Phosphorylation and regulation of a G protein–coupled receptor by protein kinase CK2

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We demonstrate a role for protein kinase casein kinase 2 (CK2) in the phosphorylation and regulation of the M_3-muscarinic receptor in transfected cells and cerebellar granule neurons. On agonist occupation, specific subsets of receptor phospho-acceptor sites (which include the SASSDEED motif in the third intracellular loop) are phosphorylated by CK2. Receptor phosphorylation mediated by CK2 specifically regulates receptor coupling to the Jun-kinase pathway. Importantly, other phosphorylation-dependent receptor processes are regulated by kinases distinct from CK2.

We conclude that G protein–coupled receptors (GPCRs) can be phosphorylated in an agonist-dependent fashion by protein kinases from a diverse range of kinase families, not just the GPCR kinases, and that receptor phosphorylation by a defined kinase determines a specific signalling outcome. Furthermore, we demonstrate that the M_3-muscarinic receptor can be differentially phosphorylated in different cell types, indicating that phosphorylation is a flexible regulatory process where the sites that are phosphorylated, and hence the signalling outcome, are dependent on the cell type in which the receptor is expressed.

Introduction

Rapid receptor phosphorylation in response to agonist stimulation is a posttranslational modification adopted by nearly all G protein–coupled receptors (GPCRs; Pierce et al., 2002). This event is generally accepted to be mediated by the GPCR kinase (GRK) family in a process that results in the recruitment of arrestin adaptor proteins to the receptor and the concomitant uncoupling of the receptor from its cognate G protein (Pierce et al., 2002). In addition, GRK phosphorylation can promote receptor activation of G protein–independent pathways such as the MAPK cascade (Wei et al., 2003).

This universal adaptive paradigm belies the complex nature of GPCR phosphorylation and regulation. There are >340 nonolfactory GPCR subtypes in the mammalian genome (Vassilatis et al., 2003) showing widespread tissue distribution and influencing nearly every biological process from sensory perception to cell growth and differentiation (Wettschureck and Offermanns, 2005). Many of these receptors are phosphorylated at multiple serine, threonine (Blaukat et al., 2001; Pollok-Kopp et al., 2003; Trester-Zedlitz et al., 2005), and occasionally tyrosine residues (Fan et al., 2001). This multisite phosphorylation has been reported in some instances to be hierarchical and mediated by more than one protein kinase (Rao et al., 1997; Kouhen et al., 2000; Blaukat et al., 2001). Most enlightening have been studies on GRK knockout animals that have suggested that the same receptor subtype expressed in different tissues may be phosphorylated by a different complement of receptor kinases (Walker et al., 2004).

It is also the case that many receptor subtypes are found in more than one tissue type (Vassilatis et al., 2003) and mediate very specialized tissue-specific responses. For example, the M_3-muscarinic receptor regulates membrane excitability in neurons (Millar et al., 2000), contraction and cell growth in smooth muscle cells (Gautam et al., 2005), and secretary vesicle priming and fusion in salivary acinar cells (Yoshimura et al., 2002; Gautam et al., 2005). It would appear intuitive that receptors expressed in different cell types, controlling specific cellular responses, would be regulated in a manner specific to that cell type.

Hence, a tantalizing alternative model of GPCR regulation is that phosphorylation is a flexible process of receptor modification where tissue-specific differences in phosphorylation would
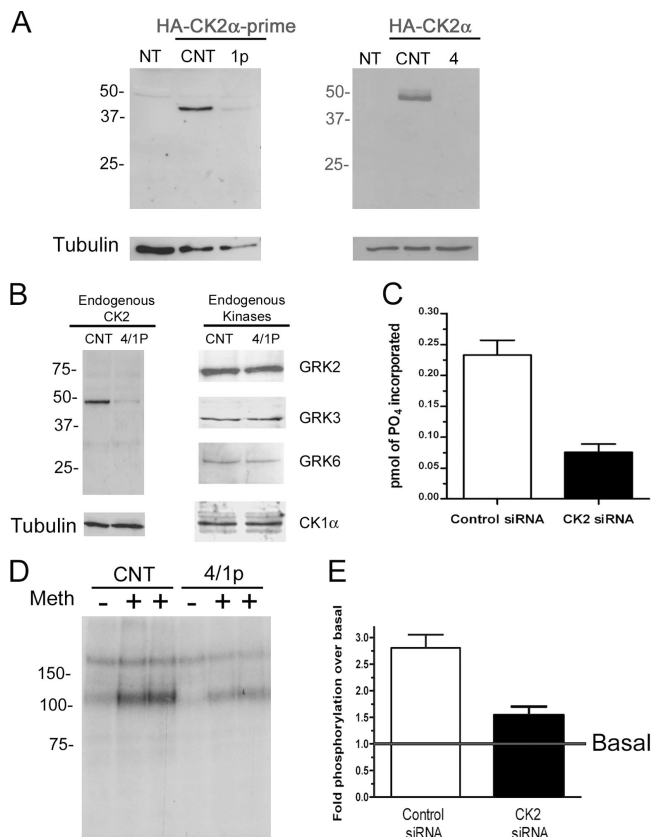
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Figure 1. **CK2 siRNAs decrease agonist-driven M₃-muscarinic receptor phosphorylation.** (A) Western blot analysis (using anti-HA antibody) of transfected HA-CK2α′ (left) and HA-CK2α (right) expression in CHO cells cotransfected with the indicated siRNA duplexes. α-Tubulin was used as a loading control. NT, nontransfected. (B) CHO cells were transfected with the indicated siRNAs and used to determine expression levels of endogenous CK2α subunit, GRK2, GRK3, GRK6, and CK1α by immunoblotting with the indicated antibodies. (C) CK2 kinase activity in lysates from the siRNA transfected CHO cells was determined by the phosphorylation of a substrate peptide. Data correspond to mean ± SD from three independent experiments. (D) Phosphorylation of the M₃ receptor in CHO-M3 cells transfected with siRNA duplexes CK2α-4 and CK2α′-1p or scrambled control siRNAs (CNT) and stimulated with 100 μM methacholine for 5 min. (E) Quantitative analysis of phosphorylation from D. Phosphate content of M₃ receptor was normalized to the basal level of phosphorylation. Data correspond to mean ± SD from three independent experiments.

underlie defined physiological functions. In this paradigm, differential deployment of receptor kinases in a tissue-selective manner would result in differential phosphorylation that would facilitate the specific physiological role of that receptor in a particular cell type.

Our work on the $G_{q/11}$-coupled M₃-muscarinic receptor has demonstrated that this receptor subtype can be phosphorylated in an agonist-dependent manner by casein kinase 1α (CK1α), and this process regulates the coupling of the receptor to the extracellular-regulated kinase (ERK) 1/2 pathway (Budd et al., 2000, 2001; Tobin, 2002). These studies established that agonist-dependent GPCR phosphorylation could be mediated by protein kinases other than the GRKs (Tobin, 2002). In the current study, we extend our investigation of the CKs in GPCR phosphorylation and provide evidence that protein kinase CK2 can also phosphorylate the M₃-muscarinic receptor. Furthermore, we

show that the M₃-muscarinic receptor is differentially phosphorylated in different cell types and that the action of specific receptor kinases can determine the signaling outcome of receptor phosphorylation.

Results

Inhibition of CK2 decreases M₃-muscarinic receptor phosphorylation

To investigate the role of the CK2 in M₃-muscarinic receptor phosphorylation, we raised siRNAs against the catalytic α and α′ subunits of CK2. The effectiveness of the siRNAs was established by cotransfection of the duplexes with plasmids expressing HA-tagged α or α′ subunits. In these experiments, we estimated the transfection efficiency of fluorescently labeled siRNAs to be ∼90% (unpublished data). The siRNAs designated CK2α-4 and CK2α′-1p effectively inhibited expression of the α and α′ subunits, respectively (Fig. 1 A). Furthermore, these siRNAs were active against the endogenously expressed kinase where the levels of the CK2α subunit fell by >85% with no subsequent change in the levels of CK1α, GRK2, GRK3, or GRK6 (Fig. 1 B). This corresponded to a fall in CK2 enzymatic activity of 68% compared with control (Fig. 1 C).

When used in phosphorylation experiments where the M₃-muscarinic receptor was immunoprecipitated from CHO-M3 cells labeled with [³²P]-orthophosphate, the CK2 siRNA duplexes reduced agonist-mediated M₃-muscarinic receptor phosphorylation by ∼72% compared with scrambled siRNA controls (Fig. 1, D and E). These results were confirmed by raising further siRNA duplexes to different regions of CK2α and -α′ (Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200610018/DC1).

The third intracellular loop of the M₃-muscarinic receptor is a serine-rich region containing several consensus sites for CK1α, CK2, and the GRKs, many of which are shared between these acidotropic kinases (Fig. 2 A). The ability of these kinases to phosphorylate the third intracellular loop is illustrated in Fig. 2 B, where GRK6 was found to phosphorylate the GST-fusion protein containing the third intracellular loop of the human M₃-muscarinic receptor (R^{253}-T^{492}, called here GST-H3iloop) with the highest efficiency followed by CK1α and then GRK2 and CK2 (Fig. 2 B). None of the kinases phosphorylated GST alone (unpublished data).

To confirm of a role for CK2 in the phosphorylation of the M₃-muscarinic receptor, the CK2-specific pharmacological inhibitors 4,5,6,7-tetrabromo-1H-benzotriazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT; Pagano et al., 2004; Sarno et al., 2005) were used. The selectively of these inhibitors was confirmed in assays using the fusion protein GST-H3iloop as a substrate for CK1, CK2, GRK2, and GRK6 (Fig. 2 C). TBB at a concentration of 1 μM was very potent against CK2, reducing the level of GST-H3iloop by ∼75% but had only a small effect on CK1, GRK2, and GRK6 (Fig. 2 C). DMAT also strongly inhibited CK2 with no significant effect on CK1 or GRK2 but was able to inhibit GRK6 by ∼45% (Fig. 2 C). These in vitro experiments were consistent with the reported selectivity of the CK2 inhibitors and established that they
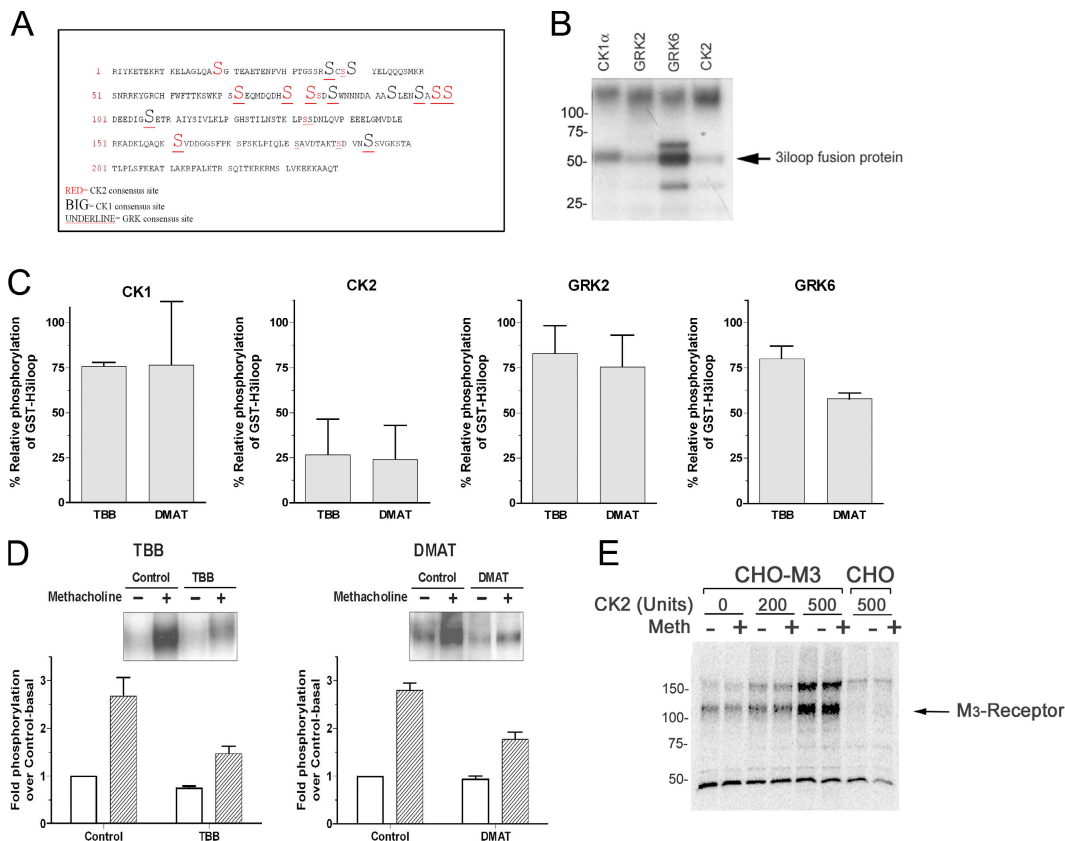
Figure 2. **Pharmacological inhibition of CK2 decreases agonist-mediated M₃-muscarinic receptor phosphorylation.** (A) The third intracellular loop of the M₃-muscarinic showing the consensus sites for CK1α, CK2, and the GRKs as predicted using GPS (http://973-proteinweb.ustc.edu.cn/gps/gps_web/; Xue et al., 2005). (B) In vitro phosphorylation of a GST-H3iloop fusion protein by CK1, CK2, GRK2, and GRK6. The position of the fusion protein as determined by Coomassie staining is shown. (C) In vitro phosphorylation of a GST-H3iloop fusion protein by CK1, CK2, GRK2, and GRK6 in the presence of 1 μM TBB and DMAT (CK2 inhibitors). Results are expressed as a percentage of controls (phosphorylation without the inhibitor). (D) M₃-muscarinic receptor phosphorylation in CHO-M3 cells in the absence (control) or presence of TBB and DMAT (20 μM, 1.5 h). Cells were stimulated with 100 μM methacholine for 5 min. The inset shows representative autoradiographs. Quantification of three independent experiments was normalized to the basal phosphorylation of control cells. Bars represent mean ± SD of at least three replicates. (E) Phosphorylation of the M₃-muscarinic receptor contained in membrane preparations from CHO-M3 cells (or as a control CHO cells) in the presence and absence of CK2 and 0.1 mM methacholine. After a 10-min phosphorylation reaction, membranes were solubilized and the M₃-muscarinic receptor was immunoprecipitated. The results shown are representative of three independent experiments.

can discriminate between the putative receptor kinases responsible for M₃-muscarinic receptor phosphorylation.

The CK2 inhibitors were then used on intact CHO-M3 cells to determine their effects on M₃-muscarinic receptor phosphorylation. Both TBB and DMAT substantially reduced agonist-mediated phosphorylation of the M₃-muscarinic receptor by 76 and 58%, respectively (Fig. 2 D). These data are consistent with the aforementioned siRNA experiments and demonstrate a role for CK2 in the phosphorylation of M₃-muscarinic receptors.

To further investigate the ability of CK2 to directly phosphorylate the M₃-muscarinic receptor, membranes prepared from CHO-M3 cells that express recombinant human receptor were reconstituted with purified CK2. We noted previously that these membranes maintain the ability to phosphorylate the M₃-muscarinic receptor (Tobin et al., 1993, 1996); hence, to reduce the endogenous kinase activity, the membranes were washed with 200 mM NaCl. Purified CK2 added to these membranes resulted in an increase in receptor phosphorylation, although this was not agonist regulated (Fig. 2 E).

M₃-muscarinic receptor phosphorylation in cerebellar granule (CG) neurons

To test whether CK2 had a role in the phosphorylation of the M₃-muscarinic receptor in a native cell type, we investigated mouse CG neuronal cultures where this receptor subtype is endogenously expressed (Fohrman et al., 1993). To facilitate these studies, we raised an antibody against the region S³⁴⁴-L⁴⁶² in the third intracellular loop of the mouse receptor that specifically immunoprecipitated the mouse M₃-muscarinic receptor when tested against all five muscarinic receptor subtypes (Fig. 3 A).

This antibody was subsequently used in phosphorylation experiments where 7-d-old mouse CG neurons were metabolically labeled with [³²P]-orthophosphate and stimulated with methacholine before being solubilized and the receptor immunoprecipitated. In CG neurons from wild-type mice, the M₃-muscarinic receptor appeared as a ∼95-kD phosphoprotein that increased in the level of phosphorylation after agonist stimulation in a manner that was inhibited by the muscarinic receptor antagonist atropine (Fig. 3 B). In CG neurons obtained from transgenic mice where the M₃-muscarinic receptor gene had
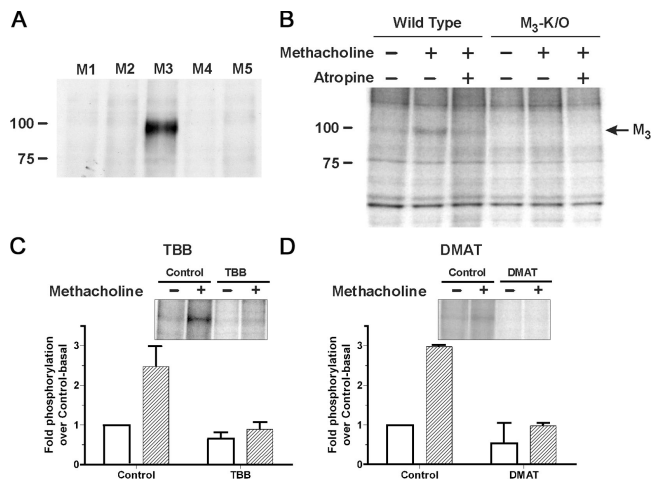
Figure 3. **Pharmacological inhibition of CK2 decreases phosphorylation of the M₃-muscarinic receptor in mouse CG neurons.** (A) Immunoprecipitation of biotinylated mouse M_1–M_5 muscarinic receptors using an in-house anti-mouse M_3 antibody. Equal amounts of receptors in each lane were ensured by parallel [³H]-NMS binding assays. (B) Phosphorylation of the M_3-muscarinic receptor in CG neurons from wild-type and M_3-knockout (K/O) mice. Neurons were metabolically labeled with [³²P]-orthophosphate and stimulated with 100 μM methacholine for 5 min in the presence or absence of 20 μM atropine as indicated. (C) Phosphorylation assays in CG neurons treated with 10 μM TBB for 30 min or 20 μM DMAT for 15 min and stimulated with 100 μM methacholine for 5 min. The inset shows representative autoradiographs. Phosphorylation of the M_3-receptor was quantified and normalized to the basal phosphorylation of the receptor in control cells without inhibitor. Data represent mean ± SD of at least three replicates.

been knocked out (Yamada et al., 2001), this ~95-kD receptor band was absent (Fig. 3 B).

To determine a role of CK2 in this phosphorylation event, the effect of the inhibitors TBB and DMAT on agonist-mediated receptor phosphorylation in CG neurons was investigated. Both TBB and DMAT reduced agonist-mediated phosphorylation from control levels of 2.47- ± 0.89-fold and 2.97- ± 0.06-fold over basal to 0.88- ± 0.37-fold and 0.98- ± 0.11-fold over basal, respectively (Fig. 3, C and D).

CK2 directs phosphorylation of a subset of sites in the intact M₃-muscarinic receptor

Chymotryptic phosphopeptide maps were prepared from the receptor immunoprecipitated from CHO-M3 cells under conditions where CK2 had been inhibited by siRNA knockdown (using CK2α-4/CK2α′-1p) or by pharmacological inhibition (with TBB). In these experiments (as before), quantification of receptor numbers by radioligand binding ensured that the same number of receptors had been immunoprecipitated from each sample. These maps revealed that CK2 siRNA and TBB inhibited the phosphorylation of the same subset of phosphopeptides (Fig. 4, arrows and asterisks) while minimally affecting the phosphorylation of other phosphopeptides (Fig. 4).

SASSDEED motif in the third intracellular loop is a CK2 phosphoacceptor site

The sites of CK2-mediated receptor phosphorylation were investigated by two-dimensional chymotryptic phosphopeptide mapping. We established previously that the M_3-muscarinic
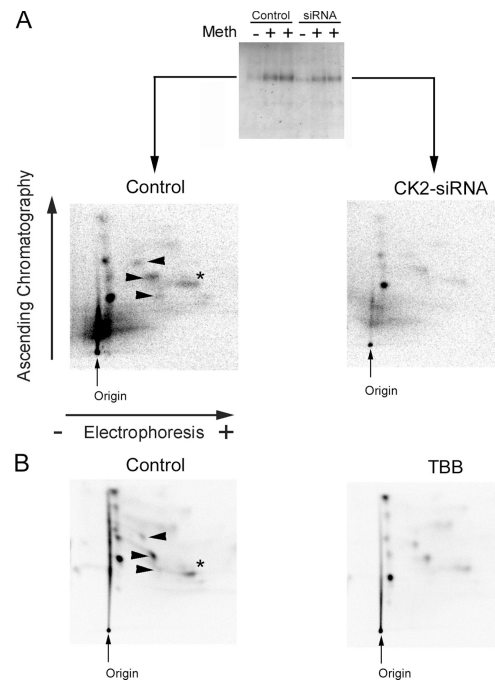

Figure 4. **CK2 inhibition decreases agonist-induced phosphorylation of only a subset of phosphoacceptor sites in the M₃-muscarinic receptor.** (A) Chymotryptic phosphopeptide maps of the M_3-muscarinic receptor immunoprecipitated from CHO-M3 cells that had been transfected with scrambled control or CK2 siRNAs (CK2α-4/CK2α′-1p) and stimulated with 100 μM methacholine for 5 min. Indicated are the origins of sample application, direction of electrophoresis, and chromatography. Spots marked by the arrowheads and asterisks are those that increase in phosphorylation in control-stimulated cells but not in CK2 siRNA–treated cells. The arrows further represent spots that are in common with the in vitro CK2 phosphopeptide map shown in Fig. 5 A. (B) Same as A, but 20 μM TBB (1.5 h) was used to inhibit CK2 activity. Data is representative of at least three experiments.

receptor is phosphorylated on serines in the third intracellular loop of the receptor (Budd et al., 2003). Here, we compared the chymotryptic phosphopeptide map of the receptor phosphorylated in a cellular context by endogenous kinases in CHO-M3 cells with the map of a bacterial fusion protein containing the third intracellular loop of the M_3-muscarinic receptor (GST-H3iloop) phosphorylated in vitro by CK2. We know from preliminary studies that this fusion protein is phosphorylated by CK2 in the muscarinic receptor portion only.

The phosphopeptide map obtained from the phosphorylated M_3-muscarinic receptor, immunoprecipitated from [³²P]-orthophosphate–labeled CHO-M3 cells, was complex, with at least 19 distinct phosphopeptides identified (Fig. 5 A, right). In contrast, the phosphopeptide map from the in vitro phosphorylated GST-H3iloop demonstrated just five major phosphopeptides (Fig. 5 A, left). Four of these phosphopeptides migrated to very similar positions to phosphopeptides in the in vivo map, suggesting that they may represent the same phosphopeptides (Fig. 5 A, asterisks). It is also of interest that the peptides that are shown to decrease in the level of phosphorylation in CK2 siRNA–treated cells (Fig. 4 A, arrows) closely correlated with peptides seen to be phosphorylated in vitro by CK2 (Fig. 5 A).

Edman degradation of peptide 1 in Fig. 5 A determined that it was phosphorylated in position 6 in both the in vitro sample

and in vivo sample (Fig. 5 B). Similarly, for peptide 2, the major phosphoacceptor site was at position 15 in the in vitro and in vivo sample (Fig. 5 B). Thus, the fact that peptides 1 and 2 run in very similar positions in the phosphopeptide maps from the in vivo and in vitro samples and that these peptides are phosphorylated at the same position (residue) indicated that they are the same phosphopeptide phosphorylated in vitro by CK2 and in vivo by endogenous receptor kinases.

The occurrence of a phosphorylated serine at position 15 in spot 2 corresponded with a predicted chymotryptic peptide where the third intracellular loop serine 351 (S^{351}) was the 15th serine (Fig. 5 B). This serine is in the motif SASS^{351}DEED, which is a classical CK2 consensus site (S-x-x-D/E/pS; Meggio and Pinna, 2003). Generation of a bacterial fusion protein 3iloop construct where the SASSDEED motif was mutated to AAAADEED resulted a fusion protein that was phosphorylated predominantly at just two sites (Fig. 5 C, marked A and B) compared with the multisite phosphorylation seen in the wide-type fusion protein. In contrast, the sites phosphorylated by CK1 were not changed in the AAAADEED mutant (Fig. 5 C). These data indicate that the SASSDEED motif was not only a phosphoacceptor site for CK2 but that phosphorylation at this motif promoted further subsequent "hierarchal" phosphorylation events, a feature that is typical of CK2-mediated phosphorylation.

Similar analysis on spot 1, where residue 6 is phosphorylated, indicated that the potential phosphoacceptor sites could be contained in one of two predicted chymotryptic peptides, which start with the following sequences: VHPTGS^{286}SRS and ELQQQS^{299}MKRS. In the first of these sequences, the serine in position 6 fits a consensus CK2 site only if position 9 is primed by phosphorylation. In the second peptide, the serine in position 6 does not fit precisely into a consensus CK2 site, although priming by phosphorylation at position 10 might be sufficient. However, there is no evidence from the Edman degradation data presented here that priming at these sites occurs. Nevertheless, CK2 might be mediating phosphorylation at these sites (even in the absence of priming), a possibility that is currently being tested using mutants lacking these sites.

Phosphorylation of the third intracellular loop by GRK2, GRK6, and CK2

As illustrated in Fig. 2 A, the predicted sites at which the GRKs and CK2 might phosphorylate the receptor show some overlap. To investigate whether phosphoacceptor sites between CK2 and the GRKs were in fact shared, we generated chymotryptic phosphopeptide maps of GST-H3iloop phosphorylated with GRK2 and GRK6 and compared these to maps generated after the phosphorylation with CK2. The phosphorylation of the third intracellular loop protein by GRK2 and GRK6 appeared to occur at very similar sites, as determined by the fact that the phosphopeptides migrated to similar positions and were phosphorylated on the same residues (i.e., spot A in GRK2 and GRK6 migrate to the same position and are both phosphorylated on residue 12; Fig. 6). In contrast, CK2 phosphorylated the receptor on very different sites, as determined by the different migration of phosphopeptides. In the single peptide that appeared to run in a position similar to that of the GRKs (i.e., spot 1 runs similarly to spot A;
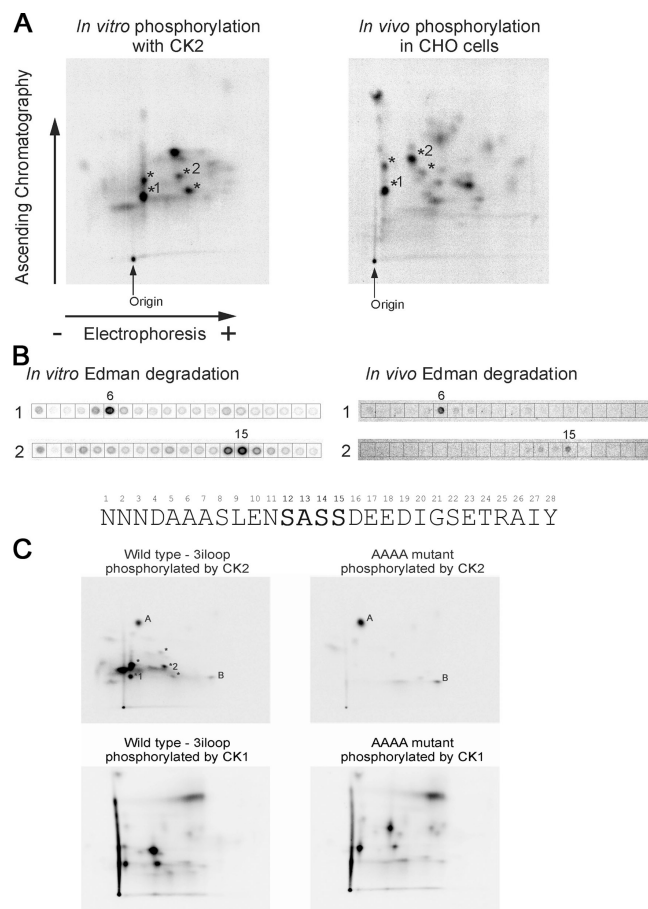

Figure 5. **CK2 phosphorylates the SASSDEED motif in the third intracellular loop of the M$_3$-muscarinic receptor.** (A) Chymotryptic phosphopeptide map of GST-H3iloop phosphorylated by CK2 in vitro (left) and of the M$_3$-muscarinic receptor phosphorylated in vivo by endogenous kinases in CHO-M3 cells (right). Indicated are the origins of sample application, direction of electrophoresis, and chromatography. Asterisks point out phosphopeptides that migrate to similar positions in both maps. Maps depicted are representative of at least three experiments. (B) Peptides 1 and 2 were subjected to Edman degradation, and the product of each cycle was spotted onto filter paper and exposed to determine which cycle contained the phosphorylated residue. The sequence shown is a predicted receptor chymotryptic peptide where the serine in position 15 is at a consensus CK2 phosphorylation site. (C) Comparison of the chymotryptic phosphopeptide map of the wild-type third intracellular loop fusion protein (left) and a fusion protein where the SASSDEED motif was been mutated to AAAADEED (right; AAAA mutant). These fusion proteins were phosphorylated with either CK2 (top) or CK1 (bottom). A representative experiment of at least three determinations is shown. The data shown were performed in parallel.

Fig. 6), Edman degradation demonstrated that this peptide was phosphorylated at a different residue (i.e., residue 6) compared with the residue phosphorylated by the GRKs (i.e., residue 12). These data indicate that CK2 phosphorylated the third intracellular loop at sites different from the GRKs.

CK2-mediated receptor phosphorylation does not regulate receptor internalization

Internalization of GPCRs has long been associated with receptor phosphorylation (Shenoy and Lefkowitz, 2003). We tested this link for the M$_3$-muscarinic receptor by removing putative phosphoacceptor sites through mutation of 16 serines in the

A GRK2   GRK6   CK2
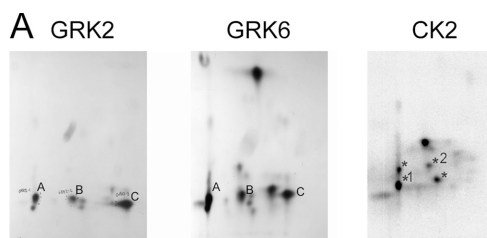
B

EDMAN DEGRADATION

GRK2
Spot A   Radioactivity in cycle 12
Spot B   Radioactivity in cycle 12
Spot C   Radioactivity in cycle 12 and 13

GRK6
Spot A   Radioactivity in cycle 12
Spot B   Radioactivity in cycle 12
Spot C   Radioactivity in cycle 12 and 13

CK2
Spot 1   Radioactivity in cycle 6
Spot 2   Radioactivity in cycle 15

Figure 6. **Comparison of the phosphorylation of GST-H3iloop with GRK2, GRK6, and CK2.** The GST fusion protein containing the third intracellular loop of the human M_3-muscarinic receptor (GST-H3iloop) was phosphorylated with GRK2, GRK6, or CK2, and chymotryptic phosphopeptide maps were generated. (A) Chymotryptic phosphopeptide maps resulting from GRK2, GRK6, or CK2 phosphorylation. (B) The phosphopeptides labeled A, B, and C from the GRK phosphorylations and the peptides labeled 1 and 2 from the CK2 phosphorylation were subjected to Edman degradation, and the cycle in which radioactivity was detected is given. Note that the CK2 data is reproduced from Fig. 5 to enable easy comparison against the GRK phosphopeptide maps.

third intracellular loop (Budd et al., 2004). This mutant receptor, termed mutant-6, was expressed normally at the cell surface, as determined by radioligand binding (unpublished data) and by biotinylation of cell surface proteins followed by immuno-precipitation of the biotinylated M_3-muscarinic receptor (Fig. 7 A). Furthermore, these receptor-biotinylation experiments demonstrated that immunoprecipitation of mutant-6 was as efficient as that of the wild-type receptor and that the efficiency of immuno-precipitation was not altered by ligand stimulation (Fig. 7 A). In subsequent phosphorylation experiments, the ability of the mutant-6 receptor to be phosphorylated in response to agonist was demonstrated to have been almost completely removed (Fig. 7 B).

The loss of receptor phosphorylation in mutant-6 correlated with a loss in the ability of the receptor to be internalized after prolonged treatment with agonist. As shown in Fig. 6 C, stimulation of CHO-M3 cells expressing the wild-type M_3-muscarinic receptor resulted in a decrease in cell surface receptor expression, as measured by radioligand binding using the non-membrane permeable antagonist [^{3}H]-N-methylscopolamine (NMS). In contrast, stimulation of cells expressing mutant-6 did not result in any significant change in the cell surface expression of the receptor (Fig. 7 C). This was confirmed using immuno-histochemistry, where staining for the wild-type receptor showed

a plasma membrane localization before agonist treatment but a characteristic endosomal localization after treatment with agonist for 30 min (Fig. 7 D). This was not the case for cells expressing mutant-6, where the receptor remains at the cell surface after exposure to agonist (Fig. 7 D).

Despite the fact that these data support a role for receptor phosphorylation in the internalization of the M_3-muscarinic receptor, inhibition of CK2-mediated receptor phosphorylation by siRNAs directed against CK2 (Fig. 7 E) or pharmacological inhibition using TBB (Fig. 7 F) did not significantly affect receptor internalization. It appears, therefore, that receptor phosphorylation mediated by CK2 is not involved in receptor internalization, but rather other kinases (presumably of the GRK family; Tsuga et al., 1998) are responsible for this process.

Role of CK2-mediated receptor phosphorylation in coupling to MAPK pathways

We tested the role of CK2-mediated receptor phosphorylation in the coupling of the M_3-muscarinic receptor to the ERK1/2 and Jun-kinase pathways. Fig. 8 A shows that siRNA-directed agonist CK2 had no effect on the ability of the receptor to activate ERK1/2. In contrast, the magnitude of the Jun-kinase response was significantly increased after inhibition of CK2-mediated receptor phosphorylation, with the maximal response being approximately twofold greater in cells transfected with siRNA against CK2 compared with control transfected cells (Fig. 8 B). Similarly, the onset of the Jun-kinase response is faster in cells transfected with CK2 siRNA, where a significant response was evident at 10 min of agonist stimulation compared with 15 min in cells transfected with control scrambled siRNA (Fig. 8 B). Importantly, the control Jun-kinase response to sorbitol was not affected by CK2 inhibition (unpublished data).

The M_3-muscarinic receptor is differentially phosphorylated in different cell types

Our study indicates that CK2 is among several protein kinases involved in the phosphorylation of the M_3-muscarinic, each able to mediate a different signaling outcome (i.e., internalization/Jun-kinase). A natural extension of these findings would be to hypothesize that cell type–specific receptor phosphorylation could contribute to tissue-specific signaling of the receptor. A first step in testing this hypothesis would be to determine if the receptor is differentially phosphorylated in different cell types. Hence, we compared the tryptic phosphopeptide maps of agonist-stimulated mouse M_3-muscarinic receptors derived from transfected CHO cells with that of maps derived from receptors expressed in mouse CG neurons.

As can be seen in Fig. 9, the receptor expressed in CHO cells was phosphorylated both basally and in response to agonist on many more sites than the receptor expressed in CG neurons. By comparing the migration of the spots, it can be seen that several of these phosphopeptides are common in the receptor maps derived from the two cell types (Fig. 9 B, marked with numbers), indicating that certain receptor phosphoacceptor sites are conserved. However, some of these common sites are differentially regulated by agonist. Hence, the phosphopeptide marked A
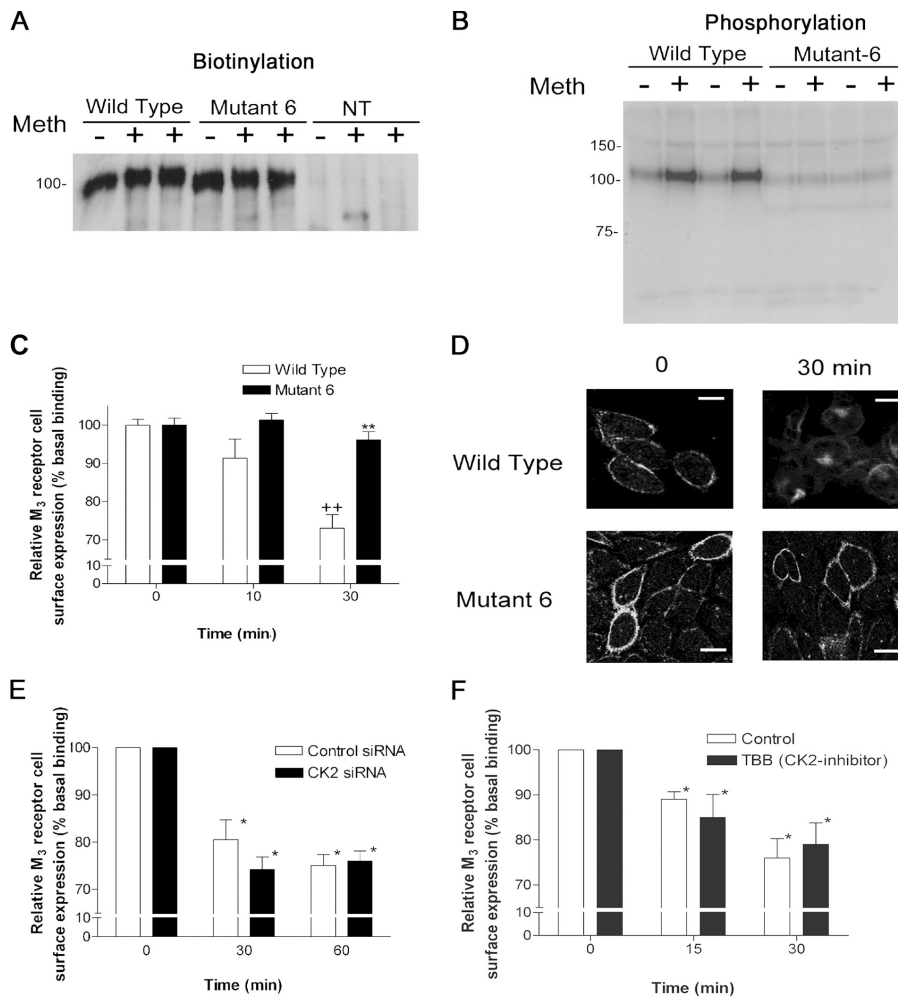
Figure 7. **Internalization of the M₃-muscarinic receptor is dependent on non–CK2-mediated receptor phosphorylation.** (A) Immunoprecipitation of biotinylated wild-type and mutant-6 M₃-muscarinic receptors from CHO cells. Equal quantities of receptor (as determined by [³H]-NMS assay) were used in the immunoprecipitation. NT, nontransfected cells. (B) Phosphorylation of wild-type and mutant-6 receptors in response to 100 μM methacholine for 5 min. (C) Internalization of the M₃-muscarinic receptor was determined by stimulation with 100 μM methacholine for the times indicated followed by the quantification of cell surface receptor expression using [³H]-NMS binding at 4°C. The histograms represent the percentage binding and correspond to mean ± SD of three experiments. Statistical comparison was performed using a one-way ANOVA with Bonferroni posttest. ⁺⁺, P < 0.001 between nonstimulated and methacholine treated cells; **, P < 0.001 between wild-type and mutant-6 cells. (D) Representative experiment of the redistribution of M₃ receptors in response to methacholine treatment. After stimulation, cells were fixed and stained with the anti-M₃ antibody followed by FITC fluorescence analysis. Bars, 10 μm. (E) Internalization of the M₃-muscarinic receptor in CHO-M3 cells transfected with scrambled control siRNA or CK2 siRNA (CK2α-4/CK2α'-1p) as measured by [³H]-NMS binding at 4°C. (F) Internalization of the M₃-muscarinic receptor in CHO-M3 cells treated with 20 μM TBB for 1.5 h. Statistical comparison was performed using a one-way ANOVA with Bonferroni posttest. *, P > 0.05 (no significant difference between treated and nontreated cells).

is agonist regulated in CG neurons but is constitutively phosphorylated in CHO cells. Furthermore, the peptide marked B is agonist regulated only in CHO cells (Fig. 9 A).

Although some of the phosphopeptides are common to the two cell types, others are cell type specific. Five examples of phosphopeptides identified only in CHO-derived receptors are marked with open arrowheads in Fig. 9 B (left), and an example of a phosphopeptide specific to CG neurons is marked with a closed arrowhead (right).

Discussion

Agonist occupation results in the multisite phosphorylation of GPCRs by receptor kinases in a manner often described as homologous phosphorylation. There is now a large body of evidence to support the role of the GRK family in this process (Pierce et al., 2002). However, it is clear from studies using phosphoacceptor site mutants (Seibold et al., 2000), peptide mapping (Blaukat et al., 2001), mass spectrometry (Trester-Zedlitz et al., 2005), phosphospecific antibodies (Pollok-Kopp et al., 2003; Tran et al., 2004), and transgenic animals (Walker et al., 2004) that the process of receptor phosphorylation is complex, suggesting the possibility of the involvement of protein kinases in addition to the GRKs (Tobin, 2002).

By use of CK2 inhibitors and siRNA against the α and α' catalytic subunits of CK2, we demonstrate that CK2 contributes to the phosphorylation of the M₃-muscarinic receptor in both a heterologous expression system and in mouse neurons. Furthermore, by using phosphopeptide maps, we show that in vitro phosphorylation of the third intracellular loop of the M₃-muscarinic receptor by CK2 results in the phosphorylation of sites that are also phosphorylated by endogenous kinases in vivo. Importantly, these in vivo sites are seen to decrease in phosphorylation after CK2 siRNA treatment. Finally, we show that purified CK2 can increase the phosphorylation state of the intact M₃-muscarinic receptor in membranes prepared from CHO-M3 cells. Thus, CK2 can be added to GRK2, GRK6, and CK1α as a protein kinase that can phosphorylate the M₃-muscarinic receptor in an agonist-dependent manner (Budd et al., 2000; Wu et al., 2000; Willets et al., 2003).

What might be the significance of multikinase receptor phosphorylation? Despite CK1α and CK2 sharing the same nomenclature, they are structurally distinct protein kinases, a fact highlighted in Hanks and Hunter's (1995) classification, where CK2 is classified as a CMGC kinase and CK1α as a member of the CK1 family. This compares with the GRKs that are classified as ACG kinases. It appears, therefore, that the M₃-muscarinic receptor can be phosphorylated in an agonist-dependent manner
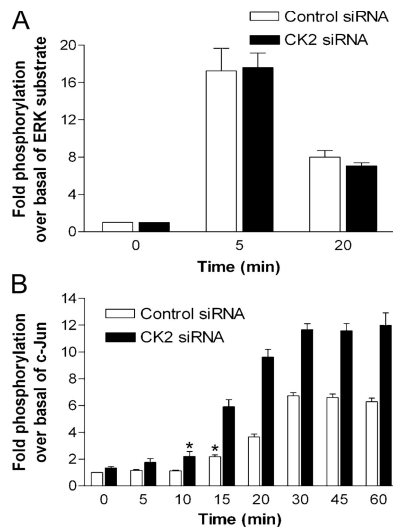
Figure 8. **CK2 regulates M$_3$-muscarinic receptor activation of Jun-kinase but not ERK1/2.** CHO-M3 cells treated with control scrambled or CK2 siRNAs (CK2α-4/CK2α-1p) were stimulated with 100 μM methacholine for the indicated times, and cell extracts were obtained and used in an ERK1/2 kinase assay where the ERK substrate was an EGFr peptide (A) or a Jun-kinase assay where GST–c-Jun fusion protein was a substrate for Jun-kinase (B). Phosphate incorporated into substrates GST–c-Jun or EGFr per milligram of protein in cell extracts was normalized to the basal level of phosphorylation (time 0). Data correspond to mean ± SD from at least three experiments. *, P < 0.05 (significant difference between stimulated levels and control).

Figure 9. **The M$_3$-muscarinic receptor is differentially phosphorylated in different cell types.** (A) CHO cells expressing the mouse M$_3$-muscarinic receptor (top) or CG neurons (bottom) were ^{32}P-labeled and treated with or without 100 μM methacholine for 5 min. The receptors were then immuno-precipitated, and a tryptic phosphopeptide map was generated. These maps are representative of three CHO and two CG neurons replicates with very similar results. (B) Comparison of receptor tryptic phosphopeptides (phosphorylation signatures) from methacholine-stimulated CHO (left) and CG cells (right). These maps are the same as shown in A except that the numbered phosphopeptides indicate those that migrate to similar positions in both maps, whereas the open arrowheads represent phosphopeptides that are specific to CHO cells and the closed arrowhead a phosphopeptide specific to receptors derived from CG neurons.

by protein kinases from very different families with distinct structural features, mechanisms of regulation and subcellular localization. This diversity would allow for a very flexible process of receptor regulation, where not only can different sites on the receptor be phosphorylated by different protein kinases but also the differential mechanisms of activation and regulation of protein kinase activity/localization could influence receptor phosphorylation and signaling.

The fact that more than one structurally distinct protein kinase family has a role in M$_3$-muscarinic receptor phosphorylation is reflected in the numerous phosphoacceptor sites determined from the proteolytic phosphopeptide maps conducted in this study. Furthermore, by comparing the in vitro CK2-mediated phosphopeptides with in vivo phosphopeptide maps, and by the analysis of phosphopeptide maps from cells where CK2 was inhibited using either siRNA or pharmacological inhibitors, we demonstrate that only a subset of the phosphoacceptor sites on the M$_3$-muscarinic receptor are phosphorylated by CK2.

This data points to the fact that the distinct receptor kinases for the M$_3$-muscarinic receptor are able to phosphorylate defined sites on the receptor. This is supported by comparisons of the phosphopeptide maps after the phosphorylation of the third intracellular loop by GRK2, GRK6, and CK2, which demonstrated that CK2 can indeed phosphorylate sites different from those phosphorylated by the GRKs. In the case of CK2, we determined that one of these sites was the SASSDEED motif in the third intracellular loop. Phosphorylation at this motif promotes further CK2-mediated phosphorylation in a process akin to hierarchal phosphorylation, which is a common feature of this protein kinase.
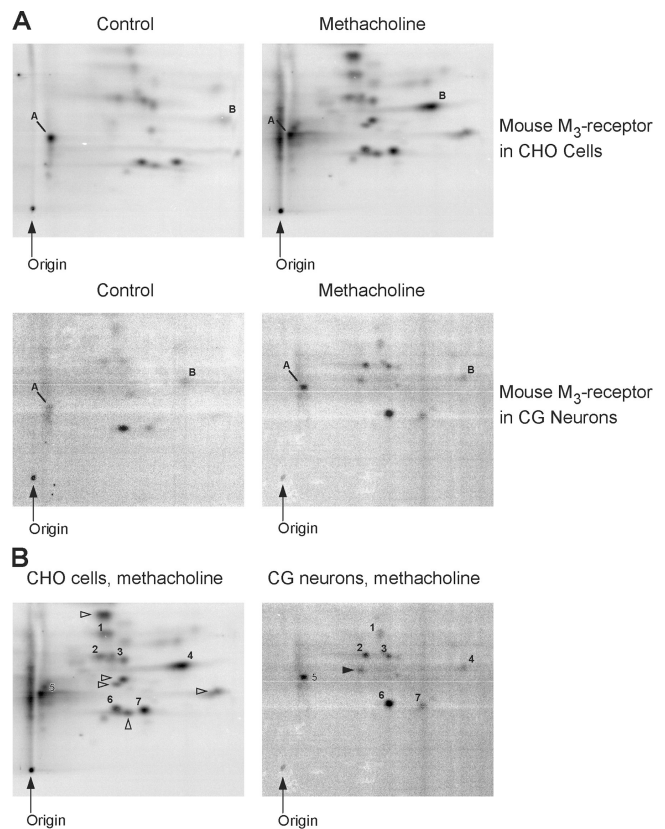
The question that arises from these observations is whether phosphorylation by different receptor kinases can result in different signaling outcomes. We addressed this here by focusing on three signaling processes well known to be regulated by receptor phosphorylation, namely, receptor internalization, activation of the ERK1/2, and activation of the Jun-kinase pathway (Pierce et al., 2002). Our study showed that inhibition of CK2-mediated phosphorylation does not affect receptor internalization. This is the case despite the fact that M$_3$-muscarinic receptor internalization is a phosphorylation-dependent process. Thus, receptor internalization must be driven by a non–CK2-dependent phosphorylation. Our unpublished data shows that CK1α inhibition similarly does not affect receptor internalization. It appears most likely that M$_3$-muscarinic receptors are internalized in a GRK-dependent manner, as has previously been reported for this receptor subtype (Tsuga et al., 1998).

Receptor coupling to the ERK1/2 pathway is similarly not affected by inhibition of CK2-mediated receptor phosphorylation. We have shown previously that this signaling response

is likely to be regulated by CK1α (Budd et al., 2001). In contrast, we show here that CK2 activity is important in coupling the receptor to the Jun-kinase pathway. Inhibition of CK2 via siRNA substantially increases both the magnitude and time course of the Jun-kinase response to muscarinic receptor stimulation but has no affect on the receptor-independent activation of the Jun-kinase pathway mediated by sorbitol. Because we show that CK2 is able to mediate receptor phosphorylation, our data point to the possibility that CK2-mediated receptor phosphorylation can regulate the coupling of the M_3-muscarinic receptor to the Jun-kinase pathway and none of the other phosphorylation-dependent signaling pathways. This supports the notion that site-specific phosphorylation mediated by a single receptor kinase can regulate a defined receptor signaling process. Our studies do not, however, completely rule out the possibility that CK2 has an indirect role on receptor coupling to the Jun-kinase pathway that is independent of receptor phosphorylation.

A logical extension of this finding is that GPCR phosphorylation might be used as a flexible adaptive process where a defined complement of protein kinases would be recruited to phosphorylate specific sites in a process that would allow for tissue-specific signaling. Hence, in the case of the M_3-muscarinic receptor, such a process would contribute to the very different physiological responses mediated by this receptor when expressed in smooth muscle cells compared with the same receptor subtype expressed in salivary acinar cells and neurons.

In the current study, we tested whether the M_3-muscarinic receptor was able to be phosphorylated in a cell type–specific fashion by comparing the tryptic phosphopeptide maps obtained from the mouse M_3-muscarinic receptor immunoprecipitated from CHO cells and mouse CG neurons. We refer to the pattern obtained from these maps as phosphorylation signatures. By comparing the receptor phosphorylation signatures obtained in CHO cells and CG neurons, it was clear that some elements of receptor phosphorylation were the same between the two cell types, whereas others were cell type specific. The common features of these maps may underlie common regulatory processes, such as receptor internalization, whereas those phosphorylation events that are unique to a given cell type might be involved in cell type–specific signaling. It was clear from these studies that at least between these two cell types the phosphorylation signatures of the M_3-muscarinic receptors were different, indicating that differential cell type–specific phosphorylation indeed occurs. Our continuing studies aimed at defining the role of these cell type–specific phosphorylation events in physiologically relevant tissues will test further whether this differential phosphorylation pattern is related to cell type–specific functional responses.

Materials and methods

Primary cell culture
Mouse CG neurons were cultured as described previously (Leist et al., 1997). In brief, cerebella from 7–8-d-old BALB/c or transgenic pups were mechanically and enzymatically (trypsin) dissociated and plated at 0.25 × 10^6 cells/cm^2 on 100 µg/ml poly-L-lysine–coated 6- or 12-well plates (Nunc). CG neurons were then incubated in Eagle's basal medium supplemented with 20 mM KCl, penicillin/streptomycin, 10% fetal calf serum,

and 10 µM cytosine arabinoside (added 48 h after plating) in a humidified atmosphere with 5% CO_2 at 37°C for 7–8 d.

siRNAs
siRNAs were chemically synthesized (Ambion). The siRNA sequences targeting CK2 were CK2α-4, 5′-AacaUUgaaUUagaUccacgU-3′; and CK2α′-1p, 5′-AagaUUcUggagaaccUUcgU-3′. These siRNAs targeted to sequences that are 100% conserved between rat, mouse, and human CKs.

Scrambled siRNA of CK2α-4 and CK2α-1p were used as nonsilencing controls. Transient transfections of siRNA duplexes were performed in 80% confluent CHO cells in 6- or 12-well plates using 120 nM siRNA and 3 µl si-PORTamine transfection reagent (Ambion) according to the manufacturer's protocol. Cells were used for experiments after 48 h.

M_3-muscarinic receptor phosphorylation and phosphopeptide mapping
The phosphorylated M_3-muscarinic receptor was immunoprecipitated from 50 µCi/ml [^{32}P]-orthophosphate–labeled cells as previously described (Budd et al., 2004). Equivalent amounts of immunoprecipitated receptor in each sample were ensured by parallel radiolabeled ligand binding experiments using the antagonist [^{3}H]-NMS (Budd et al., 2004). The immunoprecipitated receptor was resolved on 8% SDS-PAGE and visualized by autoradiography or using a phosphorimager (STORM; GE Healthcare).

In the case of CG cells, the procedure was the same, but CSS-25 incubation buffer (120 mM NaCl, 1.8 mM $CaCl_2$, 15 mM glucose, 25 mM KCl, and 25 mM Hepes, pH 7.4) containing 100 µCi/ml [^{32}P]-orthophosphate was used. The receptor was immunoprecipitated from cell lysates obtained by pooling 2 wells of a 6-well plate. In these experiments and others involving the mouse receptor, we used an in-house anti–mouse M_3-muscarinic receptor antibody (see Generation of mouse M_3-muscarinic receptor antibody).

For phosphopeptide mapping, CHO-M3 cells and CG neurons were labeled with 100 or 200 µCi/ml [^{32}P]-orthophosphate, respectively. Stimulation and immunoprecipitation were performed as described. The immunoprecipitated samples of one entire 6-well plate of CHO cells or 2 plates of CG neurons were pooled and resolved by SDS-PAGE. The gel was then electroblotted onto nitrocellulose membrane, and the phosphorylated receptor was visualized by autoradiography. The area of the membrane containing the receptor was cut out, blocked for 30 min at 37°C with 0.5% polyvinylpyrrolidone-K 30 (Sigma-Aldrich) containing 0.6% acetic acid, and washed several times with water. The M_3-muscarinic receptor contained on the membrane was digested for 20 h at 37°C in ambic solution (50 mM NH_4HCO_3 and 0.5 mM $CaCl_2$) containing 10 µg/ml of either trypsin (Promega) or chymotrypsin (Sigma-Aldrich). The supernatant was then removed, and the membrane slice washed once with water. The wash and supernatant were combined dried. Tryptic/chymotryptic peptides were then resuspended in 10 µl pH 1.9 buffer (88% formic acid/acetic acid/water, 25:78:897 vol/vol) and spotted onto cellulose-coated chromatography (TLC) plates (20 × 20 cm; Merck). The peptides were then separated in two dimensions. The first dimension was electrophoresis at 2,000 V for 30 min in pH 1.9 buffer using a Hunter HTLE-7002 system (CBS Scientific). The second dimension was ascending chromatography in isobutyric acid/n-butanol/pyridine/acetic acid/water, 1,250:38:96:58:558 vol/vol). The resolved phosphopeptides were then visualized using a STORM phosphorimager.

In vitro phosphorylation of GST-H3iloop fusion protein
The human M_3-muscarinic receptor third intercellular loop (R^{253}-T^{492}) cloned in-frame with GST (GST-H3iloop) was expressed and purified from bacteria as previously described (Tobin and Nahorski, 1993). 35 µg of fusion protein bound to glutathione–Sepharose beads was incubated with 100 units of CK1 or CK2 (New England Biolabs, Inc.) or 100 ng of purified GRK2 (provided by R. Lefkowitz, Duke University Medical Center, Durham, NC) or GRK6 (provided by J. Tesmer, University of Michigan, Ann Arbor, MI) in kinase assay buffer (10 mM $MgCl_2$, 20 mM β-glycerophosphate, and 20 mM Hepes, pH 7.4) with 50 µM ATP and 10 µCi γ-[^{32}P]ATP in a total volume of 100 µl. The reaction was continued for 30 min at 37°C. To determine the inhibition potency of TBB and DMAT (Calbiochem), protein kinases were preincubated for 10 min in the same buffer without ATP and with 1 µM of each inhibitor. Reactions were stopped by the addition of 1 ml of ice-cold buffer, and the GST-H3iloop:glutathione–Sepharose beads were pelleted and resuspended in 2× SDS-PAGE sample buffer before being resolved by SDS-PAGE. Quantification of the phosphorylation status of GST-H3iloop was determined by phosphorimager analysis. Alternatively, the phosphorylated GST-H3iloop was blotted onto nitrocellulose membrane and used in phosphopeptide mapping as described.

Phosphorylation of the M₃-muscarinic receptor on membrane preparations

Crude membranes were prepared from CHO-M3 and CHO cells and stored at −80°C as described previously (Tobin et al., 1993). Before use, membranes were washed for 15 min with kinase buffer (25 mM Tris-HCl, pH 7.4, 20 mM β-glycerophosphate, 200 mM NaCl, 10 mM MgCl₂, and 100 μg/ml BSA). Membranes were then pelleted in a microfuge (3 min at 21,000 g) and resuspended in kinase buffer before use in the in vitro phosphorylation reaction, which consisted of membranes (200 μg protein) and kinase buffer containing γ-[³²P]-ATP (2 μM; 1–4 disintegrations per minute/fmol) in the presence or absence of CK2 (200 or 500 units per reaction) in a reaction volume of 200 μl. Reactions were incubated at 37°C for 10 min. Reactions were stopped by pelting membranes in a microfuge (21,000 g for 1 min), and solubilization of the membrane pellet and immunoprecipitation of the M₃-muscarinic receptor were performed as described previously (Tobin et al., 1993).

Radioligand binding and internalization assays and immunohistochemistry

Expression of cell surface muscarinic receptors after exposure of cells to methacholine for the indicated times was determined as described previously (Budd et al., 1999) except that saturating concentrations (0.5 nM) of the muscarinic antagonist [³H]-NMS was used in incubations with whole cells for 60 min at 37°C. Washes were then conducted at 4°C (Budd et al., 1999).

To determine subcellular localization of the M₃-muscarinic receptor using immunohistochemistry, cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100 and the receptor was stained using a 1:500 dilution of an in-house anti-human M₃-muscarinic receptor antibody (Tobin and Nahorski, 1993).

MAPK assays

Cells plated onto 6-well dishes were serum starved for 1 h and stimulated with 0.1 mM methacholine for the times indicated. The cells were then lysed, and cellular ERK1/2 was immunoprecipitated and assayed in an in vitro assay using the EGFr peptide substrate as described previously (Budd et al., 2001). Alternatively, Jun-kinase was isolated from the cell lysate using a GST–c-Jun fusion protein followed by the in vitro phosphorylation of GST–c-Jun as previously described (Budd et al., 2003).

Biotinylation

Cells cultured on 6-well plates were incubated in buffer containing 1 mM biotin (Pierce Chemical Co.) for 30 min at 37°C. The cells were then lysed, and the M₃-muscarinic receptor was immunoprecipitated as described for phosphorylation experiments. Equivalent amounts of receptor were ensured by analysis of receptor levels using [³H]-NMS radiolabeling. Samples were then separated by SDS-PAGE, electroblotted to nitrocellulose membranes, and, after blocking with TBST plus 5% milk powder for 1 h, incubated with 50 ng/ml streptavidin conjugated to horseradish peroxidase (Pierce Chemical Co.) for 30 min. Biotinylated proteins were then detected with chemiluminescence reagent (ECL plus; GE Healthcare).

Generation of mouse M₃-muscarinic receptor antibody

The region S³⁴⁴-L⁴⁶² of the mouse M₃-muscarinic receptor third intracellular loop was produced as an N-terminal tagged GST/receptor fusion protein, which was used to inoculate New Zealand white rabbits (Harlan Sera-Labs). The resulting antisera was tested in immunoprecipitation studies and shown to specifically react with the mouse M₃-muscarinic receptor (Fig. 3).

CK2 activity assay

48 h after siRNA transfection, CHO cells cultured in 12-well dishes were harvested in lysis buffer (25 mM Tris-HCl, pH 7.4, 20 mM β-glycerophosphate, and 200 mM NaCl) containing 0.5% NP-40 substitute (Fluka). The kinase assay (using 10 μl of cell lysate) was performed in lysis buffer containing 1 μM ATP, 10 mM MgCl₂, 100 μg/ml BSA, 50 μM CK2 peptide substrate RRREEETEEE (Promega), and 1 μCi γ-[³²P]ATP (total volume 50 μl). After a 15-min incubation at 37°C, half of the mixture was spotted on phosphocellulose P81 filter (Whatman) and washed four times with 0.5% H₃PO₄. The filters were then transferred to vials containing scintillation fluid (Safefluor) and counted. The phosphorylation assays were performed in either the presence or absence of 1 μM of the CK2 inhibitor TBB. CK2 activity was then determined as the TBB-sensitive component of the peptide phosphorylation, which was ~20% of the total peptide phosphorylation.

Data analysis

Autoradiography densitometric analysis was performed using ImageQuant and AlphaEase FC softwares. SD of at least three determinations is presented, and significance was determined using a one-way analysis of variance (ANOVA).

Online supplemental material

Fig. S1 shows the sequences on CK2α and -α′ subunits that were targeted by siRNAs. These siRNAs were then tested for the ability to reduce expression of CK2 and effects on M₃-muscarinic receptor phosphorylation (Fig. S2). This data demonstrated that siRNAs distinct from those used in Fig. 1 were also able to reduce CK2 expression, and this correlated with a reduction in receptor phosphorylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200610018/DC1.

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