Receptor phosphorylation does not mediate cross talk between muscarinic M₃ and bradykinin B₂ receptors

GARY B. WILLARS,¹ WERNER MÜLLER-ESTERL,² AND STEFAN R. NAHORSKI¹

¹Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom; and ²Institute of Physiological Chemistry and Pathobiology, Johannes Gutenberg University at Mainz, Mainz, Germany

Willars, Gary B., Werner Müller-Esterl, and Stefan R. Nahorski. Receptor phosphorylation does not mediate cross talk between muscarinic M₃ and bradykinin B₂ receptors. Am. J. Physiol. 277 (Cell Physiol. 46): C859–C869, 1999.—This study examined cross talk between phospholipase C-coupled muscarinic M₃ and bradykinin B₂ receptors coexpressed in Chinese hamster ovary (CHO) cells. Agonists of either receptor enhanced phosphoinositide signaling (which rapidly desensitized) and caused protein kinase C (PKC)-independent, homologous receptor phosphorylation. Muscarinic M₃ but not bradykinin B₂ receptors were also phosphorylated after phorbol ester activation of PKC. Consistent with this, muscarinic M₃ receptors were phosphorylated in a PKC-dependent fashion after bradykinin B₂ receptor activation, but muscarinic M₃ receptor activation did not influence bradykinin B₂ receptor phosphorylation. Despite heterologous phosphorylation of muscarinic M₃ receptors, phosphoinositide and Ca²⁺ signaling were unaffected. In contrast, marked heterologous desensitization of bradykinin-mediated responses occurred despite no receptor phosphorylation. This desensitization was associated with a sustained component of muscarinic receptor-mediated signaling, whereas bradykinin's inability to influence muscarinic receptor-mediated responses was associated with rapid and full desensitization of bradykinin responses. Thus the mechanism of functional cross talk most likely involves depletion of a shared signaling component. These data demonstrate that receptor phosphorylation is not a prerequisite for heterologous desensitization and that such desensitization is not obligatory after heterologous receptor phosphorylation.

A GENERAL SCHEME FOR rapid desensitization of G protein-coupled receptors (GPCRs) has evolved, in which agonist occupation of a receptor results in phosphorylation of sites within the carboxy-terminal tail and/or third intracellular loop and this ultimately results in receptor/G protein uncoupling (14, 20, 26, 27). Although much of this scheme has been established through work on the Gₛₐ-coupled β₂-adrenergic receptor, there is accumulating evidence that a similar mechanism underlies the acute regulation of GPCRs coupled to other signal transduction pathways (11, 27). In the case of the β₂-adrenergic receptor, phosphorylation, particularly at high levels of receptor occupancy, is mediated via β-adrenergic receptor kinase (14, 20). This kinase is a member of a family of kinases, known as the G protein-coupled receptor kinases (GRKs), which appear to have a broad substrate specificity (17), including receptors coupled to the activation of phospholipase C (PLC) (11, 27). The physiologically relevant kinases for PLC-coupled receptors remain, however, to be defined and indeed may include kinases distinct from those of the GRK family such as casein kinase 1α (30). Receptor phosphorylation by such kinases is dependent on agonist occupation (20, 27), which ensures that the negative regulation imparted by this covalent modification is strictly homologous.

Many GPCRs are also substrates for second-messenger-activated kinases, thereby providing an alternative mechanism for receptor phosphorylation and desensitization (14, 20, 27). For a limited number of receptors, second-messenger-activated kinases such as protein kinase A (PKA) and PKC have been implicated in agonist-mediated homologous receptor phosphorylation, particularly at low agonist concentrations (1–4, 6, 20, 25). Whether this turns out to be a general phenomenon remains to be established, and the current perspective is that, for the great majority of receptors, agonist-mediated phosphorylation is predominately or exclusively via a GRK or related kinase. The phosphorylation of receptors by second-messenger-activated kinases can, however, also occur in the absence of agonist occupation (20, 27). This potentially adds an additional dimension to receptor regulation in providing a mechanism for heterologous phosphorylation between different receptors coupled to the same or distinct signal transduction pathways. It is still unclear whether such interactions are widespread amongst GPCRs and whether this provides a common and indeed obligatory mechanism for heterologous desensitization, and, if not, what other factors may provide additional or alternative means of functional regulation in a heterologous manner.

We have previously reported that stimulation of PLC-coupled muscarinic receptors (predominantly M₃), endogenously expressed in SH-SY5Y human neuroblastoma cells, is able to markedly inhibit signaling by bradykinin B₂ receptors (34). In contrast, stimulation of bradykinin receptors was unable to influence muscarinic receptor signaling. Although we provided evidence to suggest that the sustained depletion of a shared intracellular Ca²⁺ pool may be required for desensitization of both Ca²⁺ and phosphoinositide signaling (through lack of a Ca²⁺ feed-forward facilitation of PLC), the current study was designed to examine the influence of heterologous receptor phosphorylation on the functional interaction between these two receptor types. To ensure significant receptor expression to allow determination of receptor phosphorylation, we...
have developed a Chinese hamster ovary (CHO) cell line that stably coexpresses recombinant human muscarinic M3 and bradykinin B2 receptors. Although our previous study in SH-SY5Y cells implied interaction between these two receptor types, these cells express at least multiple muscarinic receptor subtypes, which has the potential to cloud interpretation. The CHO cell line used in the current study therefore has the added advantage that any observed interactions are between single receptor subtypes.

**MATERIALS AND METHODS**

**Materials**

Reagents of analytical grade were obtained from suppliers listed previously (33, 37) or from Sigma (Poole, UK). In addition, [1H]NIPC 17731 was from DuPont NEN (Stevenage, UK), [32P]orthophosphate was from Amersham International (Little Chalfont, Bucks, UK), HOE-140 ([D-Arg9,Hyp3,Thi5,D-Tic1,Oic6]bradykinin) was from Hoechst, Ro-31-8220 was from Calbiochem (Nottingham, UK), and protein A-Sepharose was from Amersham Pharmacia Biotech (St. Albans, UK). Molecular biology reagents were obtained from Amer- sham Pharmacia Biotech, Life Technologies (Paisley, UK), or Qiagen (Crawley, UK) unless otherwise stated.

**Cell Culture**

Cells were cultured in MEM α medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, and 10% (vol/vol) FCS. Cultures were maintained at 37°C in 5% CO2/humidi- fied air and passaged weekly. For experiments, cells were harvested with 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA (pH 7.4), and, with the exception of experiments in which the intracellular Ca2+ concentration ([Ca2+]i) was measured (see Measurement of [Ca2+]i), were reseeded at an approximately equivalent density into 24-well multidishes for use 1–2 days later. Cells were maintained and the experimental manipulations performed at 37°C unless otherwise stated.

**Transfection of CHO-M3 Cells With cDNA Encoding Human Bradykinin B2 Receptor**

CHO cells that had previously been transfected with the human muscarinic M3 receptor in pcDNA3 (Invitrogen, CA) containing the neomycin resistance gene (28) (CHO-M3 cells) were transfected with cDNA encoding the human bradykinin B2 receptor using a standard calcium phosphate method. The cDNA for the bradykinin B2 receptor had been cloned (Nat/ExhoI) into the pCEP4 plasmid containing the hygromycin resistance gene (Invitrogen). Cells were selected using hygro- mycin B (400 U/ml) and screened by assessing the ability of bradykinin to generate o-myo-inositol 1,4,5-triphosphate [Ins(1,4,5)P3] [see Generation and Measurement of Ins(1,4,5)P3].

**Determination of Receptor Density**

Muscarinic receptor density was determined by binding of the muscarinic agonist 1-[N-methyl-3H]isopropylmethyl chloride ([3H]NMS; 84 Ci/mmol) to intact cells. Cells in 24-well multidishes were washed and incubated for 1 h at 37°C in 1 ml of Krebs/HEPES buffer [pH 7.4, composition (in mM) 10 HEPES, 4.2 NaHCO3, 117.7 glucose, 1.2 MgSO4, 1.2 KH2PO4, 4.7 KCl, 118 NaCl, and 1.3 CaCl2] containing a range of concentrations of [3H]NMS (~10 pM to 12 nM). Nonspecific binding was determined in the presence of 10 µM atropine. Cells were washed rapidly with two 1-ml volumes of ice-cold buffer and digested with 0.5 ml of 0.1 M NaOH. This was neutralized with 0.5 ml of 1 M TCA, and [H] was determined by liquid scintillation spectroscopy in 5 ml of Florsint IV.

Bradykinin receptor density was determined by the binding of the bradykinin B2 receptor antagonist [3H]NPC 17731 ([prolyl-3,4-3H(N)]-D-Arg-Arg-Pro[3,4-3H]-Hyp-Gly-Phe-Ser-o- HypEtrans-propyl-Oic-Arg; 53.5 Ci/mmol) to intact cells. Cells in 24-well multidishes were washed and incubated for 90 min at 37°C in 1 ml of Krebs/HEPES buffer containing a range of concentrations of [3H]NIPC (~0.15–50 nM). The buffer also contained 1 mM captopril to prevent degradation of the 20 µM bradykinin used to determine nonspecific binding. After incubation, cells were processed as described above.

**Generation and Measurement of Ins(1,4,5)P3**

Media were removed from confluent cell monolayers in 24-well multidishes. Each well was washed with 1 ml Krebs/HEPES buffer and preincubated for 10 min in a further 1 ml of buffer. This buffer was then removed and the cells challenged with 200 µl of buffer containing agonist. In experiments that required pretreatment with either agonist, phor- bol ester, or PKC inhibitor, all agents were added in 100-µl aliquots. All reactions were performed in duplicate. Reactions were stopped with equivalent volumes of 1 M TCA, and a 160-µl aliquot of the acidified aqueous phase was removed, processed, and assayed for Ins(1,4,5)P3 by a radioreceptor assay as previously described (10, 34, 35).

**Measurement of Total PLC Activity**

Agonist-induced accumulation of [3H]inositol mono- and polyphosphates ([3H]InsP) was determined in cells prelabeled with myo-[3H]inositol in which inositol monophospho- phate activity was blocked with Li+. Cells were prelabeled with 3 µCi/ml of myo-[3H]inositol (117 Ci/mmol) for 48 h in 24-well multidishes. Media were then removed and the cell monolayers were washed and incubated for 10 min in 1 ml Krebs/HEPES buffer containing 10 mM Li+. Buffer was replaced with 200 µl of buffer (+Li+) containing agonist. Reactions were in duplicate and terminated with an equal volume of ice-cold 1 M TCA. [3H]InsP, were then extracted and determined exactly as previously described (37).

**Measurement of [Ca2+]i**

Single cell imaging. Cells were harvested and resuspended onto round glass coverslips (no. 1, 22 mm diameter; Chance Propper, Warley, UK). Cells were allowed to attach overnight and were washed with Krebs/HEPES buffer and incubated in this buffer for 1 h at room temperature with fura 2-AM (2 µM). Coverslips were then mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. After excitation at 340 and 380 nm, fluorescent images at wavelengths >510 nm were collected with an intensified charge-coupled device camera (Photomic Science). The 340/380 nm ratio was calculated using the Applied Imaging "Quanticell 700" system.

Population studies. Confluent monolayers of cells in 175-cm² flasks were harvested and resuspended in 2.5 ml of Krebs/HEPES buffer. A 0.5-ml aliquot of this was removed for determination of cellular autofluorescence. Fura 2-AM (5 µM) was added to the remaining 2 ml, which was then left for ~40 min at room temperature with gentle mixing. Supernatant containing extracellular fura 2-AM was then removed following gentle centrifugation of 0.5-ml aliquots. Cells were then resuspended in 3 ml of Krebs/HEPES buffer. With emission recorded at 509 nm, the 340/380 nm excitation ratio was
primary antibody was then added to 0.8 ml of the cleared carboxy-terminal tail of the human receptor (Cys 361-Gln395) required time. When the PKC inhibitor Ro-31-8220 was removed and centrifuged (3 min at 10,000 g), the resulting supernatant was used for immune precipitation with the bradykinin B2 receptor antibody. In addition, the protein A-Sepharose immune complex was washed twice with 10–50 µl of agonist.

Receptor Phosphorylation

Assessment of muscarinic M3 receptor phosphorylation in vivo was determined using a modification of a previously described protocol (29). Briefly, cells in six-well multidishes were washed with Krebs/HEPES buffer without KH2PO4 and incubated for 1 h at 37°C in 1 ml of buffer per well containing 50 µCi of [32P]orthophosphate. Test agents were then added directly to the wells and incubation continued at 37°C for the required time. When the PKC inhibitor Ro-31-8220 was used, this was added 10 min before the test agent. After the required time, buffer was aspirated and 1 ml of ice-cold solubilization buffer added (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 µg/ml iodoacetamide, and 100 µg/ml benzamidine, pH 7.4). After 30 min on ice, the solubilization buffer was removed and centrifuged (3 min at 10,000 g). The primary antibody was then added to 0.8 ml of the cleared supernatant. This antibody was raised in rabbit against a fusion protein representing a portion of the third intracellular loop of the human muscarinic M3 receptor (Ser345-Leu463) and has been previously described and characterized (designated antibody 332) (29). After 60 min on ice, immune complexes were separated by incubation with protein A-Sepharose beads (150 µl of 30 mg/ml) at 4°C under constant agitation. Beads were harvested by centrifugation (10 s at 13,000 g) and washed three times with 1 ml of ice-cold TE buffer (10 mM Tris, 10 mM EDTA, pH 7.4). Samples were extracted into 20 µl of sample buffer (100 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 200 mM dithiothreitol) by standing in water at 70°C for 5 min. Proteins were then resolved by 8% SDS-PAGE. The gels were dried and subjected to autoradiography for 1–3 days.

Bradykinin B2 receptor phosphorylation was determined in an identical fashion, with the exception that the cleared supernatant was preadsorbed (15–30 min, 4°C) with protein A-Sepharose beads (100 µl of 30 mg/ml), and 0.8 ml of the resulting supernatant was used for immune precipitation with the bradykinin B2 receptor antibody. In addition, the protein A-Sepharose immune complex was washed twice with 1 ml of ice-cold 100 mM Tris base, 1.5 M NaCl, 0.5% Tween-20 (pH 7.4) before the final washes with TE buffer. The bradykinin B2 receptor antibody was raised against a region of the carboxy-terminal tail of the human receptor (Cys301-Gln395) and has been previously described and characterized (designated antiserum anti-ID4 (AS346)) (8).

Where required, densitometric analysis of autoradiographs was performed using a Bio-Rad GS-670 imaging densitometer with Molecular Analyst v1.2 software. Local background subtraction was applied to images and data normalized to basal (nonstimulated) receptor phosphorylation.

RESULTS

Transfection of CHO cells expressing the recombinant human muscarinic M3 receptor with the CDNA for the human bradykinin B2 receptor resulted in a number of clones (CHO-M3/B2 cells) that specifically bound the radiolabeled bradykinin antagonist [3H]NPC 17731. The clone for which data are presented expressed the bradykinin B2 receptor at 900 ± 90 fmol/mg protein (n = 3) and bound [3H]NPC 17731 with a dissociation constant (Kd) of −8.46 ± 0.02 log10 M (n = 3; 3.5 nM). This compares with a Kd of 3.6 nM for this compound at the rat bradykinin B2 receptor expressed in COS-1 cells (23). The muscarinic M3 receptor was expressed at 6,117 ± 481 fmol/mg protein (n = 4) and bound [3H]NMS with a Kd of −9.48 ± 0.05 log10 M (n = 4; 0.33 nM), which is typical for muscarinic receptors (9).

Agonist-Mediated Changes in Ins(1,4,5)P3

A challenge of cells with the muscarinic agonist methacholine (1 mM) resulted in a rapid elevation of Ins(1,4,5)P3 to a peak of approximately sevenfold over basal within 5–10 s of agonist addition. In the continued presence of agonist, levels returned to approximately threefold over basal over the subsequent minute, and this was sustained throughout the remainder of the experiment (Fig. 1A). A challenge of cells with bradykinin (10 µM) also resulted in a rapid elevation of Ins(1,4,5)P3 to ~3.5-fold over basal at 5–10 s. Ins(1,4,5)P3 then fell over the subsequent minute to within 20% of basal levels and had returned to basal levels by 15 min (Fig. 1B). Figure 1C demonstrates the marked contrast between sustained Ins(1,4,5)P3 signaling mediated by muscarinic M3 receptor activation and the transient response mediated by activation of bradykinin B2 receptors. A number of other clones were examined, which displayed lower peak Ins(1,4,5)P3 responses to bradykinin but still displayed no evidence of a sustained response (data not shown). The inability of bradykinin to elicit a sustained Ins(1,4,5)P3 response is not a consequence of the metabolism of bradykinin by cell surface degradative enzymes, as further addition of bradykinin failed to elevate Ins(1,4,5)P3 levels (data not shown). Furthermore, the angiotensin-converting enzyme inhibitor captopril (1 mM) did not influence the magnitude or temporal profile of bradykinin-mediated Ins(1,4,5)P3 elevation (data not shown). Other clones also accumulated Ins(1,4,5)P3 in response to either bradykinin or methacholine, with temporal profiles identical to those shown here, although the
magnitude of bradykinin receptor-mediated responses were smaller (data not shown).

The peak elevations of Ins(1,4,5)P₃ in response to either methacholine or bradykinin were concentration dependent, with EC₅₀ values (log₁₀ M) of −6.20 ± 0.16 (n = 6; 0.63 µM) and −7.59 ± 0.04 (n = 6; 26 nM), respectively.

Agonist-Mediated Changes in [³H]InsP₃

In [³H]inositol-labeled cells, in which inositol monophosphatase activity had been blocked with Li⁺, methacholine produced an accumulation of [³H]InsP₃ that was 86% over basal after 1 min. Over the subsequent 9 min of stimulation, methacholine evoked [³H]InsP₃ accumulation at 37%/min, which represents an approximately twofold lower rate of accumulation than over the first minute of agonist challenge. Bradykinin (10 µM) evoked an accumulation of [³H]InsP₃, which was 32% over basal levels after 1 min. Between 1 and 10 min of stimulation, bradykinin evoked [³H]InsP₃ accumulation at a rate of 5.8%/min, which is more than fivefold lower than that over the first minute (Fig. 2). These data demonstrate a relative lack of sustained PLC activation during challenge with bradykinin.

Agonist-Mediated Changes in [Ca²⁺]

Single-cell imaging of fura 2-loaded cells indicated that the majority of cells (>80%) responded with an increase in [Ca²⁺], following challenge with either 1 mM methacholine or 1 µM bradykinin (data not shown). In populations of cells, 1 mM methacholine resulted in a rapid elevation of [Ca²⁺] from basal (0.85 ± 0.03; n = 3; all values are given as the 340/380 ratio) to a transient peak (3.50 ± 0.24; n = 3; Fig. 3), which is equivalent to a change in [Ca²⁺], in these cells of ~100 to 750 nM (data not shown). The [Ca²⁺] then fell quickly to a level (1.29 ± 0.06; n = 3) that was in excess of basal levels and subsequently rose gradually. The
addition of 10 µM bradykinin also caused a rapid elevation in \([\text{Ca}^{2+}]_i\) from basal (0.85 ± 0.03; \(n = 3\)) to a peak (1.71 ± 0.08; \(n = 3\)). The \([\text{Ca}^{2+}]_i\) then fell to approximately basal levels (Fig. 3).

Homologous Receptor Phosphorylation

Using the muscarinic M3 receptor antibody we show that a methacholine (1 mM, 5 min) challenge of CHO cells expressing the muscarinic M3 receptor either alone (CHO-M3) or in combination with the bradykinin B2 receptor (CHO-M3/B2) resulted in a marked increase in the amount of \([32\text{P}]\)orthophosphate associated with a band of ~100 kDa (Figs. 4 and 5). This band was not observed in CHO cells expressing only the bradykinin B2 receptor (CHO-B2) or only the muscarinic M1 receptor (CHO-M1), even when the PLC pathway was activated through challenge of the cells with the appropriate agonist (bradykinin or methacholine, respectively; Fig. 4). In cells expressing the muscarinic M3 receptor, changes in phosphorylation were sometimes observed toward the top of the gel, but it is unclear whether this represents receptor aggregates/dimers formed physiologically or during the preparative process. The increase in phosphorylation of the muscarinic M3 receptor was blocked by the muscarinic receptor antagonist atropine (Fig. 5B) but was relatively unaffected by preincubation of the cells with the PKC inhibitor Ro-31-8220 (Fig. 5A). The phorbol ester phorbol 12,13-dibutyrate (PDBu) increased the level of phosphorylation of the muscarinic M3 receptors. These data demonstrate a protein kinase C (PKC)-independent, homologous phosphorylation of muscarinic M3 receptors. There was also a marked phorbol ester (PKC-dependent) phosphorylation. Cells were either not challenged (lane 1, basal), challenged for 5 min with 1 mM methacholine (MC) without (lane 2) or with (lane 3) 10-min pretreatment with 10 µM of the PKC inhibitor Ro-31-8220 (Ro), or alternatively challenged with 1 µM of the phorbol ester phorbol 12,13 dibutyrate (PDBu) without (lane 4) or with (lane 5) 10-min pretreatment with 10 µM of Ro-31-8220. Intensity of the band representing the muscarinic M3 receptor was determined by densitometric analysis and the extent of receptor phosphorylation calculated in relation to that under basal conditions. Data in histograms represent means ± SE, \(n = 3–5\).

Fig. 5. A: homologous and phorbol ester-mediated phosphorylation of muscarinic M3 receptors. These data demonstrate a protein kinase C (PKC)-independent, homologous phosphorylation of muscarinic M3 receptors. There was also a marked phorbol ester (PKC-dependent) phosphorylation. Cells were either not challenged (lane 1, basal), challenged for 5 min with 1 mM methacholine (MC) without (lane 2) or with (lane 3) 10-min pretreatment with 10 µM of the PKC inhibitor Ro-31-8220 (Ro), or alternatively challenged with 1 µM of the phorbol ester phorbol 12,13 dibutyrate (PDBu) without (lane 4) or with (lane 5) 10-min pretreatment with 10 µM of Ro-31-8220. Intensity of the band representing the muscarinic M3 receptor was determined by densitometric analysis and the extent of receptor phosphorylation calculated in relation to that under basal conditions. Data in histograms represent means ± SE, \(n = 3–5\). B: agonist-mediated phosphorylation of the muscarinic M3 receptor was inhibited by atropine. Cells were either untreated or challenged with methacholine (1 mM) for 5 min in the absence or presence of atropine (10 µM, 5-min preincubation).

dibutyrate (PDBu) increased the level of phosphorylation of the muscarinic M3 receptor in line with the level observed following agonist treatment. Phorbol ester-mediated phosphorylation was fully blocked by inhibition of PKC with Ro-31-8220 (Fig. 5A). The current data are consistent with our previous observation of a rapid, PKC-independent phosphorylation of a band representing the muscarinic M3 receptor at 100 kDa in CHO cells, which is maximal within 1 min and sustained for at least 30 min (29).

Using the bradykinin B2 receptor antibody we show that in CHO-B2 or CHO-M3/B2 cells, challenge with bradykinin resulted in an increase in the level of phosphorylation of two diffuse bands of ~60 and 70–90 kDa (Figs. 4 and 6). No bands were observed in CHO-M3 or CHO-M1 cells, even when the PLC pathway was activated through challenge of the cells with methacholine (Fig. 4). The apparent molecular masses
B2 receptor is 41 kDa, GPCRs rarely run at their higher molecular mass form is a receptor dimer. Alternatively, but we have no evidence to suggest that the differing molecular mass represent two forms of the pressed protein. It is probable that the two bands of or expression as a recombinant vs. endogenously expressed receptor are slightly greater than that observed for the bradykinin B2 receptor expressed endogenously in HF-15 human fibroblasts in which bands were observed at 69 and 52 kDa (8). Such differences in apparent molecular mass may result from differential processing of the receptor as a consequence of expression in different cell lines or expression as a recombinant vs. endogenously expressed protein. It is probable that the two bands of differing molecular mass represent two forms of the receptor, but we have no evidence to suggest that the higher molecular mass form is a receptor dimer. Although the predicted molecular mass of the bradykinin B2 receptor is 41 kDa, GPCRs rarely run at their predicted size due to posttranslational modifications. Indeed, in human foreskin fibroblasts, experimental deglycosylation of the bradykinin B2 receptor shifts its apparent molecular mass from 69 to 44 kDa (5). Furthermore, in these cells the major ligand-binding form of the receptor was the 69-kDa band (5). Analysis of changes in the phosphorylation status of the bradykinin B2 receptor have been performed on the higher molecular weight band, although in all instances changes occurred in both bands and paralleled each other.

Challenge of the CHO-M3/B2 cells for 5 min with 10 µM bradykinin resulted in a higher level of phosphorylation of the bradykinin B2 receptor in a manner unaffected by inhibition of PKC with Ro-31-8220 (Fig. 6A) but which was blocked by the bradykinin B2 receptor antagonist HOE-140 (Fig. 6B). Incubation of cells with PDBu or both PDBu and Ro-31-8220 did not result in significant changes in the phosphorylation level of the bradykinin B2 receptor (Fig. 6A). These data are consistent with the homologous, rapid, PKC-independent phosphorylation of the bradykinin B2 receptor in HF-15 cells (8).

Heterologous Receptor Phosphorylation

Challenge of cells with 10 µM bradykinin resulted in an increase in the level of muscarinic M3 receptor phosphorylation, which was prevented by the PKC inhibitor Ro-31-8220 (Fig. 7A) or the bradykinin B2 receptor antagonist HOE-140 (Fig. 7B). This heterologous phosphorylation was rapid and maximal at −5 min (Fig. 7C). Challenge of cells with 1 mM methacholine for 5 min did not result in phosphorylation of the bradykinin B2 receptor (Fig. 8).

Sensitivity of Muscarinic M3- or Bradykinin B2-Receptor-Mediated Signaling to PKC Activation

Pretreatment of cells with 1 µM PDBu for 5 min before agonist challenge resulted in a significant (P = 0.016, t-test) reduction in the potency of methacholine-mediated peak Ins(1,4,5)P3 responses with EC50 values (log10 M) of −5.88 ± 0.09 (n = 4) and −5.41 ± 0.12 (n = 4) in the absence and presence of PDBu, respectively. PDBu did not, however, affect the magnitude of the response mediated by a maximal concentration of methacholine (Fig. 9A). In contrast, PDBu reduced the magnitude of the Ins(1,4,5)P3 responses to bradykinin (P = 0.0001, two-way ANOVA). However, this was a result of a PDBu-mediated reduction in the basal level of Ins(1,4,5)P3, and the proportional bradykinin-mediated increases in Ins(1,4,5)P3 were similar in the presence and absence of PDBu. The potency of bradykinin was unaffected with EC50 values (log10 M) of −7.28 ± 0.12 (n = 4) and −7.21 ± 0.16 (n = 4) in the absence and presence of PDBu, respectively (Fig. 9B).

Functional Interactions Between Muscarinic M3 Receptor and Bradykinin B2 Receptor Signaling

Pretreatment of cells with either bradykinin (10 µM, 5 min), Ro-31-8220 (10 µM, 10 min), or the two in combination had no significant effect on the potency or magnitude of methacholine-mediated Ins(1,4,5)P3 responses (Fig. 10).

Pretreatment of cells for 5 min with 1 mM methacholine elevated Ins(1,4,5)P3 from a basal level of 86 ± 6 (n = 4) to 254 ± 11 pmol/mg protein (n = 4). Subsequent addition of bradykinin resulted in a minor elevation of Ins(1,4,5)P3 (20 ± 10 pmol/mg protein; n = 4), which was only 22 ± 9% (n = 4; P < 0.01) of the response in the absence of pretreatment with methacholine (84 ±
In additional experiments, a 10-min preincubation of cells with 10 µM Ro-31-8220 did not restore the ability of 10 µM bradykinin to elevate Ins(1,4,5)P$_3$ 5 min after and in the continued presence of 1 mM methacholine (320 ± 65 pmol/mg protein, n = 4, vs. 295 ± 42 pmol/mg protein, n = 4, in the absence and presence of Ro-31-8220, respectively).

The addition of 10 µM bradykinin 5 min after (and in the continued presence of) 1 mM methacholine failed to produce an elevation of [Ca$^{2+}$]i (Fig. 11A). In contrast, the addition of 1 mM methacholine 5 min after (and in the continued presence of) 10 µM bradykinin resulted in a marked elevation of [Ca$^{2+}$]i. The peak elevation of [Ca$^{2+}$]i in response to methacholine (340/380 ratio: 1.54 ± 0.14; n = 3) was reduced compared with that in the absence of bradykinin (340/380 ratio: 2.65 ± 0.19; n = 3; P = 0.0495 Mann-Whitney U test; Fig. 11B). When the methacholine concentration was lowered (to 0.3 µM) to reduce the [Ca$^{2+}$]i response, challenge with bradykinin still failed to evoke an elevation of [Ca$^{2+}$]i (Fig. 11C). In contrast, this lower concentration of methacholine was still able to evoke an elevation of [Ca$^{2+}$]i following and in the continued presence of bradykinin, which was not significantly different from that in the absence of bradykinin (0.55 ± 0.07, n = 3, vs. 0.75 ± 0.08, n = 3, in the presence and absence of bradykinin respectively, P = 0.1266 Mann-Whitney U test; Fig. 11D).

**DISCUSSION**

This study demonstrates a functional interaction between two PLC-coupled GPCRs coexpressed on the same CHO cell. Despite homologous phosphorylation of both bradykinin B$_2$ and muscarinic M$_3$ receptors in these cells, using the current methodology we have only been able to demonstrate heterologous phosphorylation of the latter. Surprisingly, this PKC-dependent heterologous phosphorylation was not associated with functional desensitization. Furthermore, activation of muscarinic M$_3$ receptors markedly inhibited bradykinin-mediated...
phosphoinositide and Ca\(^{2+}\) signaling, despite the absence of heterologous phosphorylation of bradykinin B\(_2\) receptors. Thus functional desensitization is not a necessary consequence of receptor phosphorylation, and in some circumstances mechanisms other than receptor phosphorylation determine cross talk between PLC-coupled receptors.

Heterologous phosphorylation of muscarinic M\(_3\) receptors but not bradykinin B\(_2\) receptors occurred, despite greater phosphoinositide and Ca\(^{2+}\) responses following muscarinic receptor activation, thereby implying receptor specificity. Although the extent of heterologous phosphorylation was comparatively low (1.5- to 2.5-fold over basal), this is consistent with the heterologous phosphorylation of other PLC-coupled receptors (16, 21, 31). The current data also parallel that describing a degree of heterologous phosphorylation considerably less than either homologous phosphorylation or that following activation of PKC by phorbol ester (3, 16). It remains to be established whether this is related to the activation of different kinases or phosphorylation of different sites. The pattern of heterologous phosphorylation correlates with the observation that there is a substantial phosphorylation of muscarinic M\(_3\) receptors but not bradykinin B\(_2\) receptors following activation of PKC with phorbol ester. This may be a consequence of the 16 intracellular PKC consensus sites of the muscarinic M\(_3\) receptor compared with only 4 of the bradykinin B\(_2\) receptor, of which only 2 (Thr\(^{264}\) and Thr\(^{369}\)) are on the cytosolic side of the plasma membrane. It is possible, therefore, that the effect of PDBu on the potency of muscarinic receptor-mediated elevations of Ins(1,4,5)P\(_3\) and not those in response to bradykinin are a reflection of these differences in PKC-mediated receptor phosphorylation, although it is clear that activated PKC can also inhibit signaling at a postreceptor level (7, 24, 33).

We have previously demonstrated a similar unidirectional functional interaction between endogenously expressed muscarinic and bradykinin receptors of SH-SY5Y human neuroblastoma cells (34). Although these cells express predominantly muscarinic receptors of the M\(_3\) subtype, they also express other subtypes (32), and we also cannot discount the possibility of multiple bradykinin receptor subtypes. The present study not only demonstrates that cross talk is not dependent on the coexpression of multiple receptor subtypes but that the greater level of receptor expression has enabled us to examine receptor phosphorylation. In the current study we sought to match (or at least maximize) the bradykinin receptor-mediated signaling with that of the muscarinic receptor-mediated signaling (rather than matching levels of receptor expression). This has resulted in the use of a cell line expressing much greater levels of muscarinic M\(_3\) receptors than bradykinin B\(_2\) receptors. However, despite this, the phosphoinositide and Ca\(^{2+}\) signaling data (specifically the patterns of homologous and heterologous desensitization) are entirely in accord with that seen in SH-SY5Y cells (34) that express approximately equivalent (and much lower) amounts (~300 fmol/mg membrane protein) of these receptors (A. K. Martin, S. R. Nahorski, and G. B. Willars, unpublished data). In addition, in the current study, methacholine-mediated Ins(1,4,5)P\(_3\) responses were unaffected over the concentration–response curve following maximal activation of bradykinin receptors.

Fig. 9. Effects of PKC activation with PDBu on Ins(1,4,5)P\(_3\) accumulation in response to activation of either muscarinic M\(_3\) or bradykinin B\(_2\) receptors. Cells were either untreated (○, ○) or pretreated with 1 µM PDBu for 5 min (□, ○) and the cells challenged for 10 s with various concentrations of either methacholine (A) or bradykinin (B). Ins(1,4,5)P\(_3\) mass was determined by radioreceptor assay in neutralized extracts of cells. Data are means ± SE, n = 4.

Fig. 10. Functional interaction between muscarinic M\(_3\) and bradykinin B\(_2\) receptor-mediated signaling [Ins(1,4,5)P\(_3\) accumulation]. Compared with pretreatment with buffer alone (□), pretreatment of cells with either bradykinin (10 µM, 5 min; ○) or Ro-31-8220 (10 µM, 10 min; □) or the 2 in combination (●) had no significant effect (P = 0.27, two-way ANOVA) on the potency or magnitude of peak (10 s) methacholine-mediated Ins(1,4,5)P\(_3\) responses. Data are means ± SE, n = 4.
This demonstrates that the unidirectional nature of the heterologous desensitization was not due to different levels of receptor-effector coupling.

Our previous study in SH-SY5Y cells showed that the ability of muscarinic receptors to inhibit bradykinin receptor-mediated Ca\(^{2+}\) signaling is dependent on their persistent activation (34). Furthermore, recovery of the bradykinin response following removal of muscarinic receptor stimulation required extracellular Ca\(^{2+}\) and was complete at a time (2–3 min) consistent with the depletion of a shared intracellular Ca\(^{2+}\) store (34). These data support a model in which the terns of homologous desensitization is that, in the dent manner. The consequence of these different pat-

zation (8, 29), both receptor types were subject to agonist-dependent phosphorylation in a PKC-indepen-

dence manner. The consequence of these different patterns of homologous desensitization is that, in the continued presence of methacholine, intracellular Ca\(^{2+}\) stores will remain depleted and be unavailable for release on the addition of bradykinin. In contrast, the full desensitization of bradykinin B\(_2\) receptors will result in refilling of the Ca\(^{2+}\) stores, thereby allowing responses via the coexpressed muscarinic M\(_3\) receptors. The ability of bradykinin to slightly reduce the [Ca\(^{2+}\)]~i~ response to a maximal but not submaximal concentration of methacholine is most likely due to the incomplete refilling of the Ca\(^{2+}\) stores as the response to bradykinin declined. In addition to the depletion of a shared intracellular Ca\(^{2+}\) store, we have previously demonstrated that activation of muscarinic receptors expressed in other SH-SY5Y cells or CHO cells also results in rapid, marked, and sustained depletion of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P\(_2\)], which recovers over several minutes following termination of receptor activation (15, 37). Depletion of a shared substrate pool has the potential, therefore, to contribute to heterologous desensitization of bradykinin receptor-mediated responses shown here and in SH-SY5Y cells (34). However, recovery of bradykinin-mediated Ins(1,4,5)P\(_3\) responses following termination of muscarinic receptor activation is dependent on extracellular Ca\(^{2+}\) (34), whereas recovery of PtdIns(4,5)P\(_2\) is not (36). This provides evidence that depletion of a shared intracellu-
lar Ca\(^{2+}\) store by methacholine underlies inhibition of bradykinin-mediated responses and supports the idea that heterologous desensitization of Ins(1,4,5)P\(_3\) responses may occur due to lack of Ca\(^{2+}\) feed-forward activation or facilitation of PLC (34, 38). It is also possible that the muscarinic M\(_3\) receptor is able to couple to intracellular effector molecules that the brady-
kinin B2 receptor is unable to influence, thereby providing
the basis for such unidirectional interaction. We are
at present unable to address this issue.

The current data do not discount a role for phosphor-
ylation in heterologous desensitization of phosphoinosi-
tide signaling in other circumstances. Indeed this has
been suggested elsewhere (3, 16, 19, 31), although a
recent study also failed to demonstrate a functional
consequence of bradykinin on signaling by the PLC-
coupled α1B-adrenoceptor, despite heterologous recep-
tor phosphorylation (21). It is unclear whether differ-
ences are receptor or cell specific. Even in instances
where receptors are substrates for PKC, a functional
effect may depend on the presence of appropriate PKC
isoforms and on their activation being of sufficient
magnitude and duration. These latter features may in
themselves be dependent on the temporal profile of
homologous desensitization and the efficacy of coupling
of the activated GPCR responsible for the heterologous
phosphorylation.

It is possible that heterologous receptor phosphoryla-
tion regulates the nature of signaling in ways not explored
here, thereby shaping cellular responses. For example,
phosphorylation of β2-adrenoceptors by PKA switches
their coupling from Gs to Gi (12), whereas PKC-
dependent phosphorylation of 5-hydroxytryptamine2A
receptors results in uncoupling from Ca2+ mobilization
but not from inhibition of cAMP (18). The current study
does, however, show that heterologous receptor phos-
phorylation is not obligatory for heterologous desensiti-
ization of phosphoinositide and Ca2+ signaling. Cer-
tainly in some circumstances postreceptor mechanisms,
such as Ca2+ store depletion, will represent the major
factor underlying such cross talk. Indeed, this type of
desensitization is an inevitable consequence of recep-
tors sharing a common component of the signaling
pathway, which has the potential to be rate limiting,
and the duration of desensitization will be determined
by the resupply of this component. Receptor phos-
phorylation may, in some circumstances, provide an addi-
tional mechanism for heterologous interaction and
allow for a functional desensitization that outlives the
depletion of signaling components such as the intracel-
lar Ca2+ store.

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Address for reprint requests and other correspondence: G. B.
Willars, Dept. of Cell Physiology and Pharmacology, Univ. of Leices-
ter, PO Box 138, Medical Sciences Bldg., Univ. Rd., Leicester LE1
9HN, UK (E-mail: gbw2@leicester.ac.uk).

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REFERENCES

agonist-mediated phosphorylation of the metabotropic gluta-
mate receptor 1a by protein kinase C in permanently transfected

C-terminal truncation of the neurokinin-2 receptor causes en-
hanced and sustained agonist-induced signaling. J. Biol. Chem.

3. Ali, H., R. M. Richardson, E. D. Tomhave, J. R. Didsbury,
and R. Snyderman. Differences in phosphorylation of formylpep-
tide and C5a chemotractant receptors correlate with differ-

4. Ali, H., R. M. Richardson, E. D. Tomhave, R. A. Dubose,
B. Haribaru, and R. Snyderman. Regulation of stably trans-
fected platelet activating factor receptor in RBL-2H3 cells. Role
of multiple G protein and receptor phosphorylation. J. Biol.

5. Alla, S. A., J. Buschko, U. Quittner, A. Mairhof, M. Haase-
mann, G. Breipohl, J. Knolle, and W. Muller-Esterl. Struc-
tural features of the human bradykinin B2 receptor probed by
agonists, antagonists and anti-idiotypic antibodies. J. Biol.

Ball. Evidence of an important and direct role for protein kinase
C in agonist-induced phosphorylation leading to desensitization

delayed inhibitory feedback regulation of human neurokinin type
1 receptor activation of phospholipase C in UCL1 astrocy-

Ligand-induced phosphorylation/phosphorylation of the endog-
ogenous bradykinin B2 receptor from human fibroblasts. J. Biol.

coupling of m1, m2 and m3 muscarinic receptor subtypes to
inositol 1,4,5-trisphosphate and adenosine 3′,5′-cyclic monophos-
phate accumulation in Chinese hamster ovary cells. J. Pharma-

Nahorski. Heterogeneity of [3H]inositol 1,4,5-trisphosphate bind-
ing sites in adrenal-cortical membranes. Characterization and
validation of a radioreceptor assay. Biochim. J. 265: 421–427,
1990.

protein-coupled receptors: heterologous regulation of homolo-

12. Daaka, Y., L. M. Luttrell, and R. J. Lefkowitz. Switching of the
coupling of the β2-adrenergic receptor to different G proteins

attenuation of muscarinic agonist stimulated phosphoinositide
hydrolysis precedes receptor sequestration in human SH-SY-

off the signal: desensitization of β-adrenergic receptor function.

tion by lithium of phosphatidylinositol 4,5-bisphosphate supply
and inositol 1,4,5-trisphosphate generation in Chinese hamster
ovary cells expressing human recombinant m1 muscarinic recep-

16. Leeb-Lundberg, L. M. F., S. Cotecchia, A. De Blasi, M. G.
Caron, and R. J. Lefkowitz. Regulation of adrenergic receptor
function by phosphorylation. J. Biol. Chem. 262: 3098–3105,
1987.

17. Lefkowitz, R. J. G-protein coupled receptor kinases. Cell 74:

18. Lembo, P. M. C., and P. R. Albert. Multiple phosphorylation
sites are required for pathway-selective uncoupling of the
5-hydroxytryptamine2A receptor by protein kinase C. Mol.

19. Lian, M., M. G. Eason, E. A. J. Hewill-Motz, M. A. Williams,
C. T. Theiss, G. W. Dorn, and S. B. Liggett. Phosphorylation and
functional desensitization of the m3 muscarinic receptor by

20. Loehse, M. J. Molecular mechanisms of membrane receptor


