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Homologous and heterologous uncoupling of muscarinic M_3 and α_{1B} adrenoceptors to $G\alpha_{q/11}$ in SH-SY5Y human neuroblastoma cells

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1 The present study employed a [^{35}S]-GTPγS binding protocol in conjunction with immunoprecipitation (IP) of the Gα subunits to investigate the desensitization of $G_{q/11}$-coupled receptors at the level of the G-protein activation. Membranes from SH-SY5Y cells expressing the recombinant human α_{1B}-adrenoceptor (α_{1B}-AR) (and endogenously expressing the M_3 muscarinic acetylcholine receptor (M_3-AChR)) exhibited $G_{q/11}$ activation in a concentration-dependent manner in response to noradrenaline or methacholine.

2 Pre-treatment of intact cells with agonist prior to membrane preparation and use in the [^{35}S]-GTPγS IP assay demonstrated that both receptors were homologously desensitized by pre-treatment with agonist since the $G_{q/11}$ activation in response to a secondary challenge with agonist was markedly reduced. Stimulation of α_{1B}-AR was effective at heterologously desensitizing the M_3-AChR. The PKC inhibitor, Ro-31-8220 (10 μM) was ineffective at preventing the agonist-mediated receptor desensitization.

3 [^{32}P]P_i-labelled cells allowed the detection of increases in receptor phosphorylation. Phorbol 12,13 dibutyrate (PDBu) (1 μM) was effective at producing a Ro-31-8220 (10 μM)-sensitive, detectable increase in α_{1B}-AR but not M_3-AChR phosphorylation. Noradrenaline (30 μM) stimulated α_{1B}-AR phosphorylation, which could be partially inhibited by Ro-31-8220 (10 μM). The phosphorylation of M_3-AChR was increased by methacholine (100 μM) incubation and this effect appeared to be insensitive to Ro-31-8220 (10 μM).

4 These findings demonstrate that [^{35}S]-GTPγS-Gα-subunit IP can be used to estimate receptor desensitization as a decline in receptor-G-protein coupling. Both the α_{1B}-AR and M_3-AChR undergo rapid homologous desensitization that is associated with an increase in receptor phosphorylation. The heterologous desensitization of M_3-AChR produced by α_{1B}-AR stimulation is not associated with a detectable increase in M_3-AChR phosphorylation, suggesting that receptor phosphorylation is not necessarily a prerequisite for desensitization.

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Abbreviations: α_{1B}-AR, α_{1B}-adrenoceptor; β_2-AR, β_2-adrenoceptor; B_2-BK, B_2-bradykinin receptor; GPCR, G protein-coupled receptor; GRK, G-protein receptor kinase; HBS, HEPES buffered saline; IP, immunoprecipitation; KHB, Krebs' Henseleit buffer; M_3-AchR, M_3 muscarinic$_3$ acetylcholine receptor; MCh, methacholine; NA, noradrenaline, norepinephrine; [^{3}H]-NMS, [N-methyl-^{3}H]scopolamine methyl chloride; PDBu, phorbol 12,13 dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; [^{3}H]-prazosin, prazosin [7-methoxy-^{3}H]

Introduction

Many G protein-coupled receptors (GPCR) undergo a rapid desensitization in response to stimulation by agonist. The mechanism underlying this phenomenon has been most extensively characterized for the β_2-adrenoceptor (β_2-AR) (Lefkowitz, 1998). The activated β_2-AR is susceptible to phosphorylation by intracellular G-protein receptor kinase 2 (GRK2) and the binding of β-arrestin to the phosphorylated receptor produces an uncoupling of the receptor from G_s-protein activation. The β_2-AR can then be internalized either to be recycled or down-regulated (Lefkowitz, 1998). Desensitization of $G_{q/11}$-coupled receptors has been less clearly characterized (see Tobin, 1997) and the present study aimed to investigate further the relationship between phosphorylation of such receptors and its uncoupling of receptor from G-protein activation.

Since $G_{q/11}$ activation indirectly stimulates protein kinase C (PKC) (*via* the 1,2-diacylglycerol produced by increased phospholipase C activity), one possible regulatory pathway for $G_{q/11}$-coupled receptors could be from a negative feedback by PKC-mediated phosphorylation of the receptor. For example, recently García-Sáinz *et al.* (1999) demonstrated that the phosphorylation of the α_{1B}-adrenoceptor (α_{1B}-AR) expressed in mouse fibroblasts could be increased

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by the PKC activator, phorbol 12-myristate 13-acetate (PMA) and furthermore, that this phosphorylated α_{1B}-AR displayed attenuated Ca^{2+} and phospholipase C signalling.

Phosphorylation and attenuation of signalling of $G_{q/11}$-coupled receptors does not appear to be confined to a PKC dependent mechanism. Studies involving coexpression of GRKs with the α_{1B}-AR demonstrated that GRKs 2,3,5 and 6 can phosphorylate the receptor but only GRKs 2 and 3 had an effect on α_{1B}-AR-mediated inositol phosphate production (Diviani *et al*., 1996). Similarly, the type 1A angiotensin II receptor, which also couples to $G_{q/11}$, displayed reduced desensitization, assessed by GTPase activity in membranes, when co-expressed with a dominant negative GRK2 (Oppermann *et al*., 1996). Further evidence implicating GRKs in $G_{q/11}$ coupled receptor regulation has come recently from imaging studies using EGFP tagged GRK2 co-expressed with substance P receptor in HEK 293 cells. Receptor stimulation induced a translocation of GRK2 to the plasma membrane that peaked at 15–21s (Barak *et al*., 1999).

The kinases that phosphorylate $G_{q/11}$ linked receptors in an agonist sensitive manner may not be confined to GRK or second messenger activated kinases. Previous studies from this laboratory (Tobin *et al*., 1996; 1997; Waugh *et al*., 1999) have revealed that casein kinase 1α may phosphorylate the agonist-stimulated M_1- and M_3-AChRs as well as rhodopsin.

In an attempt to simplify the relationship between receptor function and phosphorylation the present study has employed a [^{35}S]-GTPγS binding assay in conjunction with immunoprecipitation (IP) of specific Gα subunits to measure receptor-G-protein uncoupling directly. Most previous studies have assessed desensitization as a waning of second messenger accumulation (Wojcikiewicz *et al*., 1993; Briddon *et al*., 1998; Willars *et al*., 1999), the results of which could be complicated by downstream factors (e.g. Ca^{2+} store depletion, modulation of effector function by second messenger activated kinases). Alternatively, investigations have used total [^{35}S]-GTPγS binding or GTPase activity as an index of receptor desensitization (Oppermann *et al*., 1996; Vázquez-Prado *et al*., 1997; García-Sáinz *et al*., 1999). However this assesses total G-protein activation and can complicate interpretation of the results. For instance, a growing number of GPCRs have now been shown to couple to more than one type of Gα-subtype (Gudermann *et al*., 1997). Using IP in conjunction with [^{35}S]-GTPγS binding it is also possible to assess the activity of several specific Gα-subtypes addressing the possibility that uncoupling from one Gα subunit may be accompanied by increased trafficking to another (see Daaka *et al*., 1997). The technique of IP of [^{35}S]-GTPγS-Gα complexes has been used in previous studies to successfully characterize receptor-G-protein coupling specificities in rat brain (Wang *et al*., 1995) and SF9 cell membranes (Barr *et al*., 1997).

The present study used the IP of [^{35}S]-GTPγS-Gα complexes, in conjunction with an estimation of receptor phosphorylation by IP of [^{32}P]-labelled receptor, to investigate receptor-G-protein coupling in a neuronal phenotypic cell line, SH-SY5Y. This cell line has been well characterized in our laboratory and has been shown to endogenously express M_3 muscarinic acetylcholine receptor (M_3-AChR) (e.g. Lambert *et al*., 1989; Lambert & Nahorski, 1990; Wojcikiewicz *et al*., 1990; 1994; Willars *et al*., 1996). We have generated a SH-SY5Y cell line that stably expresses α_{1B}-AR in order to investigate homologous and heterologous regulation between $G_{q/11}$-linked receptors.

Methods

Cell culture and transfection protocol

SH-SY5Y cells were transfected using DOTAP reagent (Roche Molecular Biochemicals, Germany) with a pcDNA3 vector containing the cDNA for human α_{1B}-adrenoceptor. SH-SY5Y wild-type were grown in minimum essential medium (Gibco) supplemented with 5%:5% foetal calf serum: newborn calf serum and antibiotics. Stable α_{1B}-AR transfected cells were selected and maintained in media containing 300 μg ml^{-1} geneticin.

Membrane preparation and radioligand saturation binding

Cells were grown to confluency in 175 cm^2 flasks prior to addition of HEPES buffered saline (HBS) (HEPES, 10 mM; NaCl, 0.9%; EDTA, 0.2%, pH 7.4). Following cell detachment the cell suspension was transferred to centrifuge tubes for homogenization (Polytron 2100 (Kinematica), speed 24, 4×5 s bursts). The cell homogenate was then centrifuged ($40{,}000 \times g$, 15 min, 4°C). The pellet was resuspended in buffer (HEPES, 10 mM; EDTA, 10 mM; pH 7.4) for a second homogenization and centrifugation. The final pellet was then resuspended in buffer (HEPES, 10 mM; EDTA, 0.1 mM, pH 7.4) at a protein concentration of 1 mg ml^{-1} and snap frozen in liquid N_2 until required.

Saturation binding assays were performed by incubation of cell membranes with [7-methoxy-^{3}H] prazosin ([^{3}H]prazosin) or 1-[N-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]-NMS) (both 10 pM–3 nM) for 1 h at 37°C. Bound radioligand was separated from free by rapid filtration through GF/B Brandell filters. Non-specific binding was determined in the presence of phentolamine (10 μM) or atropine (10 μM).

GTPγS binding followed by immunoprecipitation of Gα subunits

G-protein activation was assessed using a variation (Burford *et al*., 1995; Burford Akam *et al*., 1998) of a [^{35}S]-GTPγS binding assay (Barr *et al*., 1997; Barr & Manning, 1999). Cell membranes (50 μg of protein) were incubated with [^{35}S]-GTPγS (40 nM), GDP (1–10 μM) and agonist for 2 min, 30°C, in assay buffer (HEPES, 10 mM; NaCl, 100 mM; $MgCl_2$, 10 mM; pH 7.4). Non-specific binding was defined in the presence of 10 μM GTPγS. The incubation was terminated by dilution with ice-cold assay buffer followed by centrifugation ($20{,}000 \times g$, 4°C, 6 min). The Gα-[^{35}S]-GTPγS complex was then solubilized in buffer (Tris, 100 mM; NaCl, 200 mM, SDS, 0.2%, EDTA, 1 mM; Igepal, 1.25%; pH 7.4) for 1 h on ice prior to immunoprecipitation with anti-sera raised to specific G-protein α subunits complexed with protein A-sepharose. Substantial characterization of these anti-sera revealed their specificity under the conditions of this assay (Akam *et al*., 2001). Following washing of the immunoprecipitate with solubilization buffer lacking SDS the radioactivity content was evaluated by liquid scintillation counting.

Pre-stimulation protocol

In experiments where pre-stimulation of intact cells was performed prior to membrane preparation, cell suspensions were incubated in Krebs' Henseleit buffer (KHB) containing agonist (30 μM noradrenaline (NA) or 3 μM – 1 mM methacholine (MCh)) for 0 – 300 s, 37°C. The agonist incubation was terminated by dilution and immediate centrifugation (4 min, 900 × *g*, 4°C). In experiments involving Ro-31-8220 (10 μM), the cells were incubated with the compound for 10 min prior to vehicle or agonist incubation.

Receptor phosphorylation

These experiments utilized specific receptor anti-sera against M_3-AChR (Tobin & Nahorski, 1993) and α_{1B}-AR (Santa Cruz Biotechnology, Santa Cruz, California) to immunoprecipitate [^{32}P]-labelled receptors in SH-SY5Y cells. The assay was performed exactly as previously described by Willars *et al*. (1999) with the minor modification that the low expression level of M_3-AChR dictated a stringent washing of the protein A sepharose-anti-sera-receptor complex with two 1 ml washes of Tris buffered saline (Tris, 100 mM; NaCl, 1.5 M; pH 7.4) containing Tween-20 (0.5%) followed by three 1 ml washes with wash buffer (Tris 10 mM; EDTA 1 mM, pH 7.4). The resulting pellet was subject to SDS – PAGE and subsequent autoradiography as previously described (Willars *et al*., 1999).

Materials

Anti-sera to $G_{q/11}$ were generated by Genosys Biotechnologies (Pampisford, U.K.) by inoculation of rabbits with a decapeptide (QLNLKEYNLV) corresponding to 344 – 353 residues of the G_q sequence (which is also 100% homologous to the G_{11} sequence). Anti-sera to m_3-AChR were generated from a portion of the third intracellular loop of the human m_3-AChR and has been characterized previously (Tobin & Nahorski, 1993). G_{i3} and $G_{i1/2}$ antibodies were purchased from NEN Life Science Products (Hounslow, U.K.) whilst all other anti-sera were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). [^{35}S]-GTPγS (specific activity 1250 Ci mmol^{-1}), Prazosin [7-methoxy-^{3}H] (specific activity 75 – 79 Ci mmol^{-1}), [^{3}H]-NMS (specific activity 79 Ci mmol^{-1}) were from NEN Life Science Products (Hounslow, U.K.) whilst [^{32}P]-orthophosphate was from Amersham (Bucks, U.K.). With the exception of Ro-31-8220, phorbol 12,13-dibutyrate (PDBu) (both from Calbiochem, Nottingham, U.K.) and protein A sepharose (Amersham-Pharmacia Biotech, U.K.) all other reagents were purchased from Sigma (U.K.).

Results

Radioligand saturation binding determination of receptor density

Transfection of SH-SY5Y cells with the human α_{1B}-AR produced several α_{1B}-AR stably expressing clones. The present study focused on two that were chosen based upon their different α_{1B}-AR densities (a 'low' clone which expressed α_{1B}-AR receptor at a similar density to wild-type M_3-AChR) and G_q activation profiles (a 'high' clone which displayed similar maximal activation of $G_{q/11}$ in response to α_{1B}-AR or M_3-AChR stimulation). The B_{max} determined by [^{3}H]-prazosin saturation binding, which are indicative of α_{1B}-AR expression levels, were 306 ± 40 fmols mg^{-1} protein and 3.9 ± 0.4 pmols mg^{-1} protein for low and high expressing clones, respectively (n = 4). Wild-type SH-SY5Y did not display significant specific [^{3}H]-prazosin binding. The B_{max} determined by [^{3}H]-NMS saturation binding, which are indicative of M_3-AChR expression levels, were 326 ± 42, 114 ± 13 and 288 ± 42 for wild-type, low and high clones (n = 4).

α_{1B} adrenoceptor and M_3 muscarinic receptor stimulation increase binding of [^{35}S]-GTPγS to Gq

[^{35}S]-GTPγS binding followed by immunoprecipitation (IP) of the Gα subunit with anti-$G_{q/11}$ anti-sera was used to estimate the G-protein activation. Basal levels of [^{35}S]-GTPγS-$G_{q/11}$-immunoprecipition following the 2-min, 30°C incubation (used throughout except where stated) was in the range 1640 – 2070 c.p.m. (non-specific binding 750 – 1050 c.p.m.). In wild-type SH-SY5Y cells and the low clone, NA was ineffective at stimulating $G_{q/11}$, however MCh produced activation with an EC_{50} of 17.4 ± 6.7 μM, maximum response of 473 ± 65% basal for wild-type cells (n = 3) and an EC_{50} of 34.3 ± 3.7 μM, maximum response of 178 ± 36% basal for the low clone (n = 3) (Figure 1). In the high clone, NA was more potent than MCh at stimulating $G_{q/11}$ (EC_{50} = 100 ± 35 nM and 36.9 ± 12.5 μM, respectively, n = 3) and the maximum activation of $G_{q/11}$ by MCh and NA was 500 ± 66 and 682 ± 51% basal, respectively (n = 3) (Figure 1). Some of the data presented in Figure 1 have been reported previously to the Society in a preliminary communication (Bundey *et al*., 1999).

Application of MCh (100 μM) in conjunction with NA (30 μM) produced a $G_{q/11}$ activation not significantly different from that of NA (30 μM) alone (6090 ± 432 c.p.m., P > 0.05, Student's t-test, n = 2) suggesting both receptors accessed the same pool of $G_{q/11}$. Stimulation of cells with agonist produced no significant effect on the receptor density in membranes used for [^{35}S]-GTPγS assays over the timescale studied (0 – 300 s) (P > 0.05, Student's t-test, n = 3) (data not shown). Similarly, Western blotting revealed no detectable differences in the $G_{q/11}$ content of the membrane fraction of agonist treated cells compared to untreated cells over the timescale studied (0 – 300 s) (P > 0.05, Student's t-test, n = 3) (data not shown).

Desensitization of receptor-mediated $G_{q/11}$ activation

Since the low clone failed to produce a $G_{q/11}$ activation in response to NA, all subsequent studies employed the high clone to investigate the relationship between the M_3-AChR and α_{1B}-AR. Treatment of intact cells with agonist prior to the preparation of membranes and subsequent stimulation of G-protein activation by a secondary incubation with agonist revealed that the α_{1B}-AR-mediated stimulation of $G_{q/11}$ activation was reduced by pre-treatment with NA (30 μM, 30 s) (maximum response 55.5 ± 5.8% of control, n = 3) but not MCh (100 μM, 30 s) (Figure 2). In contrast, the M_3-

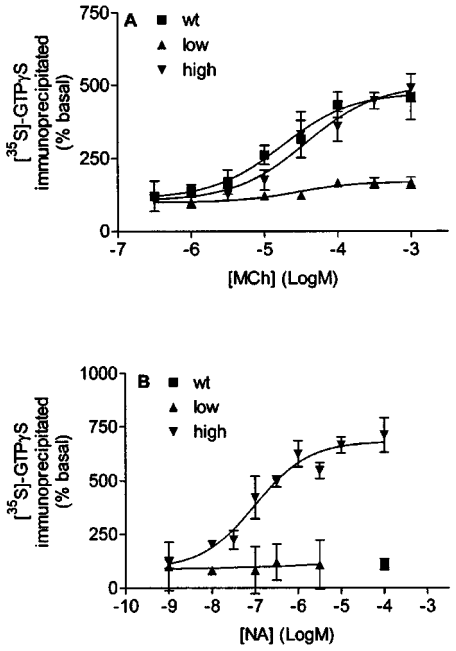

Figure 1 $G_{q/11}$ activation in response to (A), methacholine (MCh) and (B), noradrenaline (NA) in wildtype (wt), low and high expressing α_{1B}-adrenoceptor (α_{1B}-AR) transfected SY-SHSY cells. $G_{q/11}$ activation was estimated using [^{35}S]-GTPγS binding to cell membranes followed by immunoprecipitation (IP) of $G_{q/11}$ with a specific antibody. See Methods for details. Data are mean ± s.e.mean ($n=3$).

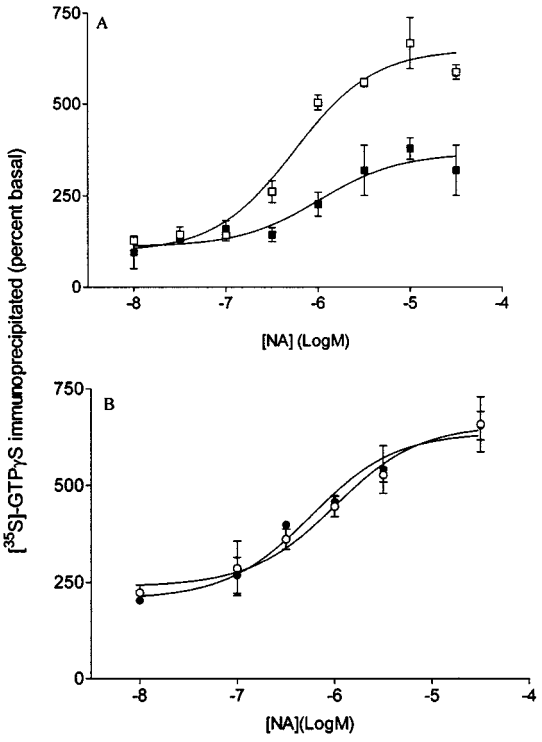

Figure 2 The α_{1B}-AR mediated activation of $G_{q/11}$ following pre-treatment with agonists. Intact human α_{1B}-AR transfected SH-SY5Y cells were treated with vehicle, (A), NA (30 μM) or (B), MCh (100 μM), for 30 s at 37°C before preparation of membranes that were then used in the [^{35}S]-GTPγS IP assay. Open and filled symbols represent vehicle- and agonist-treated cells, respectively. Pre-treatment with NA attenuates the NA stimulation of α_{1B}-AR mediated $G_{q/11}$ activation. Data are means ± s.e.mean of three or four separate experiments.

AChR-mediated stimulation of $G_{q/11}$ activation was reduced by either NA pre-treatment (30 μM, 30 s) (47.2 ± 5.7% of control, $n=3$) or MCh (100 μM, 30 s) (44.7 ± 2.1% of control, $n=4$) (Figure 3).

The desensitization effect of MCh pre-stimulation upon a subsequent MCh-induced activation of $G_{q/11}$ was concentration-dependent (pre-stimulation with MCh (30 μM) produced desensitization with $t_{1/2}=100.6\pm31.5$ s, MCh (1 mM) $t_{1/2}=20.9\pm6.2$ s, $n=3$) but the maximal desensitization effect was not significantly different for both concentrations (MCh (30 μM), 39.2 ± 4.2% control and MCh (1 mM), 41.8 ± 4.4% control, $P>0.05$, Student's t-test, $n=3$) (Figure 4).

Cells incubated with the PKC inhibitor Ro-31-8220 (10 μM) for 10 min prior to treatment with MCh (100 μM, 30 s) or NA (30 μM, 30 s) did not affect the desensitization of the $G_{q/11}$ activation observed with a subsequent stimulation with MCh (100 μM) or NA (30 μM), respectively (Figure 5). The heterologous desensitization of the MCh response by a NA pre-stimulation was also not affected by the Ro-31-8220 incubation.

Agonist stimulation of receptor phosphorylation

Agonist stimulated phosphorylation of receptors was estimated using $^{32}P_i$-labelled cells. A preliminary set of experiments attempted to measure increases in phosphorylation after a 30 s incubation with agonist (data not shown) however the results were variable and consequently not significant. In order to observe reproducible phosphorylation it was necessary to increase the incubation time to 5 min. Immunoprecipitation of α_{1B}-adrenoceptor and M_3-muscarinic receptor demonstrated that homologous phosphorylation of the receptors could be induced by MCh (100 μM) or NA (30 μM) (Figure 6). Phorbol 12,13 dibutyrate (PDBu) (1 μM) was effective at producing a phosphorylation of α_{1B}-AR that could almost be completely inhibited by the PKC inhibitor Ro-31-8220 (10 μM) ($n=3$). In contrast, Ro-31-8220 was not effective at significantly inhibiting agonist-induced homologous phosphorylation of either receptor (Figure 6) (however the effect of Ro-31-8220 on α_{1B}-AR homologous phosphorylation was approaching significance with a P value of 0.07, Student's t-test, $n=3$). α_{1B}-AR stimulation had no significant effect on the phosphorylation state of the M_3-AChR (Figure 6). The small increase in phosphorylation of the α_{1B}-AR in response to MCh (which was not significant despite being observed to some degree in every experiment) was completely abolished by pre-incubation with Ro-31-8220 (10 μM) for 10 min ($n=3$).

Discussion

The present study has utilized a direct assay of receptor catalysed [^{35}S]-GTPγS binding to $G_{q/11}$ to assess rapid desensitization of M_3-AChR and α_{1B}-AR by prior exposure to agonists. Although there is substantial evidence that PLC-linked receptors display rapid desensitization (see Tobin,

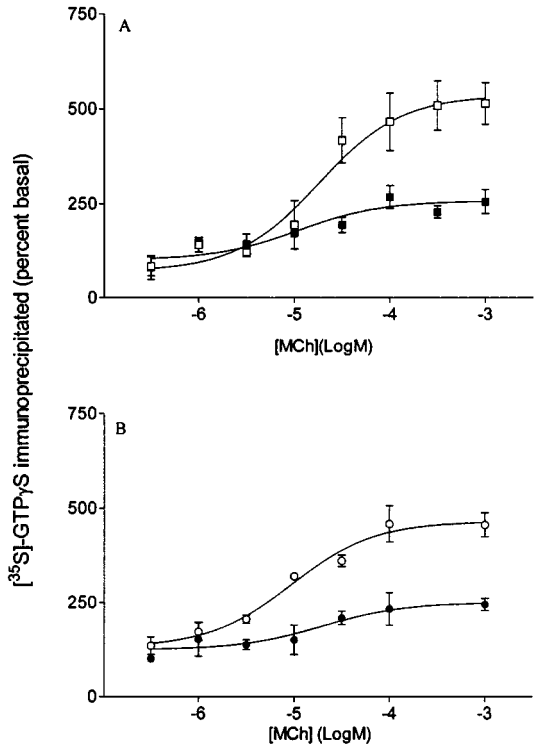

Figure 3 The M_3-muscarinic receptor (M_3-AchR) mediated activation of $G_{q/11}$ following pre-treatment with agonists. Intact human α_{1B}-AR transfected SH-SY5Y cells were treated with vehicle, (A), NA (30 μM) or (B), MCh (100 μM), for 30 s at 37°C before preparation of membranes that were then used in the [^{35}S]-GTPγS IP assay. Open and filled symbols represent vehicle- and agonist-treated cells, respectively. The M_3-AChR appears to be uncoupled from $G_{q/11}$ activation by either NA or MCh pre-treatment. Data are means$\pm$ s.e.mean of three or four separate experiments.

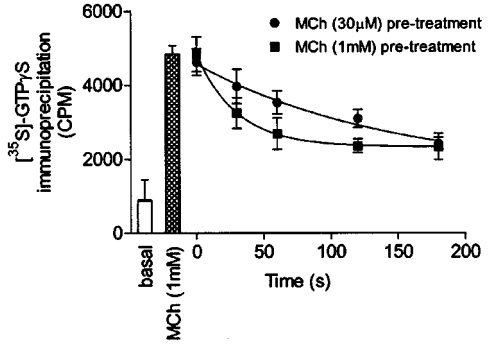

Figure 4 The homologous desensitization of the M_3-AChR is dependent on concentration and duration of the MCh pre-treatment. Two concentrations of MCh were incubated for 0–180 s with intact cells prior to membrane preparation. Membranes were then incubated for 2 min (30°C) with [^{35}S]-GTPγS and MCh (100 μM) prior to IP of the [^{35}S]-GTPγS-Gα complex. The shaded bar represents the MCh stimulation of $G_{q/11}$ activation in the absence of any pre-treatment. Data are points are means$\pm$s.e.mean of three separate experiments.

1997) this has been invariably assessed by waning phosphoinositide hydrolysis, Ca^{2+} mobilization or total [^{35}S]-GTPγS binding. The current study demonstrates the partial uncoupling of both the M_3-AChR and the α_{1B}-AR from $G_{q/11}$ activation when pre-treated with MCh or NA respectively.

To compare the $G_{q/11}$ coupling of α_{1B}-AR and M_3-AChR in the same cell we have investigated the signalling of a SH-SY5Y neuroblastoma transfected with the α_{1B}-AR that expressed the α_{1B}-AR at a similar density to the endogenous M_3-AChR of wild-type cells. This clone (termed 'low', see Figure 1) did not produce detectable $G_{q/11}$ activation in response to NA. A preliminary study (Bundey *et al*., 1999) suggested that this might be due to the inefficiency of α_{1B}-AR to couple to $G_{q/11}$ activation relative to M_3-AChR-$G_{q/11}$ coupling. However, one caveat to this conclusion is that in this particular clone the M_3-AChR density was significantly less than wild-type (114 fmols mg^{-1} protein compared to wild-type, 326 fmols mg^{-1} protein). This may be a clonal difference. For these reasons subsequent studies employed a α_{1B}-AR transfected SH-SY5Y clone (the 'high' clone) picked for its ability to produce $G_{q/11}$ activation to a similar extent when stimulated by NA or MCh. This clone selected on the basis of α_{1B}-AR function may better match the expression of functional α_{1B}-AR to M_3-AChR despite the apparent excess of α_{1B}-AR density as measured by [^{3}H]-prazosin saturation binding.

Stimulation of α_{1B}-AR was effective at heterologously desensitizing the M_3-AChR to produce an uncoupling of M_3-AChR from $G_{q/11}$ activation. In contrast, stimulation of M_3-AChR was ineffective at heterologously desensitising the α_{1B}-AR. Surprisingly, the functional heterologous desensitization appears to be mirrored by the phosphorylation state of the receptor, i.e., M_3-AChR stimulation produces a small but robust increase in the phosphorylation of the α_{1B}-AR but appears to have no effect on α_{1B}-AR-$G_{q/11}$ coupling, whilst α_{1B}-AR stimulation has no significant detectable effect on the phosphorylation state of the M_3-AChR, despite α_{1B}-AR stimulation attenuating the $G_{q/11}$ activation in response to M_3-AChR stimulation.

Medina *et al*. (1998) have shown a similar heterologous phosphorylation of α_{1B}-AR with no apparent effect on α_{1B}-AR signalling. Using a rat-1 fibroblast model expressing α_{1B}-AR they demonstrated that bradykinin, acting at an endogenous B_2-bradykinin (B_2-BK) receptor, could induce α_{1B}-AR phosphorylation without a significant effect on α_{1B}-AR mediated calcium responses (Medina *et al*., 1998). Our own studies (Willars *et al*., 1999) recently highlighted the complexity of heterologous desensitization using CHO cells co-expressing recombinant M_3-AChR ($\approx$6.1 pmol mg^{-1} protein) and B_2-BK ($\approx$900 fmol mg^{-1} protein) receptors. BK was effective at producing a phosphorylation of M_3-AChR, however, this was not associated with a desensitization of M_3-AChR-mediated Ca^{2+} responses (Willars *et al*., 1999). In the present study, a phorbol ester was far more effective at inducing a phosphorylation of α_{1B}-AR than M_3-AChR. Hence, one might predict that this PKC mediated phosphorylation of α_{1B}-AR could be mimicked by M_3-AChR stimulation. M_3-AChR stimulation produces only a small phosphorylation of α_{1B}-AR, however this effect is Ro-31-8220 sensitive. α_{1B}-AR stimulation produced a heterologous desensitization of the M_3-AChR-mediated $G_{q/11}$ activation despite no detectable increase in M_3-AChR phosphorylation. Similarly, Willars *et al*. (1999) demonstrated that M_3-AChR stimulation produced a heterologous desensitization of the BK response despite no observed B_2-BK phosphorylation.

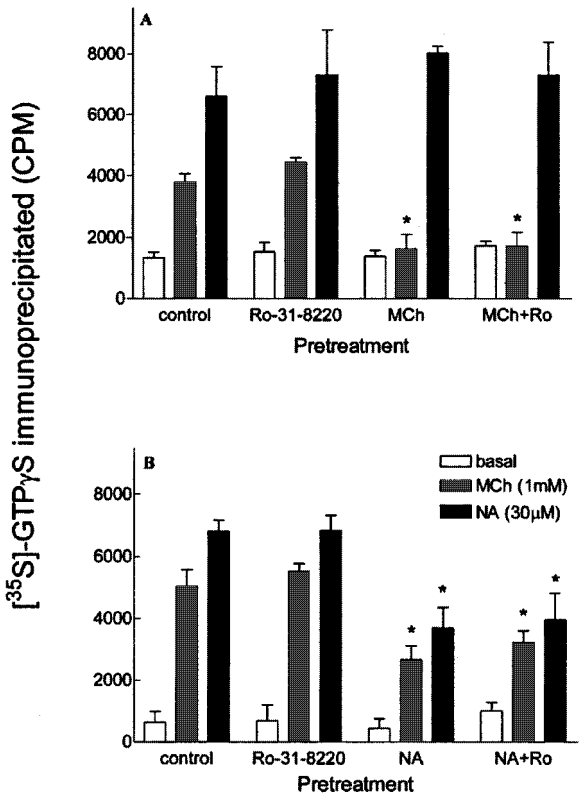

Figure 5 The effect of the PKC inhibitor, Ro-31-8220, on the receptor desensitization mediated by 30 s pre-treatment with (A) MCh (100 μM) or (B) NA (30 μM). Intact cells had one of the following pre-treatments (shown on the x-axis): control, no pre-treatment; Ro-31-8220 (10 μM) for 10 min; MCh (100 μM) for 30 s; NA (30 μM) for 30 s; MCh or NA + Ro, MCh (100 μM) or NA (30 μM) for 30 s following a 10 min incubation with Ro-31-8220 (10 μM). Agonist stimulation of $G_{q/11}$ activation was then estimated in these cells using [^{35}S]-GTPγS binding followed by $G_{q/11}$ IP as described in the Methods. *Indicates significantly different from the agonist responses of cells that have not had an agonist pre-treatment ($P<0.05$, Student's t-test, $n=3$). Data are means $\pm$ s.e.mean for three separate experiments. Note: The figure legend in B applies to both panels.

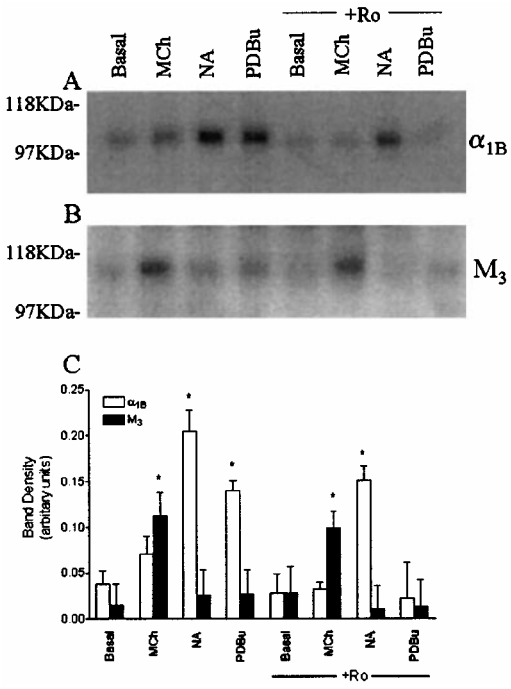

Figure 6 Effects of MCh, NA, phorbol 12, 13 dibutyrate (PDBu) and Ro-31-8220 on α_{1B}-AR and M_3-AChR phosphorylation. SH-SY5Y cells expressing α_{1B}-AR were metabolically labelled with [^{32}P]P_i and incubated with agents prior to IP with anti-sera to either (A), α_{1B}-AR or (B), M_3-AChR. The lanes represent incubation for 5 min, 37°C with: buffer (basal), MCh (100 μM), NA (30 μM) or PDBu (1 μM); in addition lanes 5 to 8 had a pre-incubation with Ro-31-8220 (10 μM) prior to the agonist incubation. Data shown are representative blots however the experiments have been performed three times with similar results. (C) Densitometric analysis of the bands representing the α_{1B}-AR and M_3-AChR produced optical density (OD) readings that have been expressed as a histogram. Data are mean $\pm$ s.e.mean from three separate experiments. *Indicates results are significantly different from basal phosphorylation ($P<0.05$, Student's t-test, $n=3$).

The density of receptor may be important in determining the extent of heterologous desensitization because, in the current study and Willars *et al.* (1999), the receptor that displayed a heterologous desensitization was at least 6 fold less abundant than the other receptor. Hence, a possible mechanism contributing to the observed α_{1B}-AR-mediated reduction in M_3-AChR-$G_{q/11}$ coupling could be by a sequestration of $G_{q/11}$ by the more abundant α_{1B}-AR relative to the M_3-AChR.

These findings suggest that receptor phosphorylation is not necessarily indicative of a functional receptor desensitization and moreover that receptor phosphorylation is not necessarily a prerequisite for desensitization. Hence there are likely to be other steps that are required to orchestrate a functional desensitization of receptor. Fonseca *et al.* (1995) reported receptor internalization in α_{1B}-AR transfected HEK 293 cells within short time frames (5 min). Szekeres *et al.* (1998) have shown a similar time-course of internalization for the endogenous M_3-AChR of SH-SY5Y cells, stimulation with carbachol (1 mM, 5 min) produced a 66% decrease in receptor density when measured by [^{3}H] NMS binding to intact cells. In the present study radioligand binding to cell membranes was used to determine receptor density at the membrane and did not detect a significant loss of receptor from the membrane over the time course of agonist stimulation studied (0–180 s). This finding suggests a direct uncoupling of receptor from G-protein activation is a more likely mechanism rather than an internalization of receptor. Arthur *et al.* (1999) showed that bradykinin stimulation of Madin-Darby canine kidney cells produced an increase in $G\alpha_{q/11}$ immunoreactivity in the cytosol that lead them to suggest that this may be a possible mechanism for desensitization. However, in the present study, Western blotting of membrane fractions of agonist stimulated SH-SY5Y cells failed to detect a significant loss of $G_{q/11}$ from the membrane. Data from Huang *et al.* (1999) showed that GTPγS-stimulated Gα subunits remain at the membrane but concentrate in subdomains and hence it may not be a loss of Gα subunit from the membrane but a restriction of Gα mobility in the membrane that may contribute to receptor desensitization.

In the present study, even at the shortest time point studied, 30 s, pre-incubation with NA followed by stimulation with a secondary NA incubation produces a $G_{q/11}$

activation reduced by approximately 50% of control values. Since agonist stimulated receptor phosphorylation is known to occur within these rapid timescales (e.g. Tobin & Nahorski, 1993) it is a potential mechanism to initiate an uncoupling of receptor from G-protein. Indeed for the β_2-adrenoceptor it is well established that the phosphorylated receptor serves as a target for β-arrestin (Lefkowitz, 1998) and evidence is accumulating that a similar mechanism may underlie $G_{q/11}$-coupled receptor desensitization. McConalogue *et al*. (1999) and Barak *et al*. (1999) have both employed green fluorescent protein tagged β-arrestin to visualize its real-time distribution upon stimulation of a neurokinin-1 receptor linked to $G_{q/11}$. More recently evidence has emerged from several sources suggesting that GRK might be able to disrupt the receptor-G-protein interaction independent of phosphorylation. Carman *et al*. (1999) have shown, using GRK affinity columns, an AlF_4^--dependent binding of $G\alpha_{q/11}$. Dicker *et al*. (1999) showed that a kinase-negative mutant of GRK2 could inhibit parathyroid receptor signalling in COS-1 cells. In support of these findings, Sallese *et al*. (2000) have demonstrated that the N terminus of GRK2 (which contains a RGS homology domain) selectively inhibits inositol phosphate production *via* the thyrotropin receptor (which couples promiscuously to several $G\alpha$ subunits but only the G_q-mediated response was affected).

The current study has demonstrated for the first time, using the [^{35}S]-GTPγS IP assay, a direct uncoupling of $G_{q/11}$ from receptor in both homologous and heterologous desensitization. Furthermore, α_{1B}-AR stimulation can produce a heterologous desensitization of the M_3-AChR however this is not concurrent with an increase in M_3-AChR phosphorylation. The recently discovered GRK2 N-terminal RGS domain association with $G_{q/11}$ (Carman *et al*., 1999; Sallese *et al*., 2000) may be fundamental to this phosphorylation independent desensitization and experiments to clarify this are a focus of future studies.

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