Differential G-protein-coupled Receptor Phosphorylation Provides Evidence for a Signaling Bar Code*

Received for publication, June 19, 2010, and in revised form, November 30, 2010 Published, JBC Papers in Press, December 21, 2010, DOI 10.1074/jbc.M110.154526

Adrian J. Butcher‡1, Rudi Prihandoko‡1, Kok Choi Kong‡, Phillip McWilliams‡, Jennifer M. Edwards‡, Andrew Bottrell‡, Sharad Mistry¶, and Andrew B. Tobin‡2

From the ‡Department of Cell Physiology and Pharmacology, ¶Protein and Nucleic Acid Chemistry Laboratory, and §MRC Toxicology Unit, University of Leicester, Hodgkin Building, Lancaster Road, Leicester LE1 9HN, United Kingdom

G-protein-coupled receptors are hyper-phosphorylated in a process that controls receptor coupling to downstream signaling pathways. The pattern of receptor phosphorylation has been proposed to generate a “bar code” that can be varied in a tissue-specific manner to direct physiologically relevant receptor signaling. If such a mechanism existed, receptors would be expected to be phosphorylated in a cell/tissue-specific manner. Using tryptic phosphopeptide maps, mass spectrometry, and phospho-specific antibodies, it was determined here that the prototypical Gq/11-coupled M3-muscarinic receptor was indeed differentially phosphorylated in various cell and tissue types supporting a role for differential receptor phosphorylation in directing tissue-specific signaling. Furthermore, the phosphorylation profile of the M3-muscarinic receptor was also dependent on the stimulus. Full and partial agonists to the M3-muscarinic receptor were observed to direct phosphorylation preferentially to specific sites. This hitherto unappreciated property of ligands raises the possibility that one mechanism underlying ligand bias/functional selectivity, a process where ligands direct receptors to preferred signaling pathways, may be centered on the capacity of ligands to promote receptor phosphorylation at specific sites.

The complexity of GPCR phosphorylation allows for the possibility that receptors might be phosphorylated differentially in different cell types (5) and thereby provide a mechanism by which the same receptor subtype would have different signaling properties depending on the cell type in which the receptor is expressed (3). This notion has been supported by recent studies on the β2-adrenergic receptor and CXCR4 receptor where receptor phosphorylation by different members of the GRK family can mediate different signaling outcomes (6, 7). Furthermore, our own studies and the studies of others on the M3-muscarinic receptor have demonstrated that phosphorylation of this receptor subtype by CK1α regulates receptor signaling to the phospholipase C and ERK pathways (8, 9), whereas phosphorylation mediated by protein kinase CK2 has no impact on ERK/phospholipase C but rather regulates the coupling of the receptor to the JNK pathway (5).

These studies suggest that one mechanism by which receptors might direct signaling to one pathway (i.e. ERK) in preference to another (i.e. ERK) might be via specific phosphorylation profiles of the receptor. By adopting a specific phosphorylation profile or “phosphorylation signature,” a receptor could favor coupling to a particular pathway. In this way, the phosphorylation profile of a receptor could act as a “bar code” that encodes a particular signaling outcome (3, 10–12). Hence, in each tissue type a GPCR might adopt a different phosphorylation profile, or bar code, and this would contribute to tissue-specific signaling related to the physiological function of the receptor.

If such a regulatory mechanism existed, then it would be expected that receptors would be differentially phosphorylated in different cell types. We test this possibility in this study and present evidence that the M3-muscarinic receptor is indeed differently phosphorylated in different cell and tissue types. Furthermore, we show that ligands can favor specific phosphorylation events that raise the possibility of ligand-specific phosphorylation and thereby a mechanism by which biased ligands could direct the preferential coupling of receptors to downstream signaling networks.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise stated, all biochemicals and reagents were from Sigma or from previously identified sources (5). Radioisotope [32P]orthophosphate (specific activity 8500–9120 Ci/mmol), myo-[3H]inositol (specific activity 25 Ci/mmol), and N-[3H]methylscopolamine (specific activity 370 Ci/mmol) were from Applied Research Products. 3-[3H]methyladenosine was a gift from Dr. S. S. Bhat (State University of New York). 11506 "Author’s Choice"—Final version full access.

‡ Author’s Choice

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed. Tel.: 116-2522935; Fax: 116-2231405; E-mail: tba@le.ac.uk.

The abbreviations used are: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; CG, cerebellar granule.
were then prepared and solubilized by addition of 5 ml of PBS containing 1% Nonidet P-40 plus a mixture of protease and phosphatase inhibitors. After centrifugation at 20,000 × g, the resulting supernatant was diluted 1:1 with PBS, and the receptor was then purified on anti-HA affinity resin (Roche Diagnostics). After extensive washing with solubilization buffer containing 0.5% Nonidet P-40, the resin was resuspended in 2 × SDS-PAGE sample buffer. The sample was resolved by SDS-PAGE on 8% gels and stained with colloidal Coomassie Blue. Purified M₃-muscarinic receptors were excised from the polyacrylamide and washed three times for 15 min with 100 mM ammonium bicarbonate. Reduction and alkylation of cysteines were performed by addition of 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate at 60 °C for 30 min followed by addition 100 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min in the dark. Gel slices were washed three times for 5 min with 50 mM ammonium bicarbonate containing 50% acetonitrile and incubated overnight at 37 °C in 50 mM ammonium bicarbonate containing 1 μg of sequencing grade trypsin (Promega).

LC-MS/MS was carried out on each sample using a 4000 Q-Trap mass spectrometer (Applied Biosystems, Warrington, UK). Peptides resulting from in-gel digestion were loaded at a high flow rate onto a reverse-phase trapping column (0.3 mm inner diameter × 1 mm), containing 5 μm of C18 300 Å Acclaim PepMap media (Dionex, UK) and eluted through a reverse-phase capillary column (75 μm inner diameter × 150 mm) containing Symmetry C18 100 Å media (Waters) that was self-packed using a high pressure packing device (Proxeon Biosystems, Odense, Denmark). The output from the column was sprayed directly into the nanospray ion source of the 4000 Q-Trap mass spectrometer. Additional mass spectrometry experiments were performed on an LTQ Orbitrap mass spectrometer by “FingerPrints” Proteomics Facility (College of Life Sciences, University of Dundee).

The resulting spectra were searched against the UniProtKB/SwissProt database using MASCOT software (Matrix Science Ltd.) with peptide tolerance set to 5 ppm for LTQ Orbitrap data or 0.1 Da for 4000 Q-Trap data, and the MS/MS tolerance was set to 0.6 Da. Fixed modifications were set as carbamidomethyl cysteine with variable modifications of phosphoserine, phosphothreonine, phosphotyrosine, and oxidized methionine. The enzyme was set to trypsin/P, and up to two missed cleavages were allowed. Peptides with a Mascot score greater than 20 and where the probability (p) that the observed match was a random event was <0.05 were included in the analysis. The spectra of peptides reported as being phosphorylated were interrogated manually to confirm the precise sites of phosphorylation.

[^{32P}]Orthophosphate Labeling and M₃-muscarinic Receptor Immunoprecipitation—In vivo[^{32P}]Orthophosphate labeling, receptor solubilization, and immunoprecipitation were conducted as described previously (5). In brief, CHO cells stably expressing the human M₃-muscarinic receptor were grown in 6-well plates, washed, and incubated for 1 h in KH₂PO₄-free Krebs buffer containing 100 μCi/ml[^{32P}]Orthophosphate (PerkinElmer Life Sciences). Cells were then stimulated with 0.1 mM methacholine for 5 min and lysed in RIPA buffer (2

83 Ci/mmol) were from PerkinElmer Life Sciences. The human muscarinic receptor antibodies used in this study were generated in-house against a glutathione S-transferase (GST) bacterial fusion protein containing the full third intracellular loop of the mouse M₃-muscarinic receptor (Arg²⁵²–Thr⁴⁹¹), and where rabbit polyclonal antibodies were raised, the antibodies were first passed through a GST-affinity column to remove antibodies to GST and then purified by affinity chromatography on a column containing the receptor-fusion protein. In the case of mouse monoclonal antibodies, affinity purification against the antigen was carried out.

Phosphorylation-specific antibodies, anti-phosphoserine 384, 412, and 577, were raised against peptide sequences NSTKLpPSSDNLQ, HKLQAQKpSMDDR, and YQQRQpS-VIFHK, corresponding to amino acid residues 378–389, 405–418, and 572–583 of the mouse M₃-muscarinic receptor in which serines 384, 412, and 577 were phosphorylated. The 87-day programs, which included four immunizations, were performed by Eurogentec. The resultant antisera were purified against the immunizing peptides.

**Generation of GST Fusion Constructs**—Mouse M₃-muscarinic receptor third intracellular loop Arg²⁵²–Thr⁴⁹¹ and the C-terminal Lys⁵⁴⁸ to Leu⁶⁸⁹ were inserted into pGEX-2t to produce N-terminal GST fusions. Escherichia coli BL21 (DE3) IRL transformed with the fusion constructs or pGEX-2t alone was grown in LB medium containing 50 μg/ml ampicillin, 50 μg/ml chloramphenicol, and 1% w/v glucose; protein expression was then induced by addition of isopropyl-β-D-galactopyranoside to a final concentration of 100 μM.

**Culture of CHO-M₃ Wild-type Stable Cell Lines**—CHO cells stably expressing the wild-type M₃-muscarinic receptor were maintained in Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), penicillin (50 units/ml), streptomycin (50 μg/ml), and geneticin G418 (500 μg/ml). Experiments were performed in Krebs/HEPES buffer (118 mM NaCl, 1.3 mM CaCl₂, 4.3 mM KCl, 1.17 mM MgSO₄, 4.17 mM NaHCO₃, 1.18 mM KH₂PO₄, 11.7 mM glucose, 10 mM HEPES (pH 7.4)) or in a modified Krebs/HEPES buffer as indicated.

**Preparation and Primary Culture of Mouse Cerebellar Granule Neurons**—Mouse CG neurons were prepared and cultured as described previously (5), trypsin- dissociated, and plated on poly-d-lysine-coated 6-well plates at a density of 2 × 10⁶ cells well. The neurons were maintained in Eagle’s basal medium (Invitrogen) supplemented with 20 mM KCl, penicillin/streptomycin, and 10% FCS. After 48 h, cytosine arabinoside (10 μM) was added to prevent glial cell proliferation, and the culture was continued for 7–8 days. Experiments were then performed on cells that were washed and then maintained in CSS-25 buffer (120 mM NaCl, 1.8 mM CaCl₂, 25 mM KCl, 15 mM glucose, 25 mM HEPES (pH 7.4)).

**M₃-muscarinic Receptor Purification and Mass Spectrometry**—For the mass spectrometry experiments, a stably transfected CHO cell line was generated that expressed a mouse M₃-muscarinic receptor HA-tagged at the C terminus. For receptor purification, 20 confluent T175 flasks were harvested and resuspended in 40 ml of Krebs/HEPES buffer and stimulated with methacholine (100 μM, 5 min). Membranes
**Tissue-specific GPCR Phosphorylation**

mm EDTA, 20 mm β-glycerophosphate, 160 mm NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 10 mm Tris (pH 7.4)). M3-muscarinic receptors were immunoprecipitated using an in-house anti-M3-muscarinic receptor polyclonal antibody (5). Immunoprecipitated proteins were resolved by SDS-PAGE on 8% gels, transferred to nitrocellulose membrane, and visualized by autoradiography. The membrane was subsequently blocked and immunoblotted with another in-house anti-mouse M3-muscarinic receptor monoclonal antibody for the detection of total receptors. To dephosphorylate the immunoprecipitated receptor, the immune complexes were washed three times with 10 mm Tris (pH 7.4) containing 0.25% n-octyl glucoside before treatment with calf intestinal phosphatase (20 units per reaction) for 2 h at 37 °C. Receptors were then resolved by SDS-PAGE on 8% gels and immunoblotted with phosphorylation-specific antibodies.

For phosphopeptide mapping, CHO-M3 cells were labeled with 100 µCi/ml of [33P]orthophosphate. Stimulation and immunoprecipitation were carried out as described above. The immunoprecipitated samples of one entire 6-well plate of CHO cells were pooled and resolved by SDS-PAGE on 8% gels. Tryptic phosphopeptide maps were prepared as described previously (5). The resolved phosphopeptides were then visualized using a STORM 820 phosphorimager (GE Healthcare) and quantified using AlphaEaseFC software (Alpha Innotech).

 substantiated by rapid aspiration of the buffer and addition of 1 M ice-cold trichloracetic acid (500 µl). Cell lysates were transferred to centrifuge tubes and incubated with 10 mm EDTA (50 µl) and 1:1,2-trichlorofluoroethane/tri-n-octylamine (1:1, v/v, 500 µl) for 15 min at room temperature. Samples were centrifuged at 20,000 × g for 4 min. An ~400-µl aliquot from the top layer was recovered and transferred to fresh tubes containing 60 mm NaHCO3. [3H]Inositol mono-, bis-, and trisphosphate ([3H]InsPn) fraction was recovered by anion exchange chromatography as follows: Dowex-1 (formate form) columns were regenerated with 10 ml of ammonium formate (2 m), formic acid (0.1 m) and washed thoroughly with distilled water. Samples were applied to the columns, and the columns washed with 10 ml of distilled water. Columns were then washed with ammonium formate (60 mM), sodium tetraborate (10 mM) solution. Total [3H]InsPn was eluted in 10 ml of ammonium formate (0.75 m), formic acid (0.1 m) and collected in large scintillation vials. A 5-ml aliquot from the eluate was mixed with 10 ml of SafeFluor scintillation mixture, and radioactivity was detected by liquid scintillation counting.

Muscarinic Receptor Expression Levels—The expression levels of the M3-muscarinic receptor were determined in membranes isolated from the hippocampus, cortex, and submandibular salivary gland by subtracting the specific binding of the muscarinic antagonist N-[3H]methylscopolamine obtained from wild-type mice from that obtained from M3-muscarinic receptor knock-out mice. N-[3H]Methylscopolamine binding was conducted as described previously (13). The M3-muscarinic receptor expression levels in hippocampus, cortex, and submandibular gland were 258, 351, and 335 fmol/mg protein, respectively.

**Immunocytochemistry of Salivary Submandibular Glands**—The submandibular glands from wild-type mice or M3-muscarinic receptor gene knock-out mice were dissected and fixed in 10% neutral buffered formalin. The fixed tissue were embedded in paraffin and processed on a Shandon Citadel 2000 where serial sections (5 µm) were cut. Sections where dewaxed in xylene, dehydrated through alcohol, and then washed with distilled water. The sections were then treated for microwave antigen retrieval in a Sanyo Shower Wave 1000-watt microwave, at 700 watts for 20 min in a 0.01 M citrate buffer (pH 6.0). Sections were then permeabilized with Triton X-100, and the M3-muscarinic receptor was stained with either anti-M3 muscarinic receptor antibodies or receptor phospho-specific antibodies used at a dilution of 1:200 using methods described previously (5, 14).

**RESULTS**

**Phosphorylation Profile of the M3-muscarinic Receptor in Three Cell Types**—The phosphorylation profile of the mouse M3-muscarinic receptor expressed in Chinese hamster ovary (CHO) cells as a recombinant protein or as an endogenously expressed protein in the mouse insulinoma...
cell line, MIN6, and mouse primary CG neurons was investigated. The receptor was immunoprecipitated from $[^{32}\text{P}]$orthophosphate-labeled cells and digested with trypsin. The tryptic phosphopeptides were then resolved by two-dimensional chromatography, and an autoradiograph was obtained to provide a phosphopeptide map (Fig. 1A).

The phosphopeptide map from the three cell types revealed a quartet of phosphopeptides labeled 1–4 that served to orient the map (Fig. 1B). These peptides were seen in all three cell types as were phosphopeptides labeled A and X (Fig. 1B). In contrast, there were a number of phosphopeptides that were observed in only one of the three cell types. Thus, phosphopeptides labeled with three asterisks were only observed in receptors derived from CHO cells (Fig. 1B). Four phosphopeptides labeled with two asterisks were only observed in CG neurons, and the phosphopeptide labeled with two plus signs was only observed in insulinoma cells. There were some phosphopeptides that were shared just between two cell types as illustrated by phosphopeptides Y and B that are shared between CHO cells and CG neurons.

These studies demonstrated that although there were common features in the phosphorylation profiles of the M3-muscarinic receptor in the three cell types investigated, there were also specific differences that make the overall phosphorylation profile of the M3-muscarinic receptor expressed in each of the cell types unique.

**Mass Spectrometry Identification of Phospho-acceptor Sites and Generation of Phospho-specific Antibodies**—Although the phosphopeptide maps described above illustrate differential receptor phosphorylation, these studies do not reveal the precise sites of phosphorylation. To do this, we conducted a mass spectrometric analysis of the phospho-acceptor sites on the M3-muscarinic receptor expressed in CHO cells. These studies revealed 13 serine and 2 threonine phospho-acceptor sites in the third intracellular loop and one serine (Ser$^{577}$) in the C-terminal tail (Fig. 2).
Tissue-specific GPCR Phosphorylation

A

Ms-Muscarinic Receptor

3rd Intracellular Loop

C-Tail

pSerine 577

QSKVFKK

b-ion series

Fragmentation Table

m/z

Relative Absorbance

pSerine 384

pSSDLQVPPD

b-ion series

Fragmentation Table

m/z

Relative Absorbance

pSerine 412

QQQKSMDDR

b-ion series

Fragmentation Table

m/z

Relative Absorbance

B

Ms-muscarinic receptor phospho-peptides

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Mascot Ion score</th>
<th>Delta PPM</th>
<th>Residue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(RES)S(L)K</td>
<td>23.99</td>
<td>0.5373</td>
<td>479-484</td>
</tr>
<tr>
<td>K(RES)S(OK)K</td>
<td>36.25</td>
<td>-0.8052</td>
<td>470-476</td>
</tr>
<tr>
<td>K(RES)S(CK)K</td>
<td>20.89</td>
<td>-0.7974</td>
<td>576-582</td>
</tr>
<tr>
<td>K(RES)S(KE)K</td>
<td>27.96</td>
<td>-1.205</td>
<td>478-484</td>
</tr>
<tr>
<td>K(RES)S(LKE)K</td>
<td>34.39</td>
<td>1.252</td>
<td>362-369</td>
</tr>
<tr>
<td>K(RES)S(LK)K</td>
<td>60.95</td>
<td>1.022</td>
<td>449-458</td>
</tr>
<tr>
<td>K(RES)S(KEK)K</td>
<td>24.65</td>
<td>-0.7566</td>
<td>478-486</td>
</tr>
<tr>
<td>K(RES)S(LKEK)K</td>
<td>46.22</td>
<td>0.06423</td>
<td>407-416</td>
</tr>
<tr>
<td>K(RES)S(L)K</td>
<td>49.22</td>
<td>-0.6567</td>
<td>382-393</td>
</tr>
<tr>
<td>K(RES)S(LQ)K</td>
<td>78.62</td>
<td>0.9672</td>
<td>288-300</td>
</tr>
<tr>
<td>K(RES)S(LQQ)K</td>
<td>66.81</td>
<td>0.9747</td>
<td>288-300</td>
</tr>
<tr>
<td>K(RES)S(LQQQ)K</td>
<td>60.23</td>
<td>0.9759</td>
<td>288-300</td>
</tr>
<tr>
<td>K(RES)S(LQQQQ)K</td>
<td>48.1</td>
<td>1.768</td>
<td>380-393</td>
</tr>
<tr>
<td>K(RES)S(LQQQQ)K</td>
<td>57.32</td>
<td>1.768</td>
<td>380-393</td>
</tr>
<tr>
<td>K(RES)S(LQQQQQ)K</td>
<td>24.65</td>
<td>0.327</td>
<td>369-382</td>
</tr>
<tr>
<td>K(RES)S(LQQQQQQ)K</td>
<td>40.75</td>
<td>-0.3186</td>
<td>407-421</td>
</tr>
<tr>
<td>K(RES)S(LQQQQQQQ)K</td>
<td>78.51</td>
<td>-5.338</td>
<td>447-465</td>
</tr>
<tr>
<td>K(RES)S(LQQQQQQQ)K</td>
<td>42.1</td>
<td>1.089</td>
<td>439-458</td>
</tr>
<tr>
<td>K(RES)S(LQQQQQQQ)K</td>
<td>38.06</td>
<td>1.089</td>
<td>439-458</td>
</tr>
</tbody>
</table>

C

Summary of Ms-muscarinic receptor phosphorylation

1 MILHSNNTTS PLFMISISSMW YHSSEAGLQ LGTVQLDOSY NISQTSNQFS
51 SNQSSDSPLG GHTINOYFPI AFLTOFLAV TIGNILIVF AYPKVQKQT
101 VINYFLISLA CADLIGYIS MLFTYIIIN NARALQHIC DMIISLIDYVA
151 SNAVVSNNLV ISFDFRTFII RPLTRFARKT TKESLQVIMG ALWISYVIRM
201 FAILFWQFTY GKRKTPPCQ IFQFSTFTI PUGAIAKFY MVPTINTL
251 KRIKTFTERK TKDLAQLQAS GTEEAEENV HILESSSST STHYQQQQK
301 RRRCKYGC HHFWVTIKSN PRAQMDQOH SSGSINNNRDD AASAEINIAS
351 SEDRGGSET PAITSVLKL HFGKISKLST KIPSNKLQY PKOKLGTDN
401 ERRALKLQG YMKIERDOQQ KMRSSQFGQL ESAYVATKTS DTMYSHTVK
451 AAILPSFKEA TLKKEFALKT YSVTTVWKR SLIEKKAQAQ TLGAILLAF
501 ITWTPFNFNQ LUNFTGCOI FRYTAYNLGN LYINSTNYVP VCYALGRXTF
551 RTTFFHLICC QCDVRRRRKQ QVDORFSVIF HRVRFQAL
Phospho-specific antibodies were raised to phosphorylated Ser\(^{384}\) and Ser\(^{412}\) in the third intracellular loop and Ser\(^{577}\) in the C-terminal tail. These were chosen because they represented phosphorylated residues that were not within a cluster of phosphorylation sites (which would have made specific antibody generation difficult) and that were sites determined to have high antigenicity. The antiserum from each of the immunizations was subjected to affinity purification that separated nonphospho-specific antibodies (structural antibodies) and phospho-specific antibodies. To test the specificity of the purified antiserum, the nonphosphorylated epitopes present in bacterial glutathione S-transferase (GST) fusion proteins containing either the third intracellular loop of the receptor or the C-terminal tail were probed in Western blots with the structural antibody and the phospho-specific receptor antibodies (Fig. 3, A – C). In these experiments, the structural antibody and the phospho-specific receptor antibodies (Fig. 3, A–C) were used as a loading control. The data shown are representative of at least two experiments.

FIGURE 3. Characterization of phospho-specific antibodies raised against serines 384, 412, and 577. Crude bacterial lysates containing either GST or GST fused to the third intracellular loop of the mouse M\(_3\)-muscarinic receptor (3i-loop) or C-terminal tail (C-tail) were probed with the following antibodies. A, antibodies against GST. This served as a loading control as did the Coomassie stain of gel. B, nonphospho-specific antibodies (structural) generated from the immunization. C, purified phospho-specific antibodies. D, CHO cells expressing the recombinant mouse M\(_3\)-muscarinic receptor were stimulated with methacholine (100 \(\mu\)M) for 5 min. The receptor was then solubilized and immunoprecipitated with an M\(_3\)-muscarinic receptor specific monoclonal antibody. The nitrocellulose was then probed with the phospho-specific antibodies to serines 384, 412, and 577. Where indicated the immunoprecipitated receptor was treated with calf intestinal phosphatase (CIAP) to remove phosphates from the receptor. The sample was also probed with an antibody against the M\(_3\)-muscarinic receptor (anti-M\(_3\)-receptor) as a loading control. The data shown are representative of at least two experiments.

Phospho-specific antibodies were raised to phosphorylated Ser\(^{384}\) and Ser\(^{412}\) in the third intracellular loop and Ser\(^{577}\) in the C-terminal tail. These were chosen because they represented phosphorylated residues that were not within a cluster of phosphorylation sites (which would have made specific antibody generation difficult) and that were sites determined to have high antigenicity. The antiserum from each of the immunizations was subjected to affinity purification that separated nonphospho-specific antibodies (structural antibodies) and phospho-specific antibodies. To test the specificity of the purified antiserum, the nonphosphorylated epitopes present in bacterial glutathione S-transferase (GST) fusion proteins containing either the third intracellular loop of the receptor or the C-terminal tail were probed in Western blots with the structural antibody and the phospho-specific receptor antibodies (Fig. 3, A – C). In these experiments, the structural anti-
bodies reacted with the appropriate fusion protein. The phospho-specific antibody fraction did not recognize the GST fusion proteins (Fig. 3, B and C). The phospho-specific antibodies did, however, identify the intact M3-muscarinic receptor immunoprecipitated from transfected CHO cells (Fig. 3D). This immunoreactivity was lost following de-phosphorylation with calf intestinal phosphatase (Fig. 3D).

**Determination of Cell Type-specific M3-muscarinic Receptor Phosphorylation Using Receptor Phospho-specific Antibodies**—The phosphorylation status of the M3-muscarinic receptor at residues 384, 412, and 577 was investigated on receptors expressed in CHO cells, CG neurons, and MIN6 insulinoma cells (Fig. 4). Receptors derived from CHO cells showed high basal levels of phosphorylation on Ser384 that fell by 82% fol-
lowing receptor stimulation with the full agonist methacholine. In contrast, agonist stimulation increased phosphorylation on Ser\(^{384}\) by 28% in receptors expressed in CG neurons (Fig. 4A). In MIN6 cells, the receptor was basally phosphorylated on Ser\(^{384}\) and was not changed following agonist stimulation (Fig. 4B). These data demonstrate that phosphorylation at Ser\(^{384}\) was regulated differentially in the three cell types.

Analysis of the phosphorylation status of the receptor at Ser\(^{577}\) also showed the importance of cell background. In CHO cells and CG neurons, Ser\(^{577}\) phosphorylation was increased following agonist stimulation by 61 and 50%, respectively (Fig. 4, A and B). Interestingly, there was no evidence of basal or agonist-mediated phosphorylation at Ser\(^{577}\) from receptors expressed in MIN6 cells (Fig. 4B).

Similarly, there was clear agonist-mediated phosphorylation at Ser\(^{412}\) from receptors derived from CHO cells and CG neurons. However, there was no evidence for Ser\(^{412}\) phosphorylation on receptors expressed in MIN6 cells (Fig. 4, A and B).

**Analysis of in Vivo M\(_3\)-muscarinic Receptor Phosphorylation**—To evaluate differential phosphorylation of the M\(_3\)-muscarinic receptor in vivo, membranes were prepared from the hippocampus, cerebral cortex, and pancreas from either wild-type mice or from mice where the M\(_3\)-muscarinic receptor gene had been knocked out. The membranes were then solubilized; the M\(_3\)-muscarinic receptor was immunoprecipitated with a receptor-specific monoclonal antibody, and the immunoprecipitate was probed in Western blots with receptor phospho-specific antibodies. In these experiments, the M\(_3\)-muscarinic receptor expressed in the hippocampus was determined to be phosphorylated equally on Ser\(^{318}\) and Ser\(^{412}\) but was phosphorylated to a lesser extent on serine\(^{577}\) (Fig. 5A). The phosphorylation profile for the receptor appeared different in the cerebral cortex where the receptor was equally phosphorylated on Ser\(^{318}\) and Ser\(^{577}\) but less phosphorylated on Ser\(^{412}\) (Fig. 5A). The receptor showed a further unique phosphorylation profile in the pancreas where the receptor was generally poorly phosphorylated, showing a weak signal for phosphorylation on Ser\(^{318}\) and Ser\(^{412}\) but no detectable phosphorylation of Ser\(^{577}\) (Fig. 5A).

The molecular weight of the M\(_3\)-muscarinic receptor immunoprecipitated from the pancreas (Fig. 5A) and from MIN6 insulinoma cells (Fig. 4B) was observed to be greater than that in the brain and CHO cells. The reason for this is likely to be differential glycosylation because the receptor was poorly phosphorylated in pancreatic cell/tissue types; hence, phosphorylation was not likely to be a major contributory factor in the apparently high molecular weight of the receptor in these experiments.

We also investigated the phosphorylation status of the M\(_3\)-muscarinic receptor in salivary submandibular glands. However, we were not able to detect the receptor in Western blots from this tissue probably because the receptor was subjected to proteolysis. We were, however, able to detect the receptor by immunocytochemistry where the M\(_3\)-muscarinic receptor was determined to be expressed in the excretory cells as well as in the cells of the excretory ducts (Fig. 5B). In these experiments weak staining for the receptor phosphorylated on Ser\(^{384}\) and Ser\(^{412}\) was detected, although there was stronger staining for phosphorylation on Ser\(^{577}\) (Fig. 5B).

Hence, the phosphorylation profile of the M\(_3\)-muscarinic receptor was different in each of the four tissues. This difference is illustrated as a bar code in Fig. 5C.

**Differential Regulation of Phosphorylation by Different Muscarinic Receptor Agonists**—The studies above provide evidence that the phosphorylation status of the M\(_3\)-muscarinic receptor is dependent on the cell and tissue type in which the receptor is expressed. We extended this analysis by investigating if different agonists working at the receptor expressed in the same cell type could mediate different receptor phosphorylation profiles. To this end, the phosphorylation profile of the human M\(_3\)-muscarinic receptor expressed in CHO-m3 cells following stimulation by the agonists methacholine, pilocarpine, and arecoline was investigated. Previous studies had established that methacholine acts as a full agonist at muscarinic receptors and pilocarpine and arecoline as partial agonists (15, 16).

In this study, assays of inositol phosphate accumulation (InsP\(_x\)) established methacholine as a full agonist at the M\(_3\)-muscarinic receptor and arecoline and pilocarpine as partial agonists (Fig. 6A). Consistent with previous studies (15, 16), the rank order of potency and maximal response (efficacy) for the InsP\(_x\) response was methacholine > arecoline > pilocarpine (Fig. 6A). In phosphorylation assays, all three agonists stimulated an increase in total receptor phosphorylation with the same rank order of efficacy as that observed in the InsP\(_x\) assay (Fig. 6B).

To determine the rank order of efficacy of the agonists at individual phosphorylation sites, phosphopeptide maps were generated following stimulation with the three agonists (Fig. 7, A–E). In these experiments, the relative intensity of four spots (labeled 1–4, Fig. 7) on the phosphopeptide map were compared. Spots 1 and 3 were significantly increased over basal when cells were stimulated with methacholine (Fig. 7, B and D). No significant changes in the phosphorylation of spot 1 or 3 were observed following stimulation with either arecoline or pilocarpine (Fig. 7B). Interestingly, spot 2 was highly phosphorylated in the basal state but became de-phosphorylated following methacholine stimulation (Fig. 7C). De-phosphorylation of spot 2 was also observed, but to a lesser extent, following stimulation with arecoline (Fig. 7C). No significant de-phosphorylation was observed in spot 2 with pilocarpine (Fig. 7C). Hence, the changes in phosphorylation observed for spots 1–3 more or less followed the expected rank order of efficacy for the three agonists. This, however, was in contrast to the phosphorylation of spot 4. Here, all three agonists increased phosphorylation at spot 4 with equivalent efficacy (Fig. 7E).

To further investigate this phenomenon, phospho-specific antibodies to Ser\(^{384}\), Ser\(^{412}\), and Ser\(^{577}\) were used to probe the effects of the three agonists on specific phosphorylation events on the M\(_3\)-muscarinic receptor in CHO-m3 cells. The receptor showed high basal levels of Ser\(^{384}\) phosphorylation, which were significantly decreased by methacholine treatment (Fig. 8A). Here, arecoline and pilocarpine also decreased phosphorylation at Ser\(^{384}\) with the following rank order of
efficacy: methacholine = arecoline > pilocarpine (Fig. 8A). A similar decrease in the phosphorylation status of spot 2 in Fig. 7 was observed suggesting that spot 2 corresponds to the phosphorylation site Ser$^{384}$.

Ser$^{412}$ phosphorylation was relatively low in the basal state and was increased equivalently by all three agonists (Fig. 8B). This is the same as the effects of the three agonists on the phosphorylation status of spot 4 in Fig. 7, suggesting that spot 4 corresponds to Ser$^{412}$.
Finally, phosphorylation of Ser577 was increased by all three agonists. The rank order of efficacy was methacholine > arecoline > pilocarpine. Thus, analysis of the phosphorylation status of the M3-muscarinic receptor with phospho-specific antibodies indicated that of the phosphorylation sites investigated only agonist-mediated phosphorylation at Ser577 followed the expected rank order of efficacy (i.e. methacholine > arecoline > pilocarpine). The rank order of efficacy for the changes in phosphorylation at the other two sites (Ser384 and Ser412) did not conform to the expected order indicating that agonists were able to direct phosphorylation preferentially at these sites.

DISCUSSION

Studies from our laboratory and from others have raised the possibility that one mechanism by which GPCRs could mediate cell/tissue-specific signaling may be through tissue-specific receptor phosphorylation (4, 5, 12). To test this notion, we addressed here whether a GPCR was phosphorylated in a cell- and tissue-specific fashion. We show that the M3-muscarinic receptor was indeed differentially phosphorylated in CHO-m3 cells, CG neurons, and MIN6 insulinoma cells. The capacity of this receptor subtype to be differentially phosphorylated was also confirmed by in vivo studies where the M3-muscarinic receptor was found to have a unique phosphorylation profile in the hippocampus, cortex, pancreas, and salivary glands. Thus, this study demonstrates that a GPCR can be phosphorylated in a cell- and tissue-specific manner consistent with the hypothesis that differential phosphorylation could contribute to tissue-specific receptor signaling.

Our study also highlights the complexity of GPCR phosphorylation with the identification of 15 phospho-acceptor sites on the M3-muscarinic receptor. In addition, we demonstrate the hitherto unobserved phenomenon of agonist-regulated receptor de-phosphorylation. Whereas receptors are known to be de-phosphorylated on withdrawal of the agonist in a process that reactivates internalized receptors (2), the notion that receptors might be phosphorylated in the basal state and then dephosphorylated on agonist stimulation has up to now not been considered. We show here that phosphorylation on Ser384 in CHO-m3 cells was decreased following agonist addition. Similarly, we show that a spot identified as spot 2 in the phosphopeptide map in CHO-m3 cells was decreased following methacholine stimulation. We are currently testing if spot 2 in the phosphopeptide map represents the same phospho-acceptor site as Ser384. Importantly, this dephosphorylation event is cell type-specific being restricted to receptors expressed in CHO-m3 cells. In neurons, Ser384 phosphorylation was seen to increase on agonist stimulation, and in MIN6 cells basal phosphorylation of Ser384 remains unchanged following agonist stimulation. Hence, the example of Ser384 reveals the true complexity and flexibility of GPCR
phosphorylation where a site can remain unchanged or can increase or decrease in its phosphorylation status following agonist stimulation depending on the cell type in which the receptor is expressed.

Are the different patterns of receptor phosphorylation identified here likely to lead to different receptor signaling outcomes? Whereas this is difficult to test in vivo, this has been extensively examined in in vitro systems. Hence, studies on the β2-adrenergic receptor have determined that the kinetics of the recruitment of arrestin to the receptor was shown to be dependent on which of the GRKs was employed in receptor phosphorylation (7). That this may impact on signaling was indicated in studies on the CCR7 chemokine receptor where GRK6-mediated phosphorylation leads to ERK1/2 activation, but GRK3 and GRK6 had to be employed in concert to drive receptor internalization and desensitization (12). That members of the GRK family can actually phosphorylate different sites on the same receptor and that these regulate different signaling outcomes have recently been demonstrated for another chemokine receptor CXCR4 (6).

In terms of the M3-muscarinic receptor, we and others have determined that this receptor subtype is phosphorylated by multiple receptor kinases, including the GRKs, protein kinase CK2, and CK1α (5, 8, 17, 18). It appears that these distinct protein kinases are able to phosphorylate different sites on the receptor and that this results in different functional outcomes (5). Hence, phosphorylation mediated by protein kinase CK2 does not appear to regulate receptor internalization.

**FIGURE 7. Muscarinic ligands drive preferential receptor phosphorylation.** A, tryptic phosphopeptide maps of the M3-muscarinic receptor expressed in CHO-m3 cells stimulated with a saturating concentration (100 μM) of methacholine (Mch), arecoline (Are), or pilocarpine (Pilo). Labeled are four spots (1–4), which were compared in intensity with the reference spot marked with an asterisk. B–E, quantification of spots labeled 1–4 in relation to the reference spot. Shown are typical autoradiographs and the cumulative mean data ± S.E. (n = 3). *, p < 0.05; **, p < 0.01 (t test).

11516 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 286 • NUMBER 13 • APRIL 1, 2011
tion nor ERK1/2 activity but rather it regulates the coupling of the receptor to the JUN kinase pathway (5). In contrast, coupling to the ERK1/2 pathway and phosphoinositide signaling is regulated by CK1α-mediated receptor phosphorylation (8, 9), and receptor desensitization and internalization are regulated by the GRKs (17, 19). Thus, these findings on the M3-muscarinic receptor, coupled to those from other GPCRs, provide compelling evidence that the signaling outcome of GPCRs can be determined by the sites on the receptor that are phosphorylated.

This realization has led to the notion that the pattern of phosphorylation of a receptor can be likened to a bar code that encodes a particular signaling outcome that could be employed in a tissue-specific manner to fine-tune the signaling outcome of a receptor to match a particular physiological role (3, 10–12). Such a concept was first proposed to account for the different signaling outcomes mediated by various members of the GRK family on the angiotensin II receptor and V2 vasopressin receptor (10, 11). If this notion were correct, then GPCRs would be expected to show cell/tissue-specific phosphorylation. Hence, the data presented here describing different phosphorylation profiles of a GPCR in different cell/tissue types support the notion of a phosphorylation bar code (Fig. 5C) being employed to drive tissue-specific receptor signaling.

It is therefore likely that at any given point a single receptor molecule will display a specific pattern of phosphorylation that is binary in a manner analogous to a bar code. It should be noted, however, that not all of the receptors in a tissue will necessarily have the same phosphorylation bar code. Thus, a population of receptors will reflect the average phosphorylation profile and as such will show different intensities at specific sites.

The phosphorylation bar code adopted by a receptor will be a reflection of the receptor conformation following agonist occupation (see below) but also the complement of protein kinases and phosphatases employed. We know that the M3-muscarinic receptor can be phosphorylated by a range of protein kinases, including GRK2, GRK6, CK1α, and protein kinase CK2 (5, 8, 17, 18). It is possible that these kinases are employed differentially to mediate tissue-specific phosphorylation (3).

We extended our studies further by asking if different ligands could mediate different patterns of phosphorylation. This study was conducted in the knowledge that there is a growing list of receptor ligands that are able to direct signaling of receptors preferentially to one signaling pathway over another (20). This phenomenon has been variously described as ligand-directed trafficking, ligand bias, and functional selectivity (21–23). The importance of this for the physiological

![Figure 8](http://www.jbc.org/)

**Figure 8.** Phospho-specific antibodies reveal the capacity of ligands to drive preferential M3-muscarinic receptor phosphorylation. CHO-m3 cells were stimulated with a saturating concentration (100 μM) of methacholine (Mch), arecoline (Are), or pilocarpine (Pilo). The cells were then lysed, and the receptor was immunoprecipitated with a receptor monoclonal antibody. The immunoprecipitate was then probed with receptor phospho-specific antibodies against phosphoserine 384 (A), phosphoserine 412 (B), and phosphoserine 577 (C). Shown are typical autoradiographs and the cumulative mean data ± S.E. (n = 3). *, p < 0.05; ***, p < 0.001 (t test).
Tissue-specific GPCR Phosphorylation

role of the M₃-muscarinic receptor has recently been highlighted in studies from our laboratory that have determined that the mechanism by which this receptor subtype regulates hippocampus-based memory and learning is via signaling through a receptor phosphorylation/arrestin-dependent pathway in preference to signaling via heterotrimeric G-proteins (13).

We show here that the efficacy of three muscarinic ligands, methacholine (full agonist), arecoline (partial agonist), and pilocarpine (partial agonist), in mediating overall receptor phosphorylation correlated with the efficacy of these ligands in the activation of inositol phosphate signaling. Similar correlations have been made for agonists at the β₂-adrenergic receptor where phosphorylation was measured in relation to the maximal response of a series of full and partial agonists (24). In this study, this correlation was not, however, maintained if individual sites of phosphorylation were measured (25). Hence, agonist-mediated phosphorylation of spot 4 in the phosphopeptide map in Fig. 7 and phosphorylation at Ser⁴¹² (Fig. 8) showed that all three agonists were full agonists in preference to signaling via heterotrimeric G-proteins (13).

In conclusion, our study supports the hypothesis that one possibility that ligands can preferentially direct receptor phosphorylation differently (6). Thus, the data from this study raise the possibility that ligands can preferentially direct receptor phosphorylation. This being the case, it would seem plausible that ligands that show a bias toward arrestin/phosphorylation signaling may do so because they have the quality of driving preferential phosphorylation of receptors in a manner that promotes signaling through this pathway in preference to signaling through other pathways.

In conclusion, our study supports the hypothesis that one mechanism that is employed by biased ligands to direct receptor signaling is the capacity of ligands to preferentially drive receptor phosphorylation. Furthermore, the data presented here also highlight the fact that GPCRs can be phosphorylated in a cell and tissue type-specific fashion in a manner that would provide a mechanism for cell/tissue-specific receptor signaling.

Acknowledgments—We thank Dr. Jurgen Wess (National Institutes of Health, Bethesda) for providing the M₃-muscarinic receptor knock-out mice.

REFERENCES
Differential G-protein-coupled Receptor Phosphorylation Provides Evidence for a Signaling Bar Code
Adrian J. Butcher, Rudi Prihandoko, Kok Choi Kong, Phillip McWilliams, Jennifer M. Edwards, Andrew Bottrill, Sharad Mistry and Andrew B. Tobin

doi: 10.1074/jbc.M110.154526 originally published online December 21, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.154526

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 17 of which can be accessed free at http://www.jbc.org/content/286/13/11506.full.html#ref-list-1