Full and Partial Agonists of Muscarinic M₃ Receptors Reveal Single and Oscillatory Ca²⁺ Responses by β₂-Adrenoceptors

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ABSTRACT

Under physiological circumstances, cellular responses often reflect integration of signaling by two or more different receptors activated coincidentally or sequentially. In addition to heterologous desensitization, there are examples in which receptor activation either reveals or potentiates signaling by a different receptor type, although this is perhaps less well explored. Here, we characterize one such interaction between endogenous receptors in human embryonic kidney 293 cells in which Gαq/11-coupled muscarinic M₃ receptors facilitate Ca²⁺ signaling by Gα₁-s-coupled β₂-adrenoceptors. Measurement of changes in intracellular [Ca²⁺] demonstrated that noradrenaline released Ca²⁺ from thapsigargin-sensitive intracellular stores only during activation of muscarinic receptors. Agonists with low efficacy for muscarinic receptor-mediated Ca²⁺ responses facilitated cross-talk more effectively than full agonists. The cross-talk required Gαq and was dependent upon intracellular Ca²⁺ release channels, particularly inositol (1,4,5)-trisphosphate receptors. However, β₂-adrenoceptor-mediated Ca²⁺ release was independent of measurable increases in phospholipase C activity and resistant to inhibitors of protein kinases A and C. Interestingly, single-cell imaging demonstrated that particularly lower concentrations of muscarinic receptor agonists facilitated marked oscillatory Ca²⁺ signaling to noradrenaline. Thus, activation of muscarinic M₃ receptors profoundly influences the magnitude and oscillatory behavior of intracellular Ca²⁺ signaling by β₂-adrenoceptors. Although these receptor subtypes are often coexpressed and mediate contrasting acute physiological effects, altered oscillatory Ca²⁺ signaling suggests that cross-talk could influence longer term events through, for example, regulating gene transcription.

Cells express a range of different receptors able to transduce extracellular signals and ultimately influence cellular behavior. Although receptor activation, intracellular signaling, and functional responses are often studied in isolation, such events in physiological settings are more likely to reflect the integration of signaling mediated by two or more different receptors that are activated either coincidentally or sequentially. For G protein-coupled receptors (GPCRs), such interactions have been explored, and there are many examples in which heterologous desensitization results in the loss of response to the challenge of one receptor type after activation of another receptor type linked to the same or a different signaling pathway. Perhaps less well explored is cross-talk in which activation of one receptor type either reveals or potentiates signaling by a different receptor type. One example of such cross-talk is in which activation of a Gαq/11-coupled GPCR facilitates Ca²⁺ signaling by either Gαo or Gα₁-s-coupled GPCRs (Werry et al., 2003a). In most instances, the facilitated Ca²⁺ signaling is dependent upon an intracellular store, but the mechanisms through which additional Ca²⁺ is released are unclear. Indeed, many mechanisms have been suggested, and where experimental evidence is available this would suggest a variety of mechanisms.
Amino acids are involved that may depend upon both the receptors and cell types involved (Werry et al., 2003a).

Some examples of cross-talk are dependent upon enhanced activation of phospholipase C (PLC) and therefore increased generation of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P$_3$] to release Ca$^{2+}$ from the intracellular stores (Dickenson and Hill, 1998; Chan et al., 2000; Werry et al., 2003b), others are independent of enhanced PLC activity, suggesting either increased sensitivity of Ca$^{2+}$ release channels or alternative release mechanisms (Jiménez et al., 1999; Tanimura et al., 1999; Short and Taylor, 2000; Yeo et al., 2001). Irrespective of the mechanisms involved, the ability of cross-talk to influence intracellular Ca$^{2+}$ signaling has profound implications for cell function given the diverse cellular events regulated by Ca$^{2+}$. Here, we have explored interactions between Go$_q$-coupled muscarinic M$_3$ receptors and Go$_a$-coupled β$_2$-adrenoceptors that result in enhanced Ca$^{2+}$ signaling, focusing particularly on the pharmacology of the cross-talk. These GPCRs are often coexpressed, for example, in airway smooth muscle, and an understanding of their potential interactions has important physiological and clinical implications.

Materials and Methods

Materials. Cell culture reagents were from Invitrogen (Paisley, UK). Cell culture plastics were from Nalgene (Hereford, UK). Poly-D-lysine-coated 96-well plates for fluorescence imaging plate reader (FLIPR) and other plate reader assays were from BD Biosciences (Oxford, UK). Cholera toxin (CTX), cAMP, Ins(1,4,5)P$_3$, pertussis toxin, fluo-3-acetoxyethyl ester (AM), fluo-4-AM, mouse γ-tubulin antibody, horseradish peroxidase-conjugated secondary antibodies, and the protein kinase inhibitor H89 were from Sigma Chemical (Poole, UK). Forskolin was from Tocris Bioscience (Bristol, UK). Myristoylated peptide protein kinase A (PKA) 14-22 amide inhibitor and myristoylated peptide protein kinase C (PKC) 20-28 inhibitor were from Merck Bioscience (Nottingham, UK). Pluronic F-127 was obtained from Invitrogen. All other reagents were of analytical grade and were purchased from Sigma Chemical or Fisher Scientific (Loughborough, UK). ECL Plus reagents, Hyperfilm, and myo-[H]inositol with PT-271 (8Ci mmol$^{-1}$) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Go$_q$ protein antibodies and antibodies against both extracellular signal-regulated kinase (ERK) 1/2 and phospho-ERK were purchased from Santa Cruz Biotechnology and cell culture plastics were from Nalgene (Hereford, UK). Poly-D-lysine-coated 96-well plate and incubated overnight. Where required, cells were plated in media with either 2 μg·ml$^{-1}$ CTX or 100 ng·ml$^{-1}$ pertussis toxin and incubated for 18 to 20 h. Cells were loaded in Hanks’ balanced salt solution (HBSS; 10 mM HEPES, 136 mM NaCl, 7.5 mM KCl, 5 mM NaHCO$_3$, 1.2 mM CaCl$_2$, and 4.1 mM NaH$_2$PO$_4$, pH 7.4) containing 5 μM fluo-3-AM and Pluronic F-127 (0.04%) for 1 h at 37°C. Before the assay, the cells were washed twice with 100 μl of HBSS to remove any excess dye. The cells were finally resuspended in 100 μl of HBSS (± CaCl$_2$ for some studies) and assayed. To determine cross-talk in HEK 293 cells, fluorescence ($\lambda_{em} =$ 488 nm and $\lambda_{ex} =$ 540 nm) was initially measured for 5 to 10 s to establish a baseline. After this, a muscarinic receptor agonist or vehicle control (HBSS) was added (30–50 μl; 30–40 μl·s$^{-1}$), and fluorescence was recorded for 130 to 180 s. When the effects of other pharmacological tools were assessed on Ca$^{2+}$ responses, these were added at the appropriate times before placing the plate in the FLIPR. In cases where the test agents were dissolved in dimethyl sulfoxide, this was included in control experiments at the appropriate concentrations and shown to be without any effect on Ca$^{2+}$ responses at the highest concentration tested. The change in fluorescence units was taken as an index of the Ca$^{2+}$ response. Concentration-response curves were fitted using nonlinear regression with a four-parameter logistic equation in Prism (GraphPad Software Inc., San Diego, CA). All data are presented as mean ± S.E.M. In a small number of experiments as indicated, population Ca$^{2+}$ signaling experiments were performed in fluo-4-loaded cells using a NOVOstar microplate reader (BMG Labtech, Aylesbury, UK).

For measurement of changes in [Ca$^{2+}$] by confocal microscopy, HEK 293 cells were plated onto 25-mm-diameter sterile borosilicate glass coverslips coated with 0.01% poly-D-lysine and incubated overnight. Cells were loaded as described above, washed twice with HBSS at 37°C, and images were collected at a rate of approximately one image per second on a laser-scanning confocal microscope (λ$_{em} =$ 488 nm, with emitted light collected at >505 nm; Olympus UK Ltd., Watford, UK). The temperature of the chamber was maintained at 37°C using a temperature controller (Harvard Apparatus Inc., Edenbridge, Kent, UK). A region of interest was chosen within the cytoplasm of each cell, using purpose written software (FluoView software, version 4.3; Olympus). Fluorescence before agonist addition was regarded as baseline fluorescence, and the data obtained from each cell during the experiment are expressed as the fold change in cytosolic fluorescence (F/F$_{0}$) relative to basal levels.

Western Blotting. To determine the effect of CTX on Go$_q$ protein expression, HEK 293 cells were either untreated or treated with 2 μg·ml$^{-1}$ CTX for 0.5, 1, 2, 4, or 20 h and then solubilized (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Igepal CA630, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 μg·ml$^{-1}$ iodoacetamide, and 100 μg·ml$^{-1}$ benzamidine, pH 7.4). Samples (30 μg of protein) were separated by SDS-PAGE with a 10% running gel. After transfer onto nitrocellulose, the membrane was blocked for 60 min at room temperature using skimmed milk powder (5%, w/v) in Tris-buffered saline/ Tween 20 (150 mM NaCl, 50 mM Tris-HCl, and 0.05% (v/v) Tween 20, pH 7.4). Nitrocellulose membranes were incubated overnight at 4°C with Go$_q$ polyclonal antibody (1:1000), and visualization was achieved using anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000) with enhanced chemiluminescence detection and Hyperfilm. To ensure equivalent protein loading the nitrocellulose membranes were stripped (0.7% 2-β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.8, at 50°C for 30 min with constant agitation), washed extensively (TBST; 30 min), and blocked and probed for γ-tubulin (1:10,000) as described above. For assessment of ERK activity, cells were cultured for 24 h in a 12-well plate and placed in serum-free media for a further 24 h to reduce basal activity. Cells were then either untreated or preincubated with PKA inhibitors (10 μM H89 or 25 μM protein kinase A amide inhibitor 14-22) for 30 min and subsequently stimulated with 100 μM forskolin for 10 min. Whole-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with phospho-ERK or total ERK antibodies using bovine serum albumin (5%, w/v) as a blocking agent and visualized as described above.

Total [H]inositol Phosphate Generation. The generation of [H]InsP$_3$ as an index of phospholipase C activity was determined as described previously (Werry et al., 2003b). In brief, cells were grown...
for 48 h in the presence of 3 μCi·mL⁻¹ myo-[³H]inositol. After washing and preincubation (20 min at 37°C) with HBSS containing 10 mM Li⁺ to inhibit inositol monophosphatase activity, cells were stimulated for the required time before the reaction was terminated by addition of an equivalent volume of ice-cold 1 M trichloroacetic acid. The reaction mix (final volume, 1 ml) was added to 250 μl of 10 mM EDTA and subsequently 1 ml of a freshly prepared 1:1 (v/v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoroethane was added. After thorough mixing, a 700-μl aliquot of the upper aqueous layer was removed and added to 50 μl of 250 mM NaHCO₃. Soluble inositol phosphates in this aqueous fraction were subsequently isolated using strongly basic Dowex chloride anion exchange columns (8% cross-linkage, 100–200 dry mesh; Sigma 1 × 8-200) and quantified using liquid scintillation counting. Data are expressed as a fold increase in [³H]InsP₄ relative to basal levels for which the cells were challenged with agonist-free buffer for the longest time point of the experiment.

Data Analysis. All data are expressed as mean ± S.E.M. of three or more experiments as indicated in parentheses. Where representative data are shown, data were performed three or more times. Concentration-response curves were fit with Prism (GraphPad Software Inc.) using a standard four-parameter logistic equation (ANCOVA), and where required either one-way or two-way analysis of variance (ANOVA). From across our study and tested for normality of distribution. There normality test, Shapiro-Wilk normality test, D’Agostino and Pearson omnibus normality test, Shapiro-Wilk normality test), supporting the use of normality test, Shapiro-Wilk normality test, D’Agostino and Pearson omnibus normality test, Shapiro-Wilk normality test), supporting the use of parametric descriptive and comparative statistics for these type of data.

Results

Demonstration of Cross-Talk. Challenge of HEK 293 cells with the muscarinic receptor agonist methacholine (1 mM) resulted in an increase in [Ca²⁺]i, consisting of a rapid transient peak, followed by a more sustained plateau phase. Addition of noradrenaline (10 μM) failed to elevate [Ca²⁺]i, (Fig. 1). However, challenge of cells with noradrenaline (10 μM) in the continued presence of methacholine (1 mM) resulted in a rapid elevation of [Ca²⁺]i, which subsided over the subsequent few minutes (Fig. 1). Preaddition of buffer rather than methacholine did not result in a Ca²⁺ response to the subsequent addition of noradrenaline (Fig. 1).

Pharmacological Characterization of Cross-Talk. To determine the subtype of adrenoceptor mediating cross-talk, cells were incubated with a range of concentrations of the nonselective α-adrenoceptor antagonist phentolamine, the β₁-adrenoceptor-selective antagonist atenolol, or the β₂-adrenoceptor-selective antagonist ICI-118,551 (Hoffmann et al., 2004; Baker, 2005). Cells were incubated with the agonists for 10 min before stimulation with a maximally effective concentration of methacholine (100 μM) and subsequently with a range of noradrenaline concentrations. In the continued presence of this fixed concentration of methacholine (100 μM), noradrenaline evoked a concentration-dependent elevation of [Ca²⁺]i, with a pEC₅₀ value (−log₁₀ of the EC₅₀ molar concentration) of 6.65 ± 0.15 (n = 4). Phentolamine, over the concentration range of 1 nM to 10 μM, had no effect on either the concentration dependence or E₅₀ values of these noradrenaline-evoked Ca²⁺ signals (e.g., at 10 μM phentolamine, the pEC₅₀ value was 6.31 ± 0.52 and the E₅₀ value was 117.6 ± 3.8% of the response in the absence of phentolamine; n = 4). Atenolol, over the range of 10 nM to 10 μM, caused a concentration-dependent dextral shift of the noradrenaline concentration-response curve in the presence of 100 μM methacholine (data not shown). However, the resulting Schild plot had a slope different from unity, perhaps as a consequence of the nonequilibrium conditions of the assay. ICI-118,551 caused a collapse of the noradrenaline concentration-response curve in the presence of 100 μM methacholine, with little effect on the pEC₅₀ values where these could be determined. Thus, at 0.01 and 0.1 μM, ICI-118,551 inhibited the maximal responses to noradrenaline by 52 and 65%, respectively, whereas at 1 μM the responses to noradrenaline were abolished. Terbutaline (10 μM), a β₂-adrenoceptor-selective agonist that has little or no efficacy at β₁-adrenoceptors (Hoffmann et al., 2004), evoked Ca²⁺ responses that were 76 ± 14 (n = 3) and 86 ± 4% (n = 3) of the response evoked by noradrenaline (10 μM) in the presence of 1 mM methacholine and oxtremorine, respectively. Together, these data suggest that noradrenaline-mediated Ca²⁺ signaling in the presence of a muscarinic receptor agonist is mediated by β₂-adrenoceptors.

Previous evidence suggests that HEK 293 cells express muscarinic M₃ receptors (Ansellin et al., 1999), although there have been some reports of muscarinic M₁ receptor-mediated effects (Mundell and Benovic, 2000). In the present study, pirenzepine and 4-diphenylacetoxy-N-methylpiperidine methiodide inhibited carbachol-mediated Ca²⁺ signaling, with pKᵯ (−log₁₀ of the Kᵯ molar concentration) values of 6.84 ± 0.04 and 9.69 ± 0.09 (n = 3), respectively, which are consistent with muscarinic M₃ receptor-mediated Ca²⁺ signaling (Dörje et al., 1991).

Partial Muscarinic Receptor Agonists Mediate Cross-Talk. In HEK 293 cells, the muscarinic receptor agonists methacholine, arecoline, and oxtremorine exhibited a range of intrinsic activities and potencies with respect to their abilities to elevate [Ca²⁺]i (Fig. 2; Table 1). Each of these muscarinic receptor agonists facilitated the elevation of [Ca²⁺]i, in response to a subsequent addition of noradrenaline (10 μM) (Fig. 3, a and b; Table 1). Despite both oxtremorine and pilocarpine being relatively weak partial agonists of the muscarinic receptor-mediated Ca²⁺ response [E₅₀ values of
erol increased [Ca\(^{2+}\)] in the continued presence of methacholine and oxotremorine both increased the proportion of cells oscillating (from 33 ± 5 to 91 ± 3%; \(n = 96\) cells in three independent experiments), and the oscillation frequency [0.10 ± 0.01 versus 0.84 ± 0.11 oscillations \(\cdot\) min\(^{-1}\) \((n = 96\) in three independent experiments); \(p < 0.01\), Student’s \(t\) test]. Addition of noradrenaline in the continued presence of oxotremorine both increased the proportion of cells oscillating (from 33 ± 5 to 91 ± 3%; \(n = 96\) cells in three independent experiments), and the oscillation frequency [0.10 ± 0.01 versus 0.84 ± 0.11 oscillations \(\cdot\) min\(^{-1}\) \((n = 96\) in three independent experiments), with each experiment consisting of more than two coverslips; \(p < 0.001\), Student’s \(t\) test).

**Further Characterization of Cross-Talk.** After stimulation of the cells in the FLIPR with a maximal concentration (1 mM) of methacholine for 150 s, the addition of the muscarinic receptor antagonist atropine (10 \(\mu\) M; 5 min) abolished Ca\(^{2+}\) responses to the subsequent addition of 10 \(\mu\) M noradrenaline (Fig. 5a). Similar data were obtained using oxotremorine, arecoline, or picolinate as the muscarinic receptor agonist (Fig. 5a; data not shown). Preincubation (5 min) of cells with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump inhibitor thapsigargin (2 \(\mu\) M) abolished responses both to the muscarinic receptor agonists (data not shown) and to the subsequent addition of 10 \(\mu\) M noradrenaline (Fig. 5b; data not shown). Removal of extracellular Ca\(^{2+}\) (by use of a nominally Ca\(^{2+}\)-free buffer) had no effect on the peak Ca\(^{2+}\) responses to either 1 mM methacholine or 1 \(\mu\) M arecoline but abolished their sustained, plateau phases (Fig. 6, a and b; data not shown). The absence of extracellular Ca\(^{2+}\) had no effect on the Ca\(^{2+}\) responses to noradrenaline (10 \(\mu\) M) after maximal concentrations of either arecoline, picolinate, or oxotremorine, whereas the response after methacholine was greater in the absence of [Ca\(^{2+}\)] (Fig. 6c). Although the use of nominally Ca\(^{2+}\)-free buffer is often sufficient to investigate the impact of extracellular Ca\(^{2+}\) on GPCR-mediated Ca\(^{2+}\) entry (as reflected here by the abolition of the plateau phase), we also performed experiments using the NOVOstar plate reader in buffer in which the extracellular [Ca\(^{2+}\)] was titrated to approximately 100 nM (determined using standard techniques with fura-2) using EGTA. Responses to noradrenaline (10 \(\mu\) M) in the presence of both arecoline (1 or 10 \(\mu\) M) or oxotremorine (1 or 10 \(\mu\) M) were not significantly different from the absence (1.3 mM [Ca\(^{2+}\)]) or presence (100 nM [Ca\(^{2+}\)]) of EGTA [an average of 92 ± 20% (\(n = 12\)) across all conditions].

Pretreatment of cells with pertussis toxin (100 ng \(\cdot\) ml\(^{-1}\); 18–20 h) to ADP-ribosylate Go\(_{q}\) and prevent GPCR-mediated activation had no effect on the magnitude or potency of either methacholine- or oxotremorine-mediated Ca\(^{2+}\) responses or Ca\(^{2+}\) responses to the addition of 10 \(\mu\) M noradrenaline in the presence of these muscarinic receptor agonists over their effective concentration ranges (data not shown). Although an activator of Go\(_{q}\), CTX on extended exposure can down-regulate Go\(_{q}\) and abolish Go\(_{q}\)-mediated signaling (Seidel et al., 1999). Treatment of HEK 293 cells for 20 h with CTX (2 \(\mu\) g \(\cdot\) ml\(^{-1}\)) was sufficient to markedly reduce levels of Go\(_{q}\) (Fig. 7a), consistent with its ability to inhibit Go\(_{q}\)-mediated signaling in this cell background (Werry et al., 2002). This pretreatment with CTX had no effect on the magnitude or
The pEC_{50} values of muscarinic receptor agonist-mediated Ca^{2+} responses and their ability to facilitate Ca^{2+} signaling by 10 μM noradrenaline are shown in Table 1.

<table>
<thead>
<tr>
<th>Muscarinic Receptor Agonist</th>
<th>pEC_{50} of Muscarinic Receptor Agonist-Mediated Ca^{2+} Responses</th>
<th>pEC_{50} of Muscarinic Receptor-Agonists Facilitation of Ca^{2+} Responses by 10 μM Noradrenaline</th>
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<tbody>
<tr>
<td>Arecoline</td>
<td>4.37 ± 0.21</td>
<td>5.03 ± 0.15</td>
<td>4</td>
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<tr>
<td>Pilocarpine</td>
<td>4.39 ± 0.16</td>
<td>5.78 ± 0.36</td>
<td>4</td>
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<tr>
<td>Methacholine</td>
<td>6.26 ± 0.52</td>
<td>5.94 ± 0.13</td>
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<tr>
<td>Oxotremorine</td>
<td>5.54 ± 0.23</td>
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Fig. 3. Partial agonists of the muscarinic receptor-mediated Ca^{2+} response facilitate greater Ca^{2+} responses to noradrenaline than the full agonist methacholine. a, cells were stimulated in a FLIPR (at 10 s) with maximal (1 mM) concentrations of muscarinic receptor agonists of different efficacies and fluorescence recorded every 1 s for a further 180 s. Noradrenaline (10 μM) was then added and fluorescence was recorded for a further 180 s, b, using the protocol described, cells were challenged with the indicated concentrations of muscarinic receptor agonist followed by noradrenaline (10 μM). The maximal change in fluorescence on addition of noradrenaline was quantified and is shown here as an index of changes in the [Ca^{2+}]_{i}. The pEC_{50} values are given in Table 1. Data are mean ± S.E.M., n = 4.

Phase determined 50 s after agonist addition (Fig. 8b). In contrast, ryanodine enhanced the oxotremorine-mediated elevation of intracellular Ca^{2+}, but it reduced its potency [E_{max} value of 330 ± 44% (n = 3)] in the presence of ryanodine; pEC_{50} values of 4.68 ± 0.25 and 6.16 ± 0.01 (n = 3) in the presence or absence of ryanodine, respectively; p < 0.001 for the difference between concentration-response curves, two-way ANOVA]. Pretreatment of cells with the putative Ins(1,4,5)P_{3} receptor inhibitor 2-aminoethoxydiphenylborane (2-APB; 100 μM; 30 min) significantly inhibited methacholine-evoked Ca^{2+} responses and abolished responses to oxotremorine (Fig. 9a). To investigate the impact of ryanodine (30 μM) and 2-APB (100 μM) on noradrenaline-mediated Ca^{2+} signaling, cells were pretreated (30 min) with these compounds alone or in combination before challenge, with a range of concentrations of either methacholine or oxotremorine and subsequently 10 μM noradrenaline. Ryanodine reduced the potency of both methacholine and oxotremorine to facilitate Ca^{2+} signaling in response to 10 μM noradrenaline (Fig. 9, b and c). However, it had little effect on the maximal Ca^{2+} responses to noradrenaline at high concentrations of the muscarinic receptor agonists. 2-APB also reduced the potency of the facilitatory action of methacholine on noradrenaline-mediated Ca^{2+} signaling (Fig. 9b). In contrast, the magnitude of the Ca^{2+} responses to 10 μM noradrenaline in the presence of oxotremorine was reduced by 2-APB, although oxotremorine potency on this facilitatory activity was unaffected (Fig. 9c). Combined pretreatment of cells with both ryanodine and 2-APB reduced the potency of the facilitatory action of methacholine on noradrenaline-mediated Ca^{2+} signaling (Fig. 9b). Furthermore, this combined treatment abolished noradrenaline-mediated Ca^{2+} signaling in the presence of oxotremorine (Fig. 9c). Preincubation of cells with xestospongin C (10 μM for 30 min) significantly reduced subsequent Ca^{2+} responses to maximal (100 μM) or approximate EC_{50} (1 μM) concentrations of either methacholine or oxotremorine when these agonists were added under conditions in which the external [Ca^{2+}] was buffered with EGTA to approximately 100 nM (Fig. 10a). Responses to the subsequent addition of 10 μM noradrenaline were also reduced with the exception of addition in the presence of 100 μM methacholine (Fig. 10b).

Cross-Talk Is Independent of Enhanced PLC Activity. As an index of total PLC activity, the accumulation of [3H]InsP_{3} in the presence of a Li^{+} block of inositol monophosphatase activity. Challenge of cells with methacholine for 20 min resulted in a concentration-dependent accumulation of [3H]InsP_{3} with a pEC_{50} value of 4.29 ± 0.24 and a maximal 4.40 ± 0.36-fold increase over basal levels (basal 2582 ± 655 dpm · well^{-1}; n = 3). Coseimulation with methacholine and 10 μM noradrenaline had no effect on the potency or magnitude of the response (pEC_{50} value of 4.05 ± 0.60 and a maximal 5.06 ± 0.69-fold increase over basal levels; n = 3). Oxotremorine (1 mM) was a weak partial.

Table 1

The pEC_{50} values of muscarinic receptor agonist-mediated Ca^{2+} responses and their ability to facilitate Ca^{2+} signaling by 10 μM noradrenaline.
agonist in respect of the [3H]InsP₃ response, generating a maximal increase of 1.34 ± 0.13 (n = 3) fold over increase basal levels that was not affected by costimulation with 10 μM noradrenaline (1.46 ± 0.13; n = 3). To examine PLC activity with greater temporal resolution, cells were transfected with the enhanced green fluorescent protein (eGFP)-tagged pleckstrin homology (PH) domain of PLCβ₁ (eGFP-PHPLCβ₁). Under basal (unstimulated) conditions eGFP-PHPLCβ₁ is localized to the plasma membrane, because it binds with high affinity and selectivity to phosphatidylinositol 4,5-bisphosphate (Nash et al., 2001). Ins(1,4,5)P₃ is able to bind to it with high affinity and displace it from the membrane (Nash et al., 2001). This can be monitored in real time by confocal imaging (Nash et al., 2001; Tovey and Willars, 2004) and increased cytosolic fluorescence reflects PLC activation and Ins(1,4,5)P₃ generation. Transfection of cells with the eGFP-PHPLCβ₁ construct resulted in fluorescence located predominantly at the plasma membrane. However, challenge of cells with either 1 mM methacholine alone or 10 μM noradrenaline in the continued presence of 1 mM methacholine did not consistently result in an increase of the cytosolic fluorescence (data not shown), presumably reflecting very low/localized changes in Ins(1,4,5)P₃. Treatment of cells with the cell-permeable PKC inhibitor myristoylated protein kinase C 20-28 (100 μM; 30 min) had no effect on the Ca²⁺ responses to either methacholine or oxotremorine across the concentration ranges of the muscarinic receptor agonists (data not shown). Furthermore, the magnitude of the subsequent responses to 10 μM noradrenaline were also unaffected by the PKC inhibitor across the range of concentrations of these muscarinic receptor agonists (Fig. 11). Cross-Talk Is Dependent on cAMP but Independent of PKA Activity. In the presence of 1 mM methacholine, treatment of cells with forskolin (100 μM) to directly activate adenylyl cyclases resulted in a Ca²⁺ response that was comparable with the responses evoked by 10 μM noradrenaline in the presence of 1 mM methacholine (Fig. 12; 1133 ± 158 versus 1877 ± 399 fluorescence units, respectively; n = 3). Addition of forskolin in the absence of methacholine did not cause a Ca²⁺ response (Fig. 12). Similar data were obtained by treating cells with forskolin in the presence of oxotremorine.

Cells were incubated with the PKA inhibitors H89 (10 μM) or 14-22 myristoylated amide PKA inhibitor (25 μM) for 30 min before stimulation with an approximate EC₅₀ concentration of oxotremorine (1 μM) and a subsequent stimulation with a maximal concentration of noradrenaline (10 μM) to evoke a robust cross-talk. Preincubation with these inhibitors did not affect the Ca²⁺ response to either oxotremorine or noradrenaline (Fig. 13). Similar data were obtained using a maximal concentration of methacholine (1 mM) to facilitate the Ca²⁺ responses to noradrenaline (data not shown). The efficacy of the PKA inhibitors was demonstrated by their ability to inhibit forskolin-mediated activation of ERK, as assessed by levels of phospho-ERK (Fig. 13e).

Discussion

Ca²⁺ responses to muscarinic receptor agonists in our HEK 293 cells are mediated by Gαo-coupled muscarinic M₃ receptors. Furthermore, noradrenaline elevates [Ca²⁺], in these cells via Gα₁₁-coupled β₂-adrenoceptors only in the presence of muscarinic receptor activation. This β₂-adrenoceptor-mediated Ca²⁺ signaling is facilitated by both full and partial muscarinic receptor agonists, with partial agonists often being more effective than full agonists. Ca²⁺ responses to noradrenaline, although dependent on Gαo, are independent of enhanced PLC activity or PKC and PKA activity.

The lack of effect of pertussis toxin on muscarinic receptor-mediated Ca²⁺ signaling or facilitated noradrenaline Ca²⁺ signaling excludes roles for Gαi-coupled muscarinic M₂ and M₄ receptors. Furthermore, pKi values of pirenzepine and
4-diphenylacetoxy-N-methylpiperidine methiodide for inhibition of muscarinic receptor-mediated Ca2+ signaling suggest that, although muscarinic M1 (Mundell and Benovic, 2000) and M3 (Ancellin et al., 1999; Tovey and Willars, 2004) receptors are reported in HEK 293 cells, muscarinic M3 receptors are responsible in our clone. The pharmacology of facilitated adrenoceptor Ca2+ signaling indicates mediation by β2-adrenoceptors. Although ICI-118,551 is considered a selective, competitive antagonist of β2-adrenoceptors (Skeberdis et al., 1997), behavior here is consistent with a noncompetitive interaction. This has been observed previously with low receptor expression, possibly due to a higher affinity of ICI-118,551 for G protein-uncoupled receptors, thereby effectively reducing receptor number and decreasing maximal responses (Hopkinson et al., 2000).

Methacholine, arecoline, oxotremorine, and pilocarpine showed intrinsic activities ranging from full agonism (methacholine) to exceptionally weak partial agonism (pilocarpine). At higher concentrations, the partial agonists arecoline and oxotremorine facilitated Ca2+ signaling by noradrenaline equivalent to or greater than that of methacholine. Thus, the extent of cross-talk is not directly related to peak Ca2+ elevations by the muscarinic receptor agonist. This may reflect competing factors; first, agonist efficacy on the cross-talk pathway and second, the remaining store Ca2+. At higher concentrations of methacholine, Ca2+ stores are likely to be substantially depleted, thereby limiting Ca2+ available for subsequent release. This might explain the reduced response to noradrenaline sometimes seen at concentrations of methacholine.

Atropine and thapsigargin abolish noradrenaline-mediated Ca2+ responses in the presence of methacholine. a, using a FLIPR, cells were challenged with the indicated muscarinic receptor agonist (1 mM) in the presence or absence of 10 μM atropine before challenge with noradrenaline (10 μM). The graph shows the responses to noradrenaline in the presence of either muscarinic receptor agonists alone or muscarinic receptor agonists and atropine. b, cells were preincubated for 5 min in the presence or absence of thapsigargin (2 μM) before challenge with muscarinic receptor agonists (1 mM) and subsequently challenged with noradrenaline (10 μM). The graph shows only the responses to noradrenaline in the presence of muscarinic receptor agonists with or without thapsigargin treatment; the initial responses to methacholine are not shown. Data are mean ± S.E.M., n = 3; *** p < 0.0001, by Student’s t test.

Fig. 5. Atropine and thapsigargin abolish noradrenaline-mediated Ca2+ responses in the presence of methacholine. a, using a FLIPR, cells were challenged with the indicated muscarinic receptor agonist (1 mM) in the presence or absence of 10 μM atropine before challenge with noradrenaline (10 μM). The graph shows the responses to noradrenaline in the presence of either muscarinic receptor agonists alone or muscarinic receptor agonists and atropine. b, cells were preincubated for 5 min in the presence or absence of thapsigargin (2 μM) before challenge with muscarinic receptor agonists (1 mM) and subsequently challenged with noradrenaline (10 μM). The graph shows only the responses to noradrenaline in the presence of muscarinic receptor agonists with or without thapsigargin treatment; the initial responses to methacholine are not shown. Data are mean ± S.E.M., n = 3; *** p < 0.0001, by Student’s t test.

Fig. 6. Effect of extracellular Ca2+ on maximal intracellular Ca2+ responses to either muscarinic receptor agonists or noradrenaline in the presence of muscarinic receptor agonists. Using a FLIPR, cells were challenged with methacholine (1 mM) in the presence or absence of [Ca2+]i, a, from such experiments, the maximal (peak) change in fluorescence immediately after addition of the indicated muscarinic receptor agonist (1 mM) in the presence or absence of [Ca2+], was determined as an index of the Ca2+ response (b). In the continued presence of the muscarinic receptor agonist, cells were stimulated with noradrenaline (10 μM) and the maximal change in fluorescence determined (c). Data are mean ± S.E.M., n = 3; ** p < 0.002, by Student’s t test.
due to different release rates, differential activation of extrusion mechanisms or oscillatory Ca\(^{2+}\)/H\(_{11001}\) signaling, the relationship between muscarinic receptor-mediated peak Ca\(^{2+}\)/H\(_{11001}\) responses and the extent of store depletion may not be identical.
for different agonists. Furthermore, at 1 μM methacholine and 100 μM arecoline, receptor occupancy will be different, and this could influence cross-talk. It is therefore unclear whether aspects other than Ca\textsuperscript{2+} store depletion contribute to greater facilitation of \textit{n}-2-adrenoceptor-mediated signaling by partial compared with full muscarinic receptor agonists.

Ca\textsuperscript{2+} signaling by \textit{n}-adrenoceptors was abolished after antagonism of muscarinic receptors with atropine, indicating that concurrent activation of both receptors is required. This is consistent with many examples of cross-talk resulting in enhanced Ca\textsuperscript{2+} signaling (Dickenson and Hill, 1998; Jiménez et al., 1999; Tanimura et al., 1999; Chan et al., 2000; Short and Taylor, 2000; Yeo et al., 2001; Werry et al., 2003b).

**Fig. 10.** Responses to muscarinic receptor agonists and noradrenaline in the presence of the Ins(1,4,5)P\textsubscript{3} Ca\textsuperscript{2+} channel blocker xestospongin C. Cells were incubated in the presence or absence of 10 μM xestospongin C for 30 min. Immediately before experimentation, cell monolayers were washed with buffer containing no added Ca\textsuperscript{2+} and in which the [Ca\textsuperscript{2+}] had been buffered with EGTA to <100 nM. Cells were then placed in this low [Ca\textsuperscript{2+}] buffer, with or without xestospongin C as appropriate and immediately stimulated with an approximate EC\textsubscript{50} or maximal concentration of either methacholine or oxotremorine in a plate reader (NOVOstar). Cells were subsequently challenged with 10 μM noradrenaline. Panels show the maximal changes in fluorescence on addition of either the muscarinic receptor agonist (a) or noradrenaline (b) as an index of the Ca\textsuperscript{2+} responses. Data are mean ± S.E.M., n = 3; *, p < 0.05; **, p < 0.01; and ***, p < 0.001, by Student’s t test.

**Fig. 11.** Cross-talk is unaffected by inhibition of PKC. Cells were incubated with 100 μM PKC inhibitor myristoylated protein kinase C 20-28 for 30 min before stimulation with the indicated concentrations of muscarinic receptor agonists, and fluorescence was recorded for 150 s. These cells were subsequently challenged with 10 μM noradrenaline, and fluorescence was recorded for an additional 150 s. Maximal changes in fluorescence on the addition of noradrenaline are shown. Data are mean ± S.E.M., n = 3.

**Fig. 12.** Muscarinic receptor activation facilitates forskolin-mediated Ca\textsuperscript{2+} signaling. Using a FLIPR, cells were challenged with either methacholine (1 mM) or buffer and ~60 s later they were challenged with forskolin (100 μM) as indicated by the arrow. Forskolin evoked a Ca\textsuperscript{2+} response only in the presence of methacholine. For comparison, cells challenged initially with methacholine and subsequently with either noradrenaline or buffer are shown. For clarity, only the responses to the second addition (noradrenaline, forskolin, or buffer) are shown. Data are representative of three or more experiments.

**Fig. 13.** Lack of effect of PKA inhibitors on muscarinic receptor- and noradrenaline-mediated Ca\textsuperscript{2+} responses. Cells were either untreated (a) or preincubated with the PKA inhibitor H89 (b) or protein kinase A amide inhibitor 14-22 (c) for 30 min. Using a plate reader (NOVOstar), cells were then stimulated with approximately an EC\textsubscript{50} concentration of oxotremorine (1 μM) and subsequently with noradrenaline (10 μM) to provide robust cross-talk. d, maximal responses to noradrenaline immediately after addition of noradrenaline in the absence or presence of the PKA inhibitors. e, cells were cultured for 24 h in a 12-well plate and placed in serum-free media for a further 24 h. Cells were either untreated (control) or preincubated with the PKA inhibitors H89 (10 μM) or protein kinase A amide inhibitor 14-22 (25 μM) for 30 min and subsequently stimulated with forskolin (100 μM) for 10 min. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with phospho-ERK and total ERK antibodies, visualized with ECL and exposure to film. Data are either representative of three independent experiments (a–c and e) or mean ± S.E.M., n = 3 (d).
Treatment of our cells with CTX for 24 h down-regulated Goβq and substantially inhibited noradrenaline-mediated Ca2+ signaling, thereby demonstrating its role in cross-talk. Furthermore, consistent with other examples of such cross-talk (Werry et al., 2002; Tovey et al., 2003; Werry et al., 2003a), noradrenaline responses during muscarinic receptor activation were independent of extracellular Ca2+. Cross-talk also required a replete, thapsigargin-sensitive intracellular Ca2+ store, and although our data suggest that Ins(1,4,5)P3 receptors are particularly involved in release, interpretation of inhibitor studies can be problematic. Thus, both 2-APB and xestospongic C inhibit a range of plasma membrane channels, including store-operated Ca2+ channels (Liu and Ambudkar, 2001; Bootman et al., 2002; Ozaki et al., 2002). However, cross-talk was independent of extracellular Ca2+, and xestospongic C reduced noradrenaline responses even when the trans-plasmalemmal [Ca2+] gradient was abolished, indicating that these compounds reduced cross-talk through inhibition of intracellular channels. The mechanism by which cross-talk enhances Ca2+ release by these receptors is not clear. Although enhanced PLC activity and increased Ins(1,4,5)P3 generation underlies cross-talk between a variety of Goq/11- and Goα-coupled receptors (Selbie et al., 1995; Yang et al., 2001), we were unable to find evidence for enhanced PLC activity. Although such measurements of PLC activity are sufficiently sensitive to demonstrate enhanced PLC activity as a means of cross-talk (Selbie et al., 1995; Yang et al., 2001; Werry et al., 2003b), we cannot exclude the possibility that localized, transient increases in Ins(1,4,5)P3 occur that are below the sensitivity of our technique. However, a lack of enhanced PLC activity is consistent with other examples of cross-talk involving Goq/11- and Goα-coupled receptors (Jiménez et al., 1999; Tanimura et al., 1999), and alternative mechanisms must be considered.

After activation of Goα and subsequent cAMP generation by adenylly cyclase, transduction occurs through PKA or an exchange protein directly activated by cAMP (EPAC), making these likely mediators of cross-talk. Although PKA-dependent sensitization of Ins(1,4,5)P3 receptors may potentiate Goq/11-coupled receptor-mediated Ca2+ responses by isoproterenol, in rat parotid cells (Tanimura et al., 1999), the cross-talk described here is independent of PKA activation. EPAC has been linked to Ca2+ signaling through either sensitization of intracellular Ca2+ channels (Kang et al., 2001, 2003, 2005; den Dekker et al., 2002) or PLCε activation (Schmidt et al., 2001). Although activation of PLCε is unlikely to be responsible (see above), sensitization of intracellular Ca2+ release channels or inhibition of the sarco(endo)plasmic reticulum Ca2+-ATPase (den Dekker et al., 2002) via EPAC and Rap-dependent mechanisms could underlie cross-talk.

Of relevance to the present study are observations in HEK 293 cells demonstrating that either ATP or the muscarinic receptor full agonist carbachol enhances Ca2+ signaling by recombinant Goα-coupled type 1 parathyroid hormone receptors independently of enhanced PLC activity or PKA (Short and Taylor, 2000; Tovey et al., 2003). Although these previous studies suggested that cAMP was not responsible, recent work by this group revealed that low-affinity binding of cAMP to the Ins(1,4,5)P3 receptor (or associated protein) increases sensitivity to Ins(1,4,5)P3 sufficiently to account for cross-talk (Tovey et al., 2008). Indeed, a specific association between adenyly cyclase 6 and the type 2 Ins(1,4,5)P3 receptor forms “cAMP junctions” that ensure sufficiently high local concentrations of cAMP to influence Ins(1,4,5)P3 receptor sensitivity. Although alternative mechanisms for cross-talk exist (Werry et al., 2003a), this is consistent with the current study, which shows lack of direct dependence on Goα (forskolin activation of adenyly cyclase is effective), independence from PKA, and requirement for coactivation of Goq/11 and Goα.

Muscarinic M3 receptors and β2-adrenoceptors are often coexpressed, for example, in smooth muscle in which muscarinic receptors evoke Ca2+-dependent contraction and adrenoceptors cause cAMP- and PKA-dependent relaxation. In airways, these receptors are critical regulators of airway diameter and drug targets in the management of respiratory diseases such as asthma. Thus, selective β2-adrenoceptor agonists are remarkably effective for acute relief of airway narrowing, and anticholinergics can also be useful. Indeed, acetylcholine release may be exaggerated in asthma, potentially contributing to the clinical efficacy of anticholinergic therapy (Hai, 2007). However, asthma is a chronic airway disease characterized by hyperactivity, inflammation, altered contractile properties, and remodelling through hypertrophy and hyperplasia. Airway smooth muscle cells play prominent roles in these pathological features, although mechanisms remain to be precisely defined (Baroillo et al., 2008). Therefore, it is noteworthy that muscarinic receptors also promote inflammatory gene expression and mitogenesis in these cells (Hai, 2007) and that this could contribute to disease development and progression, particularly with exaggerated acetylcholine release. Ca2+ is central to muscarinic receptor signaling, and prominent roles for the Ca2+-regulated transcription factors, nuclear factor of activated T cells and cAMP-response element-binding protein, have been demonstrated in smooth muscle (Barlow et al., 2006; Pulver-Kaste et al., 2006). Thus, although any Ca2+ signaling by β2-adrenoceptors during muscarinic receptor activation could offset adrenoceptor-mediated relaxation, it is important to recognize that Ca2+ plays other roles. In this respect, the pattern of Ca2+ signaling is likely to be critical as at least in other cell types, Ca2+ oscillations encode information about gene expression (Dolmetsch et al., 1998; Li et al., 1998). Thus, remodelling of Ca2+ responses by β2-adrenoceptors could contribute to altered gene expression. Such an influence of cross-talk has been established in UMR-106 rat osteosarcoma cells, in which interaction between Goqα-coupled parathyroid hormone receptors and Goq/11-coupled P2Y1 receptors enhances Ca2+ signaling, cAMP-response element-binding protein phosphorylation, and induction of the c-fos gene in a cAMP- and PKA-independent manner (Buckley et al., 2001).

It is interesting that although selective β2-adrenoceptor agonists provide acute relief of airway narrowing, long-term use may be associated with a deterioration of control and an increased morbidity and mortality (Bond et al., 2007). The mechanisms underlying this are unclear. One possibility is that this is a direct consequence of enhanced β2-adrenoceptor signaling because overexpression of β2-adrenoceptors in mice enhances tracheal sensitivity to acetylcholine through up-regulation of phospholipase Cβ (McGraw et al., 2003). Whether cross-talk as described in the present study occurs in airway smooth muscle in health and/or disease and...
whether this contributes to regulation of airway structure and function remain to be established.

References


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