 and -m2m3 cells

The robust ERK activation stimulated by incubation of CHO-m2 cells with MCh (100 μM) for 5 min was completely abolished by PTx pretreatment (Figure 9a). Therefore, it was anticipated that PTx pretreatment would result in a purely M_3 mACh receptor – $G_{q/11}$-mediated ERK activation in CHO-m2m3 cells. As can be seen in Figure 9b, PTx treatment caused a marked decrease in the maximal ERK activation elicited by MCh, and a leftward shift in the concentration-dependency (pEC_{50} values: −PTx, 7.04 ± 0.13; +PTx, 7.68 ± 0.18; $P<0.05$). In the light of this unexpected result, the effect of PTx pretreatment on the agonist-stimulated ERK response in CHO-m3 cells was also investigated. As can be seen in Figure 9c, PTx pretreatment of CHO-m3 cells caused a marked (>50-fold) leftward shift in the concentration-dependency of ERK activation by MCh (pEC_{50} values: −PTx, ± 0.16; +PTx, 7.88 ± 0.29; $P<0.05$).

Discussion

Although it is clear that in some cases signal transduction can be viewed as a series of parallel signalling pathways, activated by specific subsets of receptors that bring about distinct cellular responses, there is also considerable evidence for 'cross-talk' between signalling pathways (Gudermann *et al*., 1996; Selbie & Hill, 1998; Schaefer & Weber, 1999). Indeed, the apparent overlap between the signalling pathways activated by different extracellular stimuli can sometimes be perplexing (see Pawson & Saxton, 1999). Nevertheless, it is becoming increasingly clear that the intracellular milieu is a highly structured environment and unwanted cross-talk is efficiently eliminated *in vivo* by preventing the components of the different pathways from coming into intimate contact through compartmentation and/or the 'scaffolding' of signalling complexes (Gudermann *et al*., 1996; Burack & Shaw, 2000;

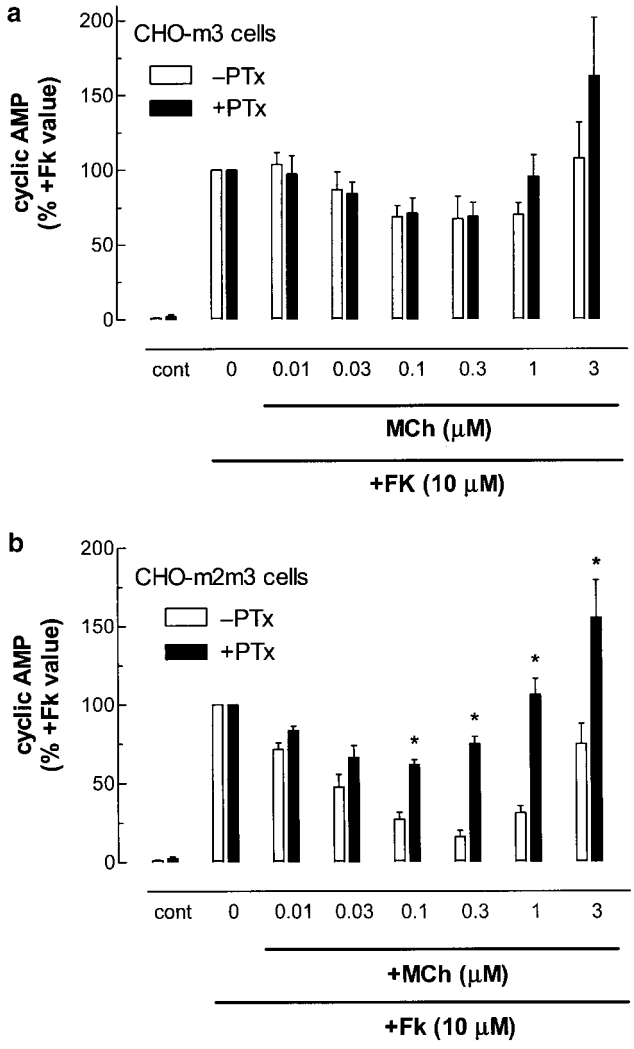

Figure 5 Effects of PTx pretreatment on the concentration-dependent modulatory actions of MCh on forskolin-stimulated cyclic AMP accumulations in CHO-m3 and -m2m3 cells. (a) CHO-m3 and (b) CHO-m2m3 cell monolayers were pretreated with PTx (100 ng ml^{-1}, 24 h) or vehicle before challenge with the indicated concentrations of MCh for 10 min before addition of forskolin (10 μM). After a further period of 10 min, incubations were terminated and cyclic AMP mass determined as described in the Methods section. Results are expressed relative to the cyclic AMP responses to forskolin alone (CHO-m3: −PTx, 1008 ± 41; +PTx, 433 ± 50; CHO-m2m3: −PTx, 908 ± 110; +PTx, 526 ± 51 pmol mg^{-1} protein), which are set to 100% for each condition. Data are shown as means ± s.e. mean for four (CHO-m3) or five (CHO-m2m3) separate experiments performed in duplicate. PTx did not significantly affect the modulatory effect of MCh in CHO-m3 cells, but significant differences were seen in toxin-treated CHO-m2m3 cells (*$P<0.05$).

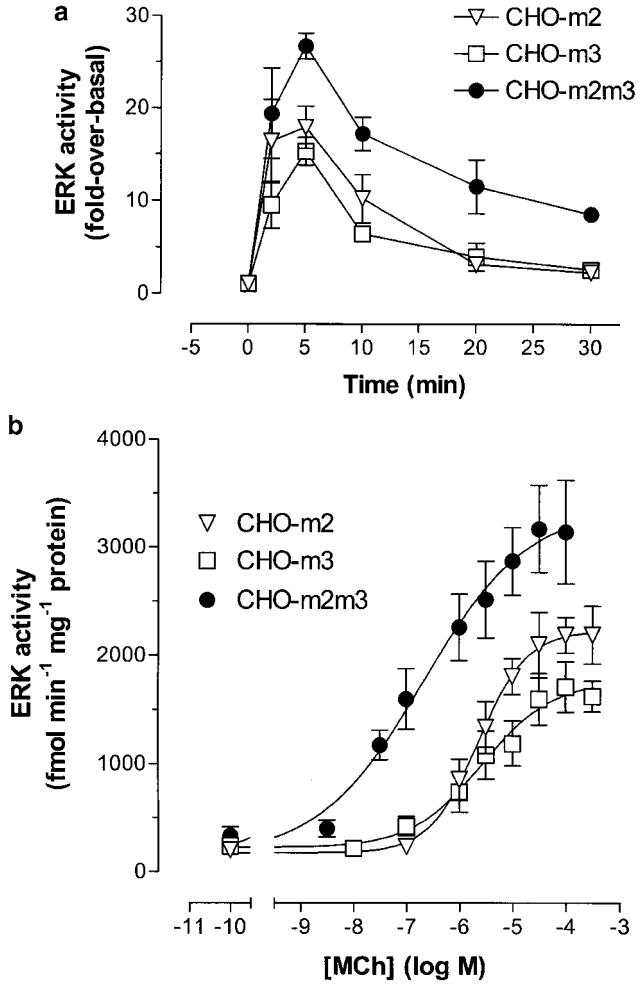

Figure 6 Time- and concentration-dependent increases in extracellular signal-regulated kinase (ERK) activity stimulated by MCh in CHO-m2, -m3 and -m2m3 cells. (a) Confluent monolayers of CHO cells were stimulated with MCh (100 μM) for the times indicated, (b) or with different concentrations of MCh for 5 min. Incubations were terminated, cell lysates prepared and kinase assays performed as described in the Methods section. Data are expressed as either a 'fold' increase over basal ERK activity (a) or as an enzymic activity (expressed as fmol phosphate incorporated into the EGF receptor fragment substrate per min per mg of cell protein (b)). Data are presented as means ± s.e. mean for four to eight separate experiments (panel a), or, for data shown in panel b, four (CHO-m2), five (CHO-m3) or six (CHO-m2m3) separate experiments.

Dumont *et al*., 2002). However, a number of physiologically important processes have been shown to depend upon cross-talk between pathways activated by distinct receptors to bring about phenomena such as coincidence detection (Sunahara *et al*., 1996; Batchelor & Garthwaite, 1997; Sweatt, 2001).

In the present study, we have investigated whether cross-talk occurs between the pathways that lie downstream of M_2 and M_3 mACh receptors stably coexpressed in CHO cell-lines. The clones isolated in this study, which demonstrated a coexpression of these receptor populations, both exhibited $M_3 > M_2$ mACh receptor densities (see Table 1). While this differs from the situation in a number of tissues, for example, the majority of smooth muscle types where the M_2 mACh receptor predominates, it does reflect the receptor distributions in some tissues (e.g. rat uterine smooth muscle; Choppin *et al*., 1999), and we considered that the CHO-m2m3 cell-lines we had isolated and characterized should provide useful information on mACh receptor cross-talk at multiple loci downstream of receptor activation.

Cross-talk at the level of second messenger generation

In both the CHO-m2m3 and -m3 cell-lines, but not in CHO-m2 cells, MCh addition caused a rapid and large increase in Ins(1,4,5)P_3. In the coexpressing cell-line, the initial peak

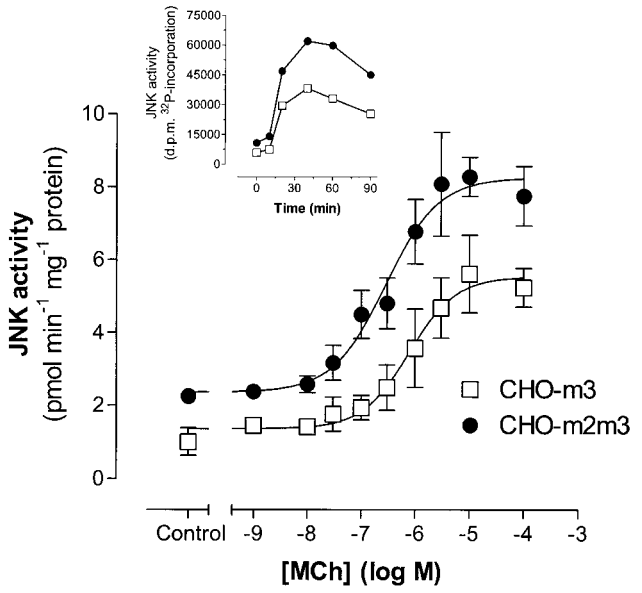

Figure 7 Concentration-dependent increases in c-Jun N-terminal kinase (JNK) activity stimulated by MCh in CHO-m3 and -m2m3 cells. Confluent monolayers of CHO cells were stimulated with the indicated concentrations of MCh for 30 min. Incubations were then terminated, cell lysates prepared and JNK assays performed as described in the Methods section. Data are expressed as enzymic activities (expressed as fmol phosphate incorporated into c-Jun fusion protein per min per mg of cell protein) and represent means ± s.e. mean for four (CHO-m3) or six (CHO-m2m3) separate experiments. The inset shows a representative experiment to illustrate a typical time-course of MCh (100 μM)-stimulated JNK activation in the two cell-lines.

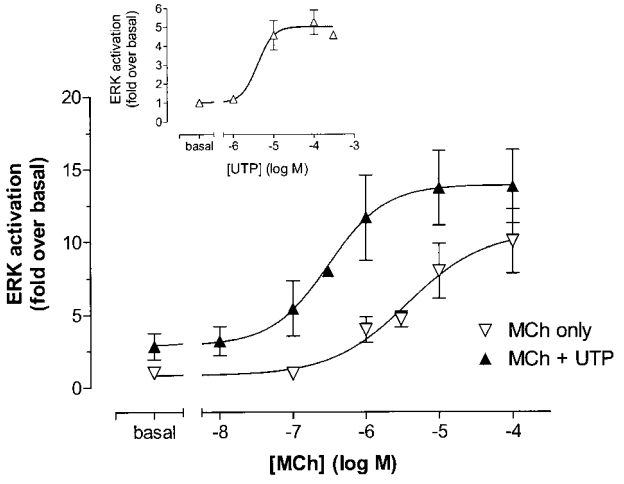

Figure 8 Effects of coincident P2Y/M_2 receptor stimulation on ERK activity in CHO-m2 cells. Confluent CHO-m2 monolayers were challenged with the indicated concentrations of MCh in the absence or presence of UTP (10 μM) for 5 min. Incubations were terminated, cell lysates prepared and kinase assays performed as described in the Methods section. Data are expressed as a 'fold' increase over basal ERK activity and are presented as means ± s.e. mean for three separate experiments. The inset shows the concentration-dependency of ERK activation by UTP ($n = 3$ for all data, except 300 μM UTP point where $n = 2$).

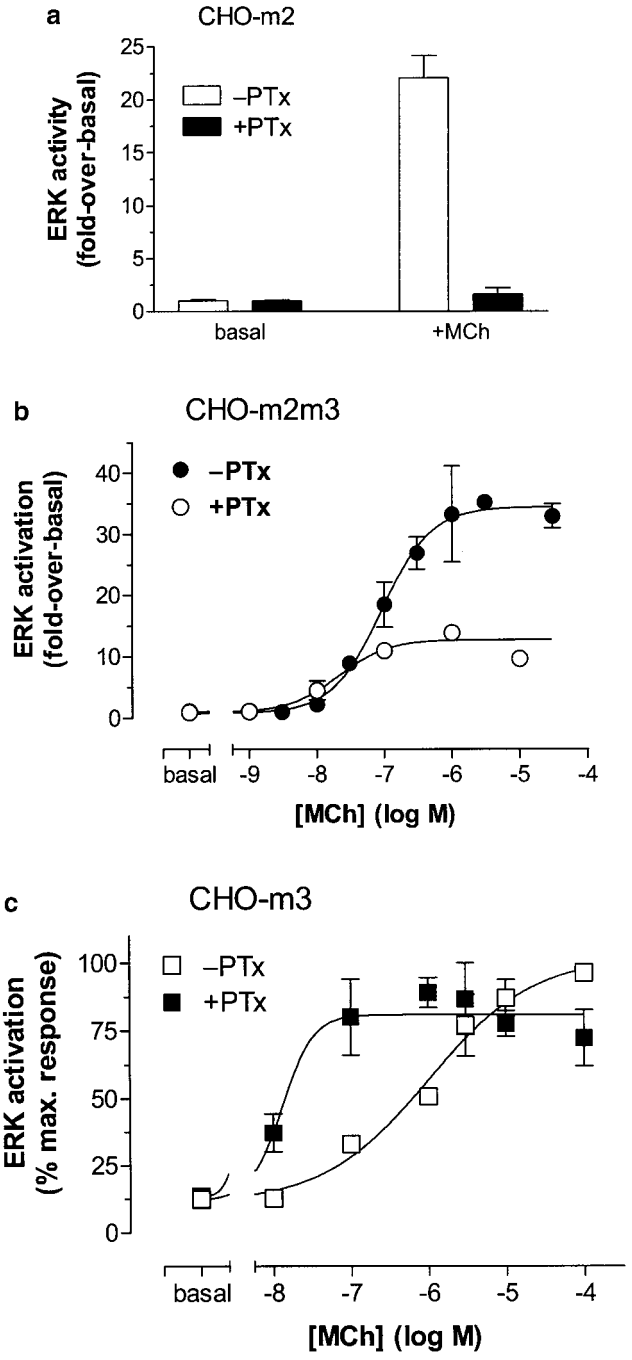

Figure 9 Effects of PTx pretreatment on the concentration-dependencies of ERK activation by MCh in CHO-m2, -m3 and -m2m3 cells. (a) CHO-m2, (b) CHO-m2m3 and (c) CHO-m3 cell monolayers were pretreated with PTx (100 ng ml^{-1}, 24 h) or vehicle before challenge with the indicated concentrations of MCh (panel a; 100 μM). Incubations were terminated, cell lysates prepared and kinase assays performed as described in the Methods section. Data are expressed either as a 'fold' increase over basal ERK activity (panels a and b), or as a percentage of the response to 100 μM MCh in vehicle-treated CHO-m3 cells (panel c). In all cases, data are shown as means ± s.e. mean for at least three separate experiments.

increase in Ins(1,4,5)P_3 was greater, and the concentration-dependency was approx three-fold to the left of the responses seen in CHO-m3 cells expressing a comparable M_3 mACh receptor density. Furthermore, elimination of productive coupling to cellular $G_{i/o}$ proteins in CHO-m2m3 cells decreased both the peak response and shifted the concentration-dependency to that seen in CHO-m3 cells. These data strongly suggest that in the CHO-m2m3 cell-line, agonist

stimulation of the M_2 mACh receptor facilitates the M_3-driven Ins(1,4,5)P_3 response. Such an effect might be brought about by a number of mechanisms, including a G_i-derived $\beta\gamma$ subunit activation of PLC-β isoenzymes that is not observed with M_2 mACh receptor activation alone as the effect may be conditional upon a $G_{q/11}\alpha$ – PLC-β binding interaction (Chan *et al*., 2000). Alternatively, the liberation of $\beta\gamma$ subunits through M_2 mACh receptor activation may facilitate $G_{q/11}\alpha$-mediated signalling by increasing the efficiency of $G_{q/11}$ heterotrimer presentation to the activated M_3 mACh receptor (Quitterer & Lohse, 1999). A further possibility is that the enhanced response seen in CHO-m2m3 cells might be mediated through an M_2-mediated enhancement of a Ca^{2+} entry pathway to facilitate phospholipase C isoenzymic activities (Rhee, 2001). However, our preliminary data on MCh-stimulated $[Ca^{2+}]_i$ responses in the CHO-m3 and CHO-m2m3 cell lines have provided no evidence in support of this possibility (Hornigold, D.C., Daniels, D. & Challiss, R.A.J., unpublished data).

As well as stimulating Ins(1,4,5)P_3 accumulation, M_3 mACh receptor activation also increased cyclic AMP accumulation by a mechanism that was attenuated by M_2 mACh receptor coactivation. Thus, PTx pretreatment of CHO-m2m3 cells resulted in a marked increase (by approx 100%) in peak cyclic AMP accumulation without affecting the concentration-dependency of this effect. Under conditions where basal cyclic AMP accumulation was increased by forskolin a rather different picture emerged. Thus, while agonist stimulation of M_2 mACh receptors resulted in a monophasic inhibition of the forskolin-stimulated response, M_3 receptor activation caused a biphasic response where low concentrations of MCh caused an inhibition, which was superseded by an enhancement of the forskolin-stimulated accumulation at higher agonist concentrations. In experiments not reported here, it has been shown that the inhibitory effect of MCh in CHO-m3 cells is dependent on the presence of extracellular Ca^{2+} and is blocked by lanthanides (La^{3+} and Gd^{3+}). These and other data suggest that receptor-gated and/or store-depletion-operated Ca^{2+}-channels cause Ca^{2+} entry that inhibits specific adenylyl cyclase (AC5/AC6) isoenzymes in CHO cells, a mechanism observed previously in other cell-types (Fagan *et al*., 1998; Wong *et al*., 2000). Irrespective of the mechanisms underlying the biphasic cyclic AMP response seen in CHO-m3 cells, an interaction between M_2- and M_3-mediated mechanisms to inhibit forskolin-stimulated adenylyl cyclase activity was clearly evident at low ($\leqslant 1\,\mu$M) MCh concentrations. This was confirmed by the demonstration that following PTx pretreatment, the magnitude and concentration-dependency of the inhibitory effects of MCh in CHO-m2m3 cells were similar to those seen in CHO-m3 cells.

Cross-talk at the level of the MAP kinases

In CHO cells, stimulation of M_2 and M_3 mACh receptors caused comparable activations of ERK, whereas JNK activity was activated only by M_3 mACh receptors, in agreement with previous work (Wylie *et al*., 1999). The pathways linking M_2/M_3 mACh receptor activation to ERK have been investigated in CHO and other cell-types. Such studies have often shown the dependence of the M_3-driven response on PKC and possibly other intermediary proteins (Kim *et al*., 1999; Wylie *et al*., 1999; Slack, 2000; Budd *et al*., 2001), while the M_2-driven ERK response has been proposed to be G$\beta\gamma$-dependent and may involve pathways distinct from, or overlapping with those seen following M_3 mACh receptor activation (Winitz *et al*., 1993; Crespo *et al*., 1994; Lopez-Ilasaca *et al*., 1997; Wylie *et al*., 1999). In the CHO-m2m3 cell-lines, the concentration-dependency of ERK activation by MCh was dramatically left-shifted (by more than 25-fold), the maximal ERK response increased, and the duration of the ERK activation prolonged, compared to the MCh responses observed in either CHO-m2 or -m3 cells. The M_2/M_3 synergism, at least with respect to the sensitivity increase for ERK activation, is unlikely to be specific to these mACh receptor subtypes, but a more general consequence of coactivating $G_{q/11}$- and $G_{i/o}$-coupled GPCRs, as activation of endogenous P2Y receptors in CHO-m2 cells also caused a significant leftward shift in the MCh concentration – effect curve for ERK activation. However, the presence of $G_{q/11}$- and $G_{i/o}$-coupled GPCRs responsive to a common hormone/neurotransmitter may optimize the synergism. In contrast to the consequences of M_2/M_3 mACh receptor coexpression for the regulation of ERK activity, the profile of JNK activation in CHO-m2m3 cells showed only modest differences from the CHO-m3 cell-line. Although constitutively active $G_i\alpha$ proteins can be shown to stimulate JNK activity (Yamauchi *et al*., 2000), in our hands M_2 mACh receptor activation did not stimulate JNK activity in CHO cells and therefore the lack of M_2/M_3 interaction to promote JNK activation was anticipated.

The observation of an altered duration of ERK activation in the receptor coexpressing cell-line may be particularly significant, as it has been shown that how well receptor-mediated ERK activation is maintained markedly affects the outcome with respect to cell fate (Marshall, 1995). Thus, a number of well-characterized examples now exist to show that the longevity of ERK activation, rather than simply the magnitude of the response, determines cellular decision-making with respect to processes such as proliferation, cell growth and transformation (Tombes *et al*., 1998; Orsini *et al*., 1999; Murphy *et al*., 2002). Therefore, if the increased agonist sensitivity and responsiveness, together with a more sustained pattern of ERK activation, are general features of cells coexpressing M_2/M_3 mACh receptors this may be of particular physiological significance.

$G_{q/11}$ and $G_{i/o}$ involvement in M_3 mACh receptor-ERK signalling

Unexpected results were obtained with respect to ERK activation profiles generated in CHO-m2m3 cells following PTx pretreatment. It had been anticipated that toxin ablation of M_2 mACh receptor-$G_{i/o}$ signalling would result in agonist concentration – effect curves that overlay those obtained in CHO-m3 cells; however, while the maximum responsiveness decreased to levels typically seen in CHO-m3 cells (i.e. from approx 30-fold down to 10 – 15 fold), no rightward shift in the concentration-dependency was observed. Interestingly, a partial explanation for these data was provided by experiments where CHO-m3 cells were PTx-treated. Under these conditions, the concentration – effect curve for the agonist-stimulated increase in ERK activity was dramatically left-shifted. Taken together these data suggest that under normal conditions M_3 mACh receptors couple to both $G_{q/11}$ and $G_{i/o}$

proteins and inactivation of the $G_{i/o}$ protein subpopulation appears to result in a much greater sensitivity (approx 100-fold) to agonist with respect to ERK activation. These data contrast with an elegant previous study by Blaukat *et al*. (2000) who demonstrated that efficient ERK activation by B_2 bradykinin, and M_1 and M_3 mACh receptors is dependent on cooperation between $G_{q/11}\alpha$ and $G_{i/o}\alpha$ signals in HEK293T cells.

We have previously shown that M_3 mACh receptors can couple to $G_{i/o}$ proteins in CHO cell membrane preparations by $G\alpha$-subtype-specific immunoprecipitation of G protein-[^{35}S]-GTPγS complexes (Akam *et al*., 2001). Using the same technique here, we have shown that PTx pretreatment did not affect receptor-$G_{q/11}$ coupling in CHO-m3 cells, and while MCh stimulated an increased maximal yield of $G_{q/11}\alpha$ protein-[^{35}S]-GTPγS complexes in CHO-m2m3 cell membranes, the concentration dependency of this stimulation was unaffected by $G_{i/o}$ inactivation. Thus, the observed effects of PTx on receptor-$G_{q/11}$ coupling in membranes derived from CHO-m3 and -m2m3 cells provide little insight into what may underlie the effects seen at the level of ERK regulation.

How is it possible to rationalize a situation where M_2 mACh receptor stimulation *per se* stimulates a robust and entirely PTx-sensitive ERK activity, while M_3 mACh receptor stimulation also appears to recruit a $G_{i/o}$ component, but in this case to attenuate the $G_{q/11}$-driven ERK response? Furthermore, in the CHO-m2m3 cell-line costimulation of the M_2-receptor must either suppress or overcome any $G_{i/o}$ protein component stimulated by the M_3-receptor such that M_2/M_3 mACh receptor coactivation results in the marked leftward curve shift and increased responsiveness. At present, we can only speculate on the mechanism(s) that underlies the data presented here. One possibility is that distinct $G_{i/o}\alpha$ protein subpopulations are activated by M_2 and M_3 receptors that respectively mediate stimulatory or inhibitory effects on the pathways regulating ERK activity. There is some evidence for this in the CHO cell-lines used here (Dell'Acqua *et al*., 1993; Akam *et al*., 2001) and in other systems (Migeon *et al*., 1995). In addition, it is possible that M_2 and M_3 receptors may facilitate the release of distinct $G_{i/o}$-derived $\beta\gamma$-subunit combinations, or there may be micro-compartmentation of mACh receptor access to $G_{i/o}$ pools (Gudermann *et al*., 1996; Albert & Robillard, 2002).

In summary, we have demonstrated a number of interactions between M_2 and M_3 mACh receptor signalling pathways that are unlikely to result from simple additivity between convergent signalling events. In particular, we report that coactivation of M_2 and M_3 mACh receptors in a CHO cell background results in a synergistic activation of ERK where both the sensitivity and responsiveness to agonist stimulation are increased. These data raise the question of whether ERK activation is similarly facilitated in tissues coexpressing these mACh receptor populations.

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