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

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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.

Agonist occupation of G-protein-coupled receptors (GPCRs) results in rapid receptor phosphorylation in a process that not only mediates the uncoupling of the receptor from its cognate G-protein but also drives G-protein-independent receptor signaling (1–3). It is now clear that GPCR phosphorylation is a complex regulatory mechanism involving an array of different protein kinases that include second messenger regulated kinases, members of the G-protein-coupled receptor kinase (GRK) family, as well as receptor tyrosine kinases and protein kinase CK2 and CK1α (2–4). The consequence of this kinase activity is the hyperphosphorylation of GPCRs at sites largely within the third intracellular loop and C-terminal tail, which in turn promotes the recruitment of arrestin proteins that mediate G-protein-independent signaling (1–4).

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 CXC4R 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. JNK) 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 differentially 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). Radiosotope [32P]orthophosphate (specific activity 8500–9120 Ci/mmol), myo-[3H]inositol (specific activity 25 Ci/mmol), and N-[3H]methylscopolamine (specific activity

* This work was supported by Wellcome Trust Grant 047600 and Heptares Inc.
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¶ The abbreviations used are: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; CG, cerebellar granule.
Tissue-specific GPCR Phosphorylation

83 Ci/mmol) were from PerkinElmer Life Sciences. The anti-
muscarinic receptor antibodies used in this study were gener-
ated in-house against a glutathione S-transferase (GST) bacte-
rial fusion protein containing the full third intracellular loop
of the mouse M3-muscarinic receptor (Arg252–Thr491).
Where rabbit polyclonal antibodies were raised, the antibo-
dies were first passed through a GST-affinity column to re-
move antibodies to GST and then purified by affinity chroma-
totography on a column containing the receptor-fusion protein.
In the case of mouse monoclonal antibodies, affinity purifica-
tion against the antigen was carried out.

Phosphorylation-specific antibodies, anti-phosphoserine
384, 412, and 577, were raised against peptide sequences
NSTKLpPSSDNLQ, HKLQAQKpsMDRDR, 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
78-day programs, which included four immunizations, were
performed by Eurogentec. The resulting antisera were puri-
fied against the immunizing peptides.

Generation of GST Fusion Constructs—Mouse M3-musca-
rinic receptor third intracellular loop Arg252 to Thr491 and the
C-terminal Lys348 to Leu589 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 expres-
sion was induced by addition of isopropyl 1-thio-β-D-galacto-
pyranoside to a final concentration of 200 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) supple-
mented with 10% fetal calf serum (FCS), penicillin (50 units/
ml), streptomycin (50 µg/ml), and genetin G418 (500 µg/ml).
Experiments were performed in Krebs/HEPES buffer
(118 mM NaCl, 1.3 mM CaCl2, 4.3 mM KCl, 1.17 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- disassociated, 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 prolifera-
tion, and the culture was continued for 7–8 days. Experi-
ments 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 sta-
bly 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 har-
vested and resuspended in 40 ml of Krebs/HEPES buffer and
stimulated with methacholine (100 µm, 5 min). Membranes
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 recep-
tor was then purified on anti-HA affinity resin (Roche Diag-
nostics). 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 cy-
teines 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 ammio-
nium bicarbonate for 30 min in the dark. Gel slices were
washed three times for 5 min with 50 mM ammonium bicar-
bonate 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 Ac-
claim PepMap media ( Dionex, UK) and eluted through a re-
verse-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 Uni-
ProtKB/SwissProt data base 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 con-
ducted 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

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M$_3$-muscarinic receptors were immunoprecipitated using an in-house anti-M$_3$-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 M$_3$-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 [$^{32}$P]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).

$M_3$-muscarinic Receptor Immunoprecipitation from Tissue Samples—To establish the phosphorylation status of $M_3$-muscarinic receptors in preparations from the hippocampus, cerebral cortex, and whole pancreas, tissues from adult wild-type or adult $M_3$-muscarinic receptor knock-out mice on a mixed genetic background (129/SvEv × CF1) (25) were extracted into ice-cold HIB buffer (25 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 9.1 mM glucose) containing protease inhibitors and phosphatase inhibitors (Complete EDTA-free, PhosStop, Roche Diagnostics). Membranes were prepared and solubilized in buffer composed of 20 mM Tris (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 1% Nonidet P-40. The receptor was immunoprecipitated with the anti-M$_3$-muscarinic receptor monoclonal antibody. Immune complexes were washed three times with 10 mM Tris (pH 7.4) containing 0.25% n-octyl glucoside and washed twice in 2000 where serial sections (5 μm) were cut. Sections where -$[^3H]$methylscopolamine bound or not were cut. Sections where -$[^3H]$methylscopolamine bound or not were cut. Sections where -$[^3H]$methylscopolamine bound or not were cut. Sections where -$[^3H]$methylscopolamine bound or not were cut.

Immunocytochemistry of Salivary Submandibular Glands—The submandibular glands from wild-type mice or $M_3$-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 $M_3$-muscarinic receptor was stained with either anti-$M_3$ muscarinic receptor antibodies or receptor phospho-specific antibodies used at a dilution of 1:200 using methods described previously (5, 14).

Data Analysis—Data presented are means ± S.E. of at least three determinations, and statistically significant differences were determined using analysis of variance followed by the Bonferroni post test. Significance was accepted at $p < 0.05$.

RESULTS

Phosphorylation Profile of the $M_3$-muscarinic Receptor in Three Cell Types—The phosphorylation profile of the mouse $M_3$-muscarinic receptor expressed in Chinese hamster ovary (CHO) cells as a recombinant protein or as an endogenously expressed protein in the mouse insulinoma...
The receptor was immunoprecipitated from $[\text{32P}]$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 (Ser⁵⁷⁷) in the C-terminal tail (Fig. 2).

FIGURE 1. Differential phosphorylation of the M₃-muscarinic receptor in CHO cells, MIN6 insulinoma cells, and CG neurons. A, schematic of the method employed in generating a tryptic phosphopeptide map. Step 1, cells are labeled with $[\text{32P}]$orthophosphate and stimulated with agonist for 5 min. The cells are then lysed and the receptors immunoprecipitated and resolved by SDS–PAGE. An autoradiograph of the gel determines the position of the radiolabeled receptor, which is excised from the gel. Step 2, excised gel band is tryptically digested. Step 3, tryptic digest is spotted onto a cellulose plate and subjected to electrophoresis and ascending chromatography to resolve the tryptic peptides. The position of the phosphopeptides is determined by exposing the gel in a phosphorimager. B, tryptic phosphopeptide maps of the mouse M₃-muscarinic receptor immunoprecipitated from CHO-m3 (CHO), CG neurons, and MIN6 insulinoma cells. For ease of viewing, the autoradiographs are divided into three sections denoted by the boxes. Phosphopeptide “spots” are labeled to designate spots that are unique to a cell type or shared between cell types (see key on the figure). Each autoradiograph is representative of at least three independent experiments.
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A

M₃-Muscarinic Receptor

3rd Intracellular Loop

C-Tail

pSerine 577

QSVWMK

Fragmentation Table

m/z

b-ion series

pSerine 384

pSSQLQVDP

b-ion series

pSerine 412

b-ion series

B

M₃-muscarinic receptor phospho-peptides

| Peptide Sequence | Mascot Ion score | Delta PPM | Residue number |
|------------------|------------------|-----------|----------------|
| KRMSHQKEET(K)    | 23.99            | 0.5373    | 479-484        |
| KITTSQGTKAR      | 36.25            | -0.8052   | 470-476        |
| RIKQSVIFHR       | 20.89            | -0.7994   | 576-582        |
| RIJKFMSUKE(K)     | 27.96            | -1.205    | 478-484        |
| RIJAVSVLKL       | 34.39            | 1.252     | 362-369        |
| KTATAALP5TKE      | 60.95            | -1.022    | 449-458        |
| RIJKRMSUKE(K)     | 24.65            | -0.7566   | 478-486        |
| KLQQQDSMQRK(D)    | 46.22            | 0.06423   | 407-416        |
| KFSQSDLQVDPK(D)   | 49.22            | 0.6567    | 382-393        |
| RIKSSTYELQQGQGT(K)    | 79.62            | 0.9672    | 288-300        |
| RIKSSTYELQQGQGK    | 66.81            | 0.9747    | 288-300        |
| RIKSSSTYELQQGQGK   | 60.23            | 0.9759    | 288-300        |
| RISSTYELQQGQGK    | 48.1             | 1.768     | 288-300        |
| RISSTYELQQGQK     | 57.32            | 1.768     | 288-300        |
| RIKQQQDSVIFHR      | 24.36            | 0.2792    | 569-582        |
| KLQQQDSMQRK(D)    | 40.75            | -0.3186   | 407-421        |
| VDIKTASLAPSDLKVLK(F)     | 78.51            | -5.338    | 447-465        |
| KTSTSNKSVKTATLPSFFK    | 42.5             | 1.089     | 439-458        |
| KSTSNNKSVKTATLPSFFK   | 38.06            | 1.089     | 439-458        |
| FINAHQKQDSMQRKQ(D)   | 22.53            | -0.0193   | 403-421        |
| KESQSDLQVDPK(D)GMORV(R) | 80.59            | -56.18    | 382-402        |
| KCIEQGQSGEAEANIFVMPSR55(S) | 49.18            | 393       | 263-287        |
| KIEQGQSGEAEANIFVMPSR555(S) | 41.89            | 1.326     | 263-287        |
| RIKELQGQSGEAEANIFVMPSR555(S) | 34.08            | -0.7901   | 261-287        |
| S3MDRRDNQENGKSR5 SLPEGLESAVDA(T) | 65.79            | -140.5    | 413-438        |

C

Summary of M₃-muscarinic receptor phosphorylation

1 MILHNSSTTS PLEFMISISSW VHSEPAGLPL GTVYQLODSY NISQTQGKFS
51 SNDFSDVPLG HTSTIOSQFI ILFOFIALVI TIGNILVIV AFNPQDSKRT
101 VNYFLLSA CADILGVIS MULFTYIIN NSKGQLHAC DIAWLSIDVA
151 SNAITYLVLV IFSDFRTFV RPLTPFTKXR KEQDQMVTLG AVISFGVLM
201 FAILFWQFVT GKRVTQPGC FQFSLKPIF TPUGAILAFY MPVTNINTLY
251 WRTKYEKR KXKELAQGAS TGQGQENY HZNSSSSTTS S3 ELYQOQQKTK
301 RRKRKYGGC HNWFYTITSKNR SRAQSQDOQN 5S5RGGNNSN RRASLSNAS
351 SGRGIDSET MAITSTVLPKLR FGORSTLST KLQKSNLQKI POKULGTMDV
401 ERMAKQGC QCQGQGQDOQ KIRSTLPIQLG ESNVQASKTS DTNLQVRKTV
451 ALPFTSYKET TLARKERALKT SRTTNTKRN SLLKXQAAQ TLLSAPLIAF
501 ITWFPMVIFM LYNVTFCQSI FYTPNGLN LGYISNTNSVNY VCYALNQTF
551 RRTIJKMLLC QCQKXRRQKQ YQDOQSVIF NKYQFQAL
Phospho-specific antibodies were raised to phosphorylated Ser\textsuperscript{384} and Ser\textsuperscript{412} in the third intracellular loop and Ser\textsuperscript{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 experiment, the structural anti-

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![FIGURE 2. Determination of the sites of phosphorylation on the M\textsubscript{3}-muscarinic receptor by mass spectrometry. A, recombinant mouse M\textsubscript{3}-muscarinic receptor expressed in CHO cells was stimulated with methacholine (100 \textmu M) for 5 min. Membranes were then prepared, and the receptor was purified. The receptor was subjected to tryptic digestion, and the peptides were analyzed by mass spectrometry. Shown is a schematic indicating the phospho-acceptor sites in the third intracellular loop and C-terminal tail. Also shown are typical LC-MS/MS traces that identify serines 384, 412, and 577 as phosphoserines. B, list of the phosphopeptides identified by LC-MS/MS. The phosphorylated residue is highlighted in red. Note that where residues are labeled with an asterisk, it was not possible to determine which of the residues were phosphorylated. All the peptides shown were observed in at least two independent experiments. C, schematic of the full amino acid sequence of the mouse M\textsubscript{3}-muscarinic receptor indicating the position of the phosphorylated residues in red and underlined.](image-url)
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).

**FIGURE 4.** Phospho-specific antibodies reveal differential phosphorylation of the M3-muscarinic receptor. A, either nontransfected CHO cells (NT) or CHO cells expressing the mouse M3-muscarinic receptor (CHO-m3) were stimulated with or without methacholine (Meth) for 5 min. The cells were then lysed, and the lysate was probed in a Western blot with receptor phospho-specific antibodies. Shown also is a loading control probed for tubulin. Graphs representing quantification of the blots are shown below the autoradiographs. B, MIN6 insulinoma cells and primary cultures of CG neurons derived from wild-type mice (WT) or mice where the M3-muscarinic receptor was knocked out (KO) were processed as described in A above. As a loading control, the lysate was probed for total M3-muscarinic receptor content (anti-M3-receptor). Shown are representative blots and below each blot graphical quantification. Graphs present the means ± S.E. of three independent experiments. *, p < 0.05 (t test).

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 Ser<sup>384</sup> 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 M3-muscarinic Receptor Phosphorylation**—To evaluate differential phosphorylation of the M3-muscarinic receptor in vivo, membranes were prepared from the hippocampus, cerebral cortex, and pancreas from either wild-type mice or from mice where the M3-muscarinic receptor gene had been knocked out. The membranes were then solubilized; the M3-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 M3-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 M3-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 M3-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 M3-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 M3-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 M3-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 M3-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 M3-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 when 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 M3-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<sup>384</sup>.

Ser<sup>412</sup> 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<sup>412</sup>. 

FIGURE 5. Differential phosphorylation of the M<sub>3</sub>-muscarinic receptor in the central nervous system, pancreas, and salivary glands. A, membranes from cerebral cortex, hippocampus, and pancreas were prepared from wild-type (WT) and M<sub>3</sub>-muscarinic receptor gene knocked out (KO) mice. Membranes were solubilized, and the M<sub>3</sub>-muscarinic receptor was immunoprecipitated using a receptor monoclonal antibody. The immunoprecipitate was probed in Western blots with M<sub>3</sub>-muscarinic receptor phospho-specific antibodies against phosphoserine 384, 412, and 577. Shown also is a loading control probed for total M<sub>3</sub>-muscarinic receptor (anti-M<sub>3</sub>-receptor). Data are representative of three independent experiments.

B, submandibular glands from wild-type and M<sub>3</sub>-muscarinic receptor knock-out mice were fixed in 10% formalin and processed for immunocytochemistry. Sections were stained with antibodies against the total M<sub>3</sub>-muscarinic receptor population (anti-M<sub>3</sub>-receptor) or receptors phosphorylated on serines 384, 412, and 577. Also shown are the DAPI-stained nuclei and the merge of the receptor staining and DAPI. C, results shown in A and B are represented as a bar code describing the relative intensities of receptor phosphorylation on serines 384, 412, and 577 in the different tissues.
Finally, phosphorylation of Ser\(^{577}\) was increased by all three agonists. The rank order of efficacy was methacholine > arecoline > pilocarpine.

Thus, analysis of the phosphorylation status of the M\(_3\)-muscarinic receptor with phospho-specific antibodies indicated that of the phosphorylation sites investigated only agonist-mediated phosphorylation at Ser\(^{577}\) 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 (Ser\(^{384}\) and Ser\(^{412}\)) 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 M\(_3\)-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 M\(_3\)-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 M\(_3\)-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 Ser\(^{384}\) 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 Ser\(^{384}\). Importantly, this dephosphorylation event is cell type-specific being restricted to receptors expressed in CHO-m3 cells. In neurons, Ser\(^{384}\) phosphorylation was seen to increase on agonist stimulation, and in MIN6 cells basal phosphorylation of Ser\(^{384}\) remains unchanged following agonist stimulation. Hence, the example of Ser\(^{384}\) 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.
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.** 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).
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 either in phosphopeptide maps or by use of phospho-specific antibodies. 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 stimulating an increase in phosphorylation at these sites.

Hence, the break from the expected rank order of efficacy observed for Ser⁴¹² and spot 4 indicates that the impact that the ligands have on phosphorylation is not equivalent for all sites. That this is the case is further supported by studies on the CXCR4 and somatostatin receptor where agonists have been identified that are able to regulate 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.

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