Phosphorylation of Human m1 Muscarinic Acetylcholine Receptors by G Protein-coupled Receptor Kinase 2 and Protein Kinase C*

Kazuko Haga, Kimihiko Kameyama, Tatsuya Haga, Ushio Kikkawa, Kazumasa Shiozaki, and Haruaki Uchiyama

From the Department of Biochemistry, Institute for Brain Research, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, the $Biological Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657, the $Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, and the $Department of Neurosurgery, Hamamatsu Red Cross Hospital, 1-5-30 Takabayashi, Hamamatsu, Shizuoka, Japan

Human muscarinic acetylcholine receptor m1 subtypes (m1 receptors) were expressed in and purified from insect Sf9 cells and then subjected to phosphorylation by G protein-coupled receptor kinase 2 (GRK2) expressed in and purified from Sf9 cells and by protein kinase C purified from rat brain (a mixture of α, β, and γ types, PKC). The m1 receptor was phosphorylated by either GRK2 or PKC in an agonist-dependent or independent manner, respectively. G protein βγ subunits stimulated the phosphorylation by GRK2 but did not affect the phosphorylation by PKC. The number of incorporated phosphates was 4.6 and 2.8 mol/mol of receptor for phosphorylation by GRK2 and PKC, respectively. The number of incorporated phosphates was 7.5 mol/mol receptor for phosphorylation by GRK2 followed by PKC, but was 5.8 mol/mol of receptor for the phosphorylation by PKC followed by GRK2. Major sites phosphorylated by GRK2 and PKC were located in the third intracellular loop and the carboxyl-terminal tail, respectively. These results indicate that GRK2 and PKC phosphorylate different sites of m1 receptors and that the phosphorylation by PKC partially inhibits the phosphorylation by GRK2, probably by affecting activation of GRK2 by agonist-bound receptors.

Muscarinic acetylcholine receptors consist of five subtypes. The m1, m3, and m5 subtypes are linked to Gαq family G proteins and the m2 and m4 subtypes to Gi/Go family G proteins (for reviews, see Refs. 1–3). Muscarinic receptors as well as other G protein-coupled receptors are known to undergo desensitization following exposure to agonists (4–6). Desensitization of receptors is generally believed to be mediated by receptor phosphorylation. Homologous desensitization is thought to be linked to agonist-dependent phosphorylation of receptors by G protein-coupled receptor kinase (GRK)1 and heterologous desensitization to agonist-independent phosphorylation of receptors by second messenger-activated protein kinases such as CAMP-dependent protein kinase and protein kinase C (PKC).

GRK is a subfamily of serine and threonine kinases and is characterized by phosphorylation of only the stimulated forms of G protein-coupled receptors (for reviews, see Refs. 7–9). The GRK family includes rhodopsin kinase (GRK1), β-adrenergic receptor kinases 1 and 2 (GRK2, GRK3) and GRK4, GRK5, GRK6. GRK1 and GRK2/3 have been characterized much more extensively than the other members. GRK1 phosphorylates rhodopsin in a light-dependent manner, and the light dependence is at least partly due to the activation of GRK1 by light-absorbed rhodopsin (10, 11). GRK2/3 are different from other GRKs in that GRK2/3 are activated by G protein βγ subunits and have longer carboxy termini, which are the sites that interact with βγ subunits (12–15). GRK2 is synergistically activated by βγ subunits and mastoparan (16) and by βγ subunits and agonist-bound receptors (17). Recently GRK2/3 have been reported to be activated by phospholipids as well as βγ subunits, although there are some discrepancies among authors (18, 19). GRK2 was originally isolated as a kinase that phosphorylates β-adrenergic receptors in an agonist-dependent manner, but is now known to phosphorylate different kinds of G protein-coupled receptors, including muscarinic m2 (17, 20–22) and m3 (23), α2-adrenergic (α2A and α2B) (24), and substance P (25) receptors. On the other hand, α1- (26) and α2C (24)-adrenergic receptors are reported not to be phosphorylated by GRK2. It remains to be known how the substrate specificity of GRK2 is determined.

Some contradictory results have been reported concerning the phosphorylation of m1 receptors. Muscarinic receptors purified from porcine brain, which contain m3 receptors as a major component, were phosphorylated in an agonist-dependent manner by a muscarinic receptor kinase that was purified from porcine brain and had properties similar to GRK2 (27). On the other hand, human m1 receptors expressed in and purified from insect Sf9 cells were not phosphorylated in an agonist-dependent manner by either the muscarinic receptor kinase or GRK2 under the same conditions where m2 receptors purified from Sf9 cells were phosphorylated in an agonist-dependent manner by the muscarinic receptor kinase or GRK2 (28). Richardson et al. (29) also reported that human m2 receptors expressed in Sf9 cells were phosphorylated in an agonist-dependent manner by an endogenous kinase, but human m1 receptors were not phosphorylated under the same conditions.

The m1 receptor as well as the m2 and α2 receptors have a long third intracellular loop that contains sequences similar to the putative phosphorylation sites in m2 (30) and α2 receptors (31). In fact, a peptide corresponding to the homologous se-
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In m1 receptors can be phosphorylated by GRK2, and the phosphorylation was markedly stimulated by G protein βγ subunits and mastoparan (6). In addition, human m1 receptors were found to be phosphorylated by GRK2 in an agonist-dependent manner in the presence of βγ subunits and m2 or phosphorylation site-deleted m2 receptors (6). These results indicate that the phosphorylation of m1 receptors by GRK2 is enhanced when GRK2 is activated by βγ subunits and mastoparan or by βγ subunits and agonist-bound m2 receptors. This raises the question whether m1 receptors are unable to activate GRK2 even in the presence of agonist or the ability was impaired during the preparation of m1 receptors from SF9 cells. We have extensively examined the purification procedure of m1 receptors and found that m1 receptors can be phosphorylated in an agonist-dependent manner by GRK2 after removing unknown factor(s) that copurify with m1 receptors.

PKC are known to phosphorylate muscarinic receptors purified from porcine brain (32), human m1 receptors expressed in and purified from SF9 cells (33), and muscarinic receptors purified from chick heart (34). The present experiments were undertaken to determine whether GRK2 and PKC phosphorylation sites in human m1 receptors are independent or shared and whether the phosphorylation by one kinase affects the phosphorylation by another. We report here that different sites in m1 receptors are phosphorylated in an agonist-dependent and -independent manner by GRK2 and PKC, respectively, and that the phosphorylation by PKC reduces subsequent phosphorylation by GRK2.

EXPERIMENTAL PROCEDURES

Materials—Human m1 muscarinic receptors were expressed in and purified from SF9 cells by single step affinity chromatography as described (37). Purified m1 receptors in a typical experiment were 15 nmol of membrane receptors and 3–4 nmol of purified receptors starting from 10 liters of culture. GRK2 was expressed and purified from SF9 cells, as described previously (17). Antibody against a synthetic peptide corresponding to the carboxyl terminus of m1 receptors (residue 436–460) was prepared as described (37). A fusion protein (I3-GST) of glutathione S-transferase and a peptide corresponding to the central part of the third intracellular loop of human m1 receptors (residues 226–320) was expressed in and purified from Escherichia coli and was used as an antigen to produce rabbit antibodies. PKC was purified from rat or porcine brain as described previously (38), and an antibody against a peptide corresponding to the central part of the third intracellular loop of human m1 receptors (residues 435–460). An aliquot of the suspension was subjected to SDS-PAGE, followed by autoradiography. Molecular weights of small peptides were estimated from the mobility of marker proteins and a phosphorylated synthetic peptide corresponding to the carboxyl terminus of m1 receptors (residue 435–460).

RESULTS

Phosphorylation by GRK2—Human m1 receptors purified from SF9 cells were reconstituted with G proteins in lipid vesicles and then subjected to phosphorylation by GRK2. Phosphorylation of m1 receptors was detected but the phosphorylation was not affected by the presence of carbachol or atropine (Fig. 1, the upper figure). This phosphorylation was inhibited by 0.1 μM heparin, a potent inhibitor of GRK2 (41), indicating that the phosphorylation was due to GRK2 and not to contaminating kinases (data not shown). The agonist-independent phosphorylation of m1 receptors by GRK2 contrasts with the agonist-dependent phosphorylation of m2 receptors by GRK2 under the same experimental conditions (14). We have attempted to remove putative contaminants that may inhibit the agonist-dependent phosphorylation of m1 receptors from m1 and GRK2 preparations. In one of such trials, we found that m1 receptors became to be phosphorylated in an agonist-dependent manner when m1 receptors were precipitated with PEG after reconstitution into lipid vesicles (Fig. 1, the lower panel). The stimulation by carbachol was dose-dependent and antagonized by atropine, confirming that the effect of carbachol was mediated by its binding to m1 receptors.

A standard assay tube for phosphorylation of m1 receptors by GRK2 contained the reconstituted vesicle (1 μl, 1.5–4.0 nm m1 receptors and 9–40 nm Gβ, in final concentrations), 1 nm carbachol, or 10 μM atropine, 100 μM GTP, and purified GRK2 (13 nm) in a medium of 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.2 mM EDTA, 0.5 mM EGTA (total volume, 40 μl). Gα and GTP were added to supply G protein βγ subunits and were precipitated by GRK2 in some experiments. A standard assay tube for phosphorylation of m1 receptors by PKC contained the same components as the above except that 2 nm EDTA and 0.5 mM EGTA were replaced by 0.2 mM CaCl2, and the reaction was precipitated by PKC (42 nm). The phosphorylation reaction was carried out at 30 °C and was terminated by addition of 20 μl of 5% sodium dodecyl sulfate (SDS) solution containing medium for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. The band of m1 receptors was cut out and counted by the use of a Phosphorlmager.
Phosphorylation by PKC—Phosphorylation of m1 receptors by PKC was not increased by addition of agonist, whether or not the m1 receptors in vesicles were subjected to precipitation with PEG (Fig. 3). A slightly higher level of phosphorylation of m1 receptors was reproducibly observed in the presence of atropine compared with the phosphorylation in the presence of carbamylcholine, although detailed statistical analysis was not performed. The phosphorylation by PKC of m1 receptors was not affected by G protein \( \beta \gamma \) subunits, in contrast to the phosphorylation by GRK2 (Fig. 4).

Phosphorylation by GRK2 and PKC—Fig. 5 shows the time courses of phosphorylation of m1 receptors which were incubated with GRK2 and PKC. The phosphorylation by GRK2 and PKC was shown to be dependent on carbamylcholine and atropine, respectively.
bated with GRK2 or PKC alone, with GRK2 and then PKC or with PKC and then GRK2. The amounts of phosphates incorporated in m1 receptors were significantly increased by addition of another kinase, indicating that the phosphorylation sites by GRK2 and PKC are different from each other. Unexpectedly, the amounts of phosphates incorporated into m1 receptors were dependent on the order of addition of the two kinases and were apparently greater when m1 receptors were phosphorylated by GRK2 and then PKC, compared with when m1 receptors were phosphorylated by PKC and then GRK2 (Fig. 5). This observation was further confirmed by phosphorylation of m1 receptors in the presence of different concentrations of ATP (Fig. 6). Amounts of phosphates incorporated in m1 receptors were greater when phosphorylated in the order GRK2 then PKC, compared with the reverse order. Table I summarizes data concerning the amount of phosphates incorporated into m1 receptors (moles of [32P]P/mol of [3H]quinuclidinyl benzylate binding site), when m1 receptors were subjected to phosphorylation in the presence of 50 μM ATP at 30 °C for 120 min with a single kind of kinase or with two kinases applied in different orders. The amount of phosphates incorporated into m1 receptors was estimated to be 4.6, 2.8, or 7.6, for GRK2 alone, PKC alone, and for GRK2 and then PKC, respectively. The value of 7.6 is virtually the same as the sum (7.4) of 4.6 and 2.8, indicating that the phosphorylation sites by GRK2 and PKC are mutually exclusive. On the other hand, the amount of phosphate incorporated into m1 receptors incubated with PKC and then GRK2 was estimated to be 5.8 and significantly lower than the value of 7.4. This result indicates that the phosphorylation by PKC suppresses the further phosphorylation by GRK2.

Very recently, Tsu et al. (42) reported that GRK2 is phosphorylated by PKC and that the phosphorylated GRK2 has a 31% lower K_m and 10% higher V_max for phosphorylation of rhodopsin. We have also examined if the activity of GRK2 to phosphorylate m1 receptors is affected by preincubation of GRK2 with PKC, but have not found significant increase of the activity of GRK2. It remains to be determined if this is due to the difference in substrates or experimental conditions.

Location of Phosphorylation Sites—To locate phosphorylation sites on m1 receptors, we determined the location of the phosphorylation sites using the following procedures. Purified m1 receptors were reconstituted with G protein, precipitated with PEG, and then subjected to phosphorylation by PKC in the presence of 0.1 mM GTP and 1 mM carbamylcholine or 10 mM atropine. Procedures for preparation of m1 receptors and phosphorylation assay were the same as described in the legend to Fig. 1, except that 2 mM EDTA and 0.5 mM EGTA in the reaction medium were replaced by 0.2 mM CaCl_2. In some experiments, phosphatidylinositol and diolein were also included in the incubation medium in addition to the lipid mixture, but no significant difference was found in the level of phosphorylation.
higher concentrations of trypsin, molecular sizes of 32P-labeled
located in the 14-kDa carboxyl-terminal fragment.

[i]tail part, and that the major PKC phosphorylation sites are
includes the third intracellular loop but not the carboxyl-ter-
phosphorylation sites reside in the 38-kDa fragment, which
contrast, the 14-kDa band was stained with anti-C tail but not
immunoprecipitated with anti-C tail antibodies also became
bands were detected on SDS-PAGE (data not shown). Bands

**TABLE I**

| Kinases   | Number of phosphorylation sites a |
|-----------|----------------------------------|
| 1) GRK2   | 4.6 ± 0.3 (4)                    |
| 2) PKC    | 2.8 ± 0.5 (4)                    |
| 3) GRK2 then PKC | 7.5 ± 1.3 (3)       |
| 4) PKC then GRK2 | 5.8 ± 0.3 (3)       |
| 5) GRK2 and PKC | 7.0 ± 0.3 (3)       |

a Values in parentheses indicate number of experiments.

By treatment of m1 receptors phosphorylated by GRK2 with
higher concentrations of trypsin, molecular sizes of 32P-labeled
bands decreased from 38 to 13 and finally to 3 and 2 kDa (Fig.
All of these phosphorylated bands were immunoprecipitated
with anti-i3 but not with anti-C tail antibodies. Bands with 3
and 2 kDa were resistant to treatment with higher concentra-
tions of trypsin. The recovery of radioactivity in the 3- and
2-kDa bands obtained by treatment of the receptor with 50

μgl/ml trypsin was 50–70% of the initial amount in the intact
receptor. This result indicates that these bands contain the
major phosphorylation sites, although they may not be the sole
phosphorylation sites. In contrast, when m1 receptors phos-
phorylated by PKC were treated with higher concentrations of
trypsin, the 14-kDa peptide with phosphorylation sites by PKC
was broken down to smaller fragments, and no 32P-labeled
bands were detected on SDS-PAGE (data not shown). Bands
immunoprecipitated with anti-C tail antibodies also became
undetectable following treatment with higher concentrations of
trypsin under conditions where bands precipitated with anti-i3
antibodies could still be detected. These results indicate that
major PKC phosphorylation sites are located in the carboxyl-
terminal portion, which is easily broken down by treatment
with trypsin, and that major GRK2 phosphorylation sites are in
peptides which are resistant to trypsin treatment and contain
at least part of the third intracellular loop.

**FIG. 6. Effect of ATP concentrations on phosphorylation of m1
receptors by GRK2 and PKC.** Experimental procedures were the
same as described in the legend to Fig. 5, except that m1 receptors
were subjected to phosphorylation in the presence of different concentrations of
[32P]ATP (2.35 cpm/2 μl) for 120 min with GRK2 alone (●) or PKC
alone (○) or for 60 min with GRK2 and then for another 60 min after
addition of PKC (GRK2 then PKC) (□) or for 60 min with PKC and then
for 60 min after addition of GRK2 (PKC then GRK2) (□). The sum of
phosphorylation by GRK2 alone and that by PKC alone is shown as
"Calc. (PKC + GRK2)" (△).

**FIG. 7. Digestion with a low concentration of trypsin of m1
receptors phosphorylated by GRK2 or PKC.** PEG-treated m1 recep-
tors (3.6 pmol as original amount in total volume of 300 μl) were phosphorylated
by GRK2, then treated with indicated concentrations of trypsin for 5
min at 30 °C. An aliquot of the reaction mixture (33 μl) was directly
subjected to SDS-PAGE followed by autoradiography. Digitonin (0.1%
in a final concentration) and then anti-i3 or anti-C tail antibodies (10 μl
of anti-serum) were added to a portion of the reaction mixture (100 μl).
After incubation at 4 °C overnight, Pansorbin was added and the sus-
pension was centrifuged. The pellet was subjected to SDS-PAGE fol-
lowed by autoradiography.

**FIG. 8. Digestion with high concentrations of trypsin of m1
receptors phosphorylated by GRK2.** PEG-treated m1 receptors (3.6
pmol as original amount in total volume of 300 μl) were phosphorylated
by GRK2, then treated with indicated concentrations of trypsin for 5
min at 30 °C. An aliquot of the reaction mixture (33 μl) was directly
subjected to SDS-PAGE followed by autoradiography. Digitonin (0.1%
in a final concentration) and then anti-i3 or anti-C tail antibodies (10 μl
of anti-serum) were added to a portion of the reaction mixture (100 μl).
After incubation at 4 °C overnight, Pansorbin was added and the sus-
pension was centrifuged. The pellet was subjected to SDS-PAGE fol-
lowed by autoradiography.
DISCUSSION

In the present paper we have shown that human m1 receptors are phosphorylated in an agonist-dependent manner by GRK2 and independent manner by PKC. The number of phosphorylation sites were estimated to be 4-5 for phosphorylation by GRK2 and 2-3 for phosphorylation by PKC. These phosphorylation sites appear to be different from each other, because the sum of sites phosphorylated by GRK2 and PKC was not significantly different from the number of sites phosphorylated following sequential phosphorylation by GRK2 and PKC. This conclusion was further supported by the finding that major phosphorylation bands obtained by trypsin treatment of m1 receptors phosphorylated by GRK2 are different from those obtained by the same treatment of m2 receptors phosphorylated by PKC. This conclusion is consistent with the fact that the consensus sequence for phosphorylation by PKC should include basic amino acids around serine and threonine residues (43) but phosphorylation sites by GRK2 should be flanked by acidic amino acids rather than basic amino acids (44).

Major phosphorylation sites by PKC have been located in a sequence within 14 kDa from the carboxyl-terminal segment, consistent with previous results using muscarinic receptors purified from porcine brain (37). The 14-kDa peptide is thought to contain residues 333-460 based on the assumption that the peptide ends at the carboxyl terminus, and the average molecular mass of each residue is 110 Da. The 14-kDa peptide labeled with 32P was sensitive to trypsin treatment and was broken down to small peptides undetectable following SDS-PAGE, consistent with the fact that there are numerous lysine and arginine residues in the carboxyl-terminal portion. There are two serine and two threonine residues in the carboxyl-terminal tail (R^*DT^246FR*, K^*R^PGS^335VHR^*T^459D*S^457R* QC-OH) and one serine and two threonine residues in the carboxyl-terminal portion of the third intracellular loop (K^*R^PT^339R*K*, K^*R^K^T^354S^356VK^EK^*K*K*), which are flanked by basic amino acids and are therefore good candidates for PKC phosphorylation sites. In fact, peptides corresponding to the carboxyl-terminal tail (sequence 345-460) and to the carboxyl-terminal part of the third intracellular loop (sequence 346-365) were phosphorylated by protein kinase C, although a peptide corresponding to the sequence 422-434 was not phosphorylated (6). Thus, Thr^354, Ser^356, Ser^451, Thr^455 and Ser^457 are likely candidates for sites phosphorylated by PKC.

Major GRK2 phosphorylation sites have been recovered in 3- and 2-kDa bands, which interact with anti-i3 and are resistant to treatment with trypsin. It is interesting to note that the amino-terminal half of the third intracellular loop contains fewer basic and many more acidic amino acid residues than the carboxyl-terminal half. Serine and threonine residues in the amino-terminal half of the third intracellular loop are not flanked by basic amino acid residues, in contrast with serine and threonine residues in the carboxyl-terminal tail, the carboxyl-terminal half of the third intracellular loop, and the second intracellular loop. In particular, serine and threonine residues in the sequence from 275 to 303, K^E^E^E^E^D^E^GS^284M^E^S^287L^T^289S^293E^G^E^E^D^GS^298E^VVIK*, are most likely to be GRK2 phosphorylation sites, because 1) the sequence contains basic amino acid residues only at both ends and the corresponding peptide with a molecular mass of 3 kDa is expected to be resistant to trypsin treatment, 2) a peptide corresponding to the sequence 279-293 was phosphorylated in vitro by GRK2 (6), and 3) sequence 286-291 fits to a consensus sequence for phosphorylation in vivo by GRK2, which was proposed from studies on a2-adrenergic receptors (31). In addition, the replacement by alanine of serine and threonine residues in the sequence 284-291 is reported to cause the attenuation of sequestration of m1 receptors (45). The kinase involved in the sequestration of m1 receptors has not been identified, but may be GRK2 or related kinases, because the phosphorylation by GRK2 of serine and threonine residues in the third intracellular region of m2 receptors is known to facilitate their sequestration (22). These results taken together suggest that the primary GRK2 phosphorylation sites reside in the 276-303 amino acid sequence of the third intracellular loop, although this does not exclude the possibility that the serine and threonine residues in the other parts of intracellular loops might be phosphorylated by GRK2.

The number of sites phosphorylated by GRK2 was estimated to be 2.9 when m1 receptors were subjected to phosphorylation by GRK2 following phosphorylation by PKC, a value significantly lower than the value of 4.6 obtained following phosphorylation with GRK2 alone. This result indicates that the phosphorylation of m1 receptors by GRK2 is inhibited by prior phosphorylation by PKC.

GRK2 is known to be synergistically activated by G protein βγ subunits and mastoparan or related peptides (16) and by βγ subunits and agonist-bound receptors (17). Peptides corresponding to the second intracellular loop, the carboxyl-terminal end of the third intracellular loop and the carboxyl-terminal tail of m2 receptors activated GRK2 (16). GRK2 was also strongly activated by a peptide corresponding to the carboxyl-terminal tail of m1 receptors and weakly activated by a peptide corresponding to the carboxyl-terminal end of the third intracellular loop of m1 receptors.2 All these peptides contain many basic amino acid residues. It is tempting to speculate that the phosphorylation by PKC of serine and threonine residues in the carboxyl-terminal tail or in the carboxyl-terminal end of the third intracellular loop of m1 receptors reduces the ability of the receptor to interact with and activate GRK2. Another possibility is that the interaction between βγ subunits and m1 receptors is impaired by phosphorylation of m1 receptors by PKC. The carboxyl-terminal tail of rhodopsin has been reported to be the site for interaction with βγ subunits (46).
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