Stimulus-dependent Phosphorylation of G-protein-coupled Receptors by Casein Kinase 1α*

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We have previously demonstrated that the phospholipase C-coupled m3-muscarinic receptor is phosphorylated in an agonist-sensitive manner by a protein kinase of ~40 kDa purified from porcine cerebellum (Tobin, A. B., Keys, B., and Nahorski, S. R. (1996) J. Biol. Chem. 271, 3907–3916). This kinase, called muscarinic receptor kinase (MRK), is distinct from second messenger-regulated protein kinases and from β-adrenergic receptor kinase and other members of the G-protein-coupled receptor kinase family. In the present study we propose that MRK is casein kinase 1α (CK1α) based on the following evidence: 1) the amino acid sequence from two proteolytic peptide fragments derived from purified MRK corresponded exactly to sequences within CK1α. 2) Casein kinase activity co-eluted with MRK activity from the final two chromatography steps in the purification of porcine brain MRK. 3) Recombinant CK1α expressed in NIH3T3 cells is able to phosphorylate both casein and the bacterial fusion protein, Ex-m3, that contains a portion of the third intracellular loop of the m3-muscarinic receptor downstream of glutathione S-transferase. 4) Partially purified CK1α increased the level of muscarinic receptor phosphorylation in an agonist-sensitive manner when reconstituted with membranes from Chinese hamster ovary-m3 cells expressing the human recombinant m3-muscarinic receptor. 5) Partially-purified CK1α phosphorylated rhodopsin, contained in urea-treated bovine rod outer segment membranes, and the extent of phosphorylation was increased in the presence of light. These data demonstrate that the kinase previously called MRK is CK1α, and that CK1α offers an alternative protein kinase pathway from that of the G-protein-coupled receptor kinase family for the stimulus-dependent phosphorylation of the m3-muscarinic receptor, rhodopsin, and possibly other G-protein-coupled receptors.

Intensive research over the last decade have revealed that many GPCR1 subtypes are phosphorylated in response to agonist stimulation (1). These receptors include those coupled to either the adenylate cyclase or phospholipase C (PLC) pathways and suggests that receptor phosphorylation is a common regulatory mechanism employed by all but a few GPCR’s (1). For the majority of these receptors the cellular protein kinases involved in agonist-mediated receptor phosphorylation have yet to be determined. However, this is not the case for the extensively studied β-adrenergic receptor where agonist-dependent phosphorylation and receptor desensitization is mediated by the receptor-specific kinase, β-adrenergic receptor kinase (β-ARK) (2, 3).

Studies using purified or partially purified receptor preparations reconstituted in phospholipid vesicles with purified β-ARK, have demonstrated that β-ARK is also able to phosphorylate both cyclase-coupled (e.g. m2-muscarinic (4)) and PLC-coupled (e.g. substance P receptor (5)) receptors in an agonist-dependent manner. Furthermore, the use of dominant negative mutants of β-ARK to inhibit endogenous β-ARK activity (6) has suggested that β-ARK is the endogenous kinase responsible for the phosphorylation of recombinant PLC-coupled α2-adrenergic receptors expressed in COS-7 cells and rat-1 fibroblasts (7), angiotensin II receptors in HEK 293 cells (8), and the cyclase-coupled δ-opioid receptors in HEK 293 cells (9). These studies have indicated that β-ARK may have a broad receptor substrate specificity that extends beyond β-adrenergic receptors. β-ARK-1 and β-ARK-2 are members of a protein kinase family called the G-protein-coupled receptor kinase (GRK) family (10). The newly cloned members of the GRK family, namely IT-11 (GRK-4), GRK-5, and GRK-6, phosphorylate rhodopsin in reconstituted systems (11–14). Furthermore, GRK-5 is able to phosphorylate purified α2- and β2-adrenergic, and m2-muscarinic receptors (11–13) and GRK-6 to phosphorylate β2-adrenergic and m2-muscarinic receptors, in an agonist-dependent manner (14). Therefore, it appears that β-ARK and other members of the GRK family are able to phosphorylate a number of GPCR subtypes in reconstituted systems lending support to the proposed role of this protein kinase family in phosphorylation of GPCRs.

However, recent evidence suggests that receptor phosphorylation mediated by β-ARK and the GRKs may be inhibited by the PLC signaling pathway. For example, increased intracellular calcium concentrations and depletion of the phospholipid, phosphoinositide 4,5-bisphosphate (PIP2), may contribute to a reduction in the activity of GRKs (see “Discussion”). This raises the possibility that agonist-dependent phosphorylation of PLC-coupled receptors may be mediated by an alternative protein kinase pathway from that of β-ARK and the other GRKs.

Our early studies on the PLC-coupled m3-muscarinic receptor indicated that the rapid serine phosphorylation observed

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1 The abbreviations used are: GPCR, G-protein-coupled receptor; CK1α, casein kinase 1α; CHO, Chinese hamster ovary; MRK, muscarinic receptor kinase; PLC, phospholipase C; β-ARK, β-adrenergic receptor kinase; GRK, G-protein coupled receptor kinase; PIP2, phosphoinositide 4,5-bisphosphate; NIH3T3 cells, Spodoptera frugiperda cells; PAGE, polyacrylamide gel electrophoresis.

2 β-ARK-1 and β-ARK-2 are also known as GRK-2 and GRK-3, respectively.
following agonist stimulation (15) was mediated by a receptor kinase that was distinct from β-ARK (15, 16). We have recently purified a 40-kDa protein kinase from porcine cerebellum that is able to phosphorylate a glutathione S-transferase bacterial fusion protein containing a portion of the third intracellular loop of the m3-muscarinic receptor (17). Furthermore, this 40-kDa protein kinase was able to enhance the agonist-dependent phosphorylation of the muscarinic receptor present in membranes obtained from CHO-m3 cells transfected with the human m3-muscarinic receptor cDNA (17). The molecular weight, chromatographic properties, and protein kinase inhibitor studies demonstrated that the 40-kDa protein kinase was distinct from the second messenger-regulated protein kinases (e.g. protein kinase C) and from β-ARK and other members of the GRK family (17). These findings indicated that the 40-kDa protein kinase, called muscarinic receptor kinase (MRK), represents a previously unidentified receptor-specific kinase that offers an additional/alternative protein kinase pathway for the phosphorylation of the m3-muscarinic receptor and possibly other GPCRs.

In the present paper we present evidence that MRK is a member of the casein kinase I family, namely casein kinase 1α (CK1α), and reveal the ability of the recombinant kinase to phosphorylate m3-muscarinic receptors and rhodopsin in an agonist/stimulus-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—CHO (Chinese hamster ovary) cells culture stably transfected with human m3-muscarinic receptor cDNA (CHO-m3 cells), a kind gift from Dr. N. J. Buckley, Dept. Pharmacology, University College, London, UK) contained ~2100 fmol of receptor/mg of protein. These cells were routinely maintained in 0.1% minimal essential medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (2.5 µg/ml), and fetal calf serum (10% v/v). Spodoptera frugiperda (SF9) cells were maintained as suspension cultures in SF900 II medium (Life Technologies, Inc.).

**Purification of Muscarinic Receptor Kinase (MRK) from Porcine Brain**—The procedure used for purification of MRK from porcine cerebellum has been previously described (17).

**Preparation of the Bacterial Fusion Protein Ex-m3**—Preparation of the bacterial fusion protein, Ex-m3, where amino acids Ser48-Leu66 of the human m3-muscarinic receptor third intracellular loop are fused with glutathione S-transferase has previously been described (15).

**Amino Acid Sequencing**—The excised Coomassie-stained protein band corresponding to MRK was digested with trypsin. Peptides were extracted for 2 h in a sonicating water bath. After concentration, the peptides were resolved using a Relasil C18 column with a guard pre-column. These fractions were combined and passed through a 1-ml heparin-Sepharose column equilibrated with 0.3 M NaCl in TE buffer and stored at 4 °C. The kinase in this preparation was ~4 ng/µl, and represents ~3.5% of the total protein in the preparation.

Control purification protocol involved identical purification steps from Sf9 cells infected with the m3-muscarinic receptor baculovirus. The total protein in fractions obtained from the heparin purification of the control and CK1α-infected cells were very similar, 100 µg/ml total protein in the control, and 112 µg/ml from the CK1α-infected cells.

**Assay for Muscarinic Receptor Kinase and Casein Kinase Activity**—Assay for the muscarinic receptor kinase involved using the muscarinic receptor fusion protein Ex-m3 as a substrate for the kinase (17). Samples from chromatography fractions (10 µl) or from supernatant fractions of infected Sf9 cells were incubated with Ex-m3 (3.5 µg) or dephosphorylated α-casein (15 µg) in kinase buffer (20 mM Tris-HCl, 10 mM MgCl2, 1 mM EGTA, pH 7.4) containing 50 µM [γ-32P]ATP (0.4–1.0 cpm/mg) for 10 min at 37 °C (final volume = 110 µl). Where Ex-m3 was used, the reaction was terminated by addition of 1 ml of ice-cold TE buffer. Glutathione-Sepharose (20 µl, Pharmacia) was added and collected by centrifugation (150,000 × g, 10 s) and washed twice with 1 ml of TE buffer. Bound fusion protein was dissociated by boiling in 2 × SDS-PAGE sample buffer (20 µl).

Where α-casein was used, the reaction was stopped by the addition of 100% trichloroacetic acid (11 µl). The precipitated proteins were pelletized by centrifugation in a Microfuge for 10 min at 13,000 × g. The protein pellet was washed with acetone (~20 °C) and resuspended in 20 µl of 2 × SDS-PAGE sample buffer.

The proteins from the Ex-m3 or α-casein assay were resolved by 12% SDS-PAGE. To ensure the equal recovery of the protein substrates and to confirm their relative positions, gels were stained with Coomassie Blue. Gels were then dried and autoradiographs obtained and/or bands corresponding to the peptide substrates were excised and counted.

**Phosphorylation of m3-muscarinic Receptors in Membrane Preparations**—Porcine brain membranes (1 mg of protein/ml) were incubated with [γ-32P]ATP (1–4 cpm/fmol ATP), then centrifuged at 13,000 × g for 30 s. The supernatant was applied to a 1-ml Resource S (Pharmacia) column which was eluted using a linear gradient of 0–1.0 M NaCl over 20 bed volumes (flow rate = 2 ml/min). 1-ml fractions were collected. The kinase activity eluted as a single peak at ~0.3 M NaCl. These fractions were combined and passed through a 1-ml heparin-Sepharose column equilibrated with 0.32 M NaCl. The column was eluted using a linear gradient of 0.32–1.75 M NaCl over 15 ml. The kinase activity eluted as a single peak at ~0.94 M NaCl. Total peak activity was dialyzed against 0.3 M NaCl in TE buffer and stored at 4 °C. The kinase in this preparation was ~4 ng/µl (0.1 pmol/µl), and represents ~3.5% of the total protein in the preparation.

Control purification protocol involved identical purification steps from Sf9 cells infected with the m3-muscarinic receptor baculovirus. The total protein in fractions obtained from the heparin purification of the control and CK1α-infected cells were very similar, 100 µg/ml total protein in the control, and 112 µg/ml from the CK1α-infected cells.

**Preparation of Recombinant Baculovirus**—The coding sequence for bovine CK1α (a kind gift from Dr. Melanie Cobb, Department of Pharmacology, University of Wurzburg, Versbacher Strasse 9, D-97078 Wurzburg, Germany) was added to kinase buffer containing 50 µM of membranes (~0.1 pmol of receptor) were used in a phosphorylation reaction mixture that contained final concentrations of 20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM EGTA, 100 µM [γ-32P]ATP (1–4 cpm/mg ATP), ≤ 1 mM carbobol and ≤20 µF ATP. To this reaction mixture 10 µl of partially purified CK1α (0.5–1 pmol) or control extract was added. Total volume was 100 µl. Reactions were started by the addition of ATP and continued at 32 °C for 10 min. Reactions were stopped by centrifugation at 13,000 × g for 30 s. The supernatant was removed by aspiration and membranes solubilized with 1 ml of solubilization buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate) for 30 min on ice. m3-Muscarinic receptors were then immunoprecipitated with a specific antisemur (323) as described previously (15).

**Phosphorylation of Rod Outer Segment Membranes**—Urea-treated bovine rod outer segment membranes (30 pmol of rhodopsin/reaction; a kind gift from Dr. Martin Lohse, Institute of Pharmacology and Toxicology, University of Wurzburg, Versbacher Strasse 9, D-97078 Wurzburg, Germany) were added to kinase buffer containing 50 µM ATP (1 cpm/mmol). To this partially purified CK1α (0.3 pmol), purified bovine β-ARK (0.3 pmol; a gift from Dr. Martin Lohse), or control extract were added. Total volume was 30 µl. The above reagents were combined at 4 °C (where necessary under a safe light). Reactions were started by placing the tubes in a water bath at 32 °C either under a safe light or in room fluorescent light for a given time period. Reactions were stopped by the addition of 10 µl of 2 × SDS-PAGE sample buffer and then resolved on a 12% gel. Gels were stained with Coomassie Blue, dried, and autoradiographs obtained. Bands corresponding to rhodopsin were excised and counted.

**Miscellaneous Procedures**—Determination of relative intensities of phosphorylated bands was carried out using a Bio-Rad GS 670 densitometer. Western blotting of whole cell extracts from Sf9 cells used a...
commercially available antiserum (FLAG-M2, Kodak) that recognizes the FLAG epitope cloned onto the N terminus of CK1α.

RESULTS

Amino Acid Sequence Analysis—Proteolytic fragments of MRK purified from porcine brain were tested for MRK activity (i.e. the ability to phosphorylate the m3-muscarinic receptor fusion protein, Ex-m3) and casein kinase activity. The amino acid sequences derived from two peptides were determined to be: 1, WYGQEK; and 2, IEYVHTK. These sequences matched exactly to sequences found exclusively within CK1α (peptide 1, Trp 78–Lys83; and peptide 2, Ile124–Lys130). Furthermore, the predicted molecular mass of CK1α is 37.5 kDa, which corresponds closely with the 40-kDa mass suggested for MRK (17).

Co-purification of MRK Activity and Casein Kinase Activity—To determine if the kinase we previously defined as MRK was CK1α, samples from fractions obtained from the purification of MRK were assayed for both MRK activity, which is defined by the ability of fractions to phosphorylate the bacterial fusion protein Ex-m3 (glutathione S-transferase:m3-third intracellular loop; see “Experimental Procedures”), and casein kinase activity. Fractions from the final two column purification steps, namely the Resource S and heparin-Sepharose fractionation (see Ref. 17), were analyzed. The casein kinase activity was found to elute from the Resource S (data not shown) and heparin-Sepharose columns in an identical manner to MRK activity (Fig. 1). The peak of MRK and casein kinase activity co-eluted from the Resource S and heparin-Sepharose columns at ~0.37 and ~0.87 M NaCl, respectively.

Expression of Recombinant CK1α in Sf9 Cells—Attempts to express recombinant bovine CK1α in mammalian cells (COS-7, HEK 294, and CHO cells) have not proven successful. The reason for this is unclear and is currently under investigation. To obtain a source of recombinant CK1α, we turned to the insect cell baculovirus expression system.

Recombinant CK1α was expressed in infected Sf9 cells as determined by Western blotting of whole cell extract (30 μg of protein/lane) from cells infected with control baculovirus or CK1α baculovirus. The antiserum used was a commercially available monoclonal antiserum against a FLAG epitope engineered on the N terminus of CK1α. B, high speed supernatant extracts from Sf9 cells infected with control virus or CK1α virus were tested for the ability to phosphorylate the m3-muscarinic receptor fusion protein Ex-m3 (3.5 μg) and casein (15 μg). The relative positions of casein and Ex-m3 as determined by Coomassie Blue staining are indicated. C, high speed supernatant extracts from Sf9 cells infected with control virus or CK1α virus were fractionated on a Resource S column and fractions tested for the ability to phosphorylate Ex-m3 and casein.

insect cell baculovirus expression system.

To confirm that the recombinant CK1α contained in infected
cell extracts was able to phosphorylate both casein and Ex-m3, the cell extracts were fractionated on a Resource S column. The peak of casein kinase activity contained in CK1α-infected cells co-eluted with the peak of MRK activity (Fig. 2C). The peak of recombinant kinase activity eluted at ~0.3 M NaCl. Note, the presence of endogenous casein kinase activity is evident in the control fractionation (Fig. 2C). MRK activity and casein kinase activity also co-eluted during heparin-Sepharose chromatography (data not shown).

Phosphorylation of m3-Muscarinic Receptors and Rhodopsin by Recombinant CK1α—Previous studies have demonstrated that membranes from CHO-m3 cells containing an endogenous protein kinase able to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner (17). In these earlier studies, addition of cerebellum-derived MRK to the CHO-m3 membrane preparation resulted in an enhancement of agonist-sensitive m3-muscarinic receptor phosphorylation (17). A parallel experiment was conducted here using partially purified extracts from infected Sf9 cells as the source of exogenous kinase. Fig. 3 shows that in the presence of control extract, purified on the Resource S column, the m3-muscarinic receptor (~0.1 pmol of receptor/reaction) contained in CHO-m3 membranes undergoes agonist-sensitive phosphorylation which can be inhibited by the muscarinic antagonist atropine. Addition of Resource S purified extract from Sf9 cells infected with CK1α baculovirus (0.5–1 pmol of CK1α/reaction) resulted in a ~2.3-fold increase in agonist-sensitive m3-muscarinic receptor phosphorylation with no significant change in the basal phosphorylation of the receptor (Fig. 3). Furthermore, the increase in agonist-sensitive muscarinic receptor phosphorylation, mediated by CK1α, was inhibited by the antagonist atropine (Fig. 3).

To further test the ability of CK1α to phosphorylate GPCR’s, rhodopsin, contained in urea-treated rod outer segments, was used as substrate for CK1α. Heparin-Sepharose-purified CK1α (~300 fmol) was incubated with rod outer segment membranes (containing rhodopsin at 30 pmol/reaction) either under a safe light or under room fluorescent lights. Rhodopsin phosphorylation was observed only in the extract obtained from cells infected with CK1α baculovirus and not from control extracts (Fig. 4A). The CK1α extract did phosphorylate rhodopsin under dark conditions and this phosphorylation was enhanced in the presence of light (118 fmol of phosphate incorporated/5-min reaction) (Fig. 4A). In comparison, β-ARK (~300 fmol/reaction) did not phosphorylate rhodopsin in the absence of light but in the presence of light rhodopsin was phosphorylated to a similar extent as that seen for light-mediated CK1α phosphorylation (122 fmol of phosphate incorporated/5-min reaction) (Fig. 4A).

The time course for CK1α-mediated phosphorylation of rhodopsin was found to be similar to the time course for β-ARK-mediated phosphorylation (Fig. 4B).

**DISCUSSION**

The present study has revealed that the 40-kDa protein kinase purified previously from porcine cerebellum, called MRK due to its ability to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner (17), can be identified as CK1α. These data, together with the ability of recombinant CK1α to phosphorylate the m3-muscarinic receptor and rhodopsin in a stimulus-dependent manner, indicate that CK1α offers an alternative protein kinase pathway, from that of β-ARK and the other GRKs, for the agonist-sensitive phosphorylation and the potential regulation of GPCR’s.

Due to the presence of endogenous receptor kinase activity in membranes from CHO-m3 cells, the enhancement of muscarinic receptor phosphorylation observed in the presence of CK1α may be due to an activation of the endogenous kinase rather than a direct phosphorylation of the receptor. This, however, appears unlikely since CK1α is also able to phosphorylate rhodopsin in a stimulus-dependent manner, in urea-treated rod outer segment membranes where there is no endogenous kinase activity. These data also suggest that like β-ARK, CK1α acts predominantly on the activated form of the receptor. This conclusion is supported by dose-response analysis of m3-muscarinic receptor phosphorylation in intact...
CHO-m3 cells which have demonstrated a close correlation between receptor occupancy and receptor phosphorylation (19). Furthermore, the ability of CK1α to mediate light-dependent phosphorylation of rhodopsin suggests that the substrate specificity of CK1α is broader than just the m3-muscarinic receptor. Experiments are presently in progress to test the receptor-substrate specificity of CK1α against a range of cyclase- and PLC-coupled receptors.

Casein kinase activity was one of the first protein kinase activities identified in mammalian cells and is attributed to two enzymes called casein kinase I and casein kinase II (20, 21). The true “biological” casein kinase responsible for phosphorylating casein in mammary glands is a transmembrane protein kinase of the Golgi apparatus that bears no relationship to casein kinase I or II. In this regard casein kinase I and II are misnomers since the biological substrates for these kinases is not casein. The casein kinase I gene family consists of at least six members; CK1α, CK1β (22), CK1γ1–3 (23), and CK1δ (24). A number of in vitro substrates for this kinase family have been identified including: glycogen synthase, SV40 large T antigen (20), p53 tumor suppressor protein (25), and DARPP-32 (26). However, it is not clear which are the biologically relevant substrates. The present study is the first to demonstrate that CK1α is able to phosphorylate GPCR’s in a stimulus-dependent manner.

The broad receptor substrate specificity of β-ARK (5, 27) and other members of the GRK family (11–14), as determined in reconstituted systems, has implicated this protein kinase family in the phosphorylation of PLC-coupled receptors. Furthermore, expression of the dominant negative mutant of β-ARK (28) results in a 80% fall in the levels of PIP2 within the first 10 s of agonist (31, 32). Similar rapid falls in the level of PIP2 could have a profound influence on the activation and translocation of β-ARK and GRK-5 with the plasma membrane (38, 39). β-ARK and GRK-5 are among the proteins that may mediate light-dependent calcium entry across the plasma membrane, could again discourage GRK activity.

Overall, it could be anticipated that the intracellular environment following signal transduction via PLC-coupled receptors would not be conducive for GRK translocation and receptor phosphorylation. We would suggest, on the basis of the present study, that CK1α offers an attractive alternative route for the phosphorylation of PLC-coupled receptors. In this regard it is interesting to note that casein kinase I isolated from erythrocytes is inhibited by PIP2 (40). Therefore, in contrast to the GRK’s, it is possible that PLC-coupled receptors may increase the activity of CK1α by the hydrolysis of membrane PIP2. Other regulatory features may also be important such as autophosphorylation which is reported to occur on both tyrosine and serine residues on CK1α (41) classifying this kinase as a dual kinase. We have identified autophosphorylation of MRK purified from porcine brain (17) and are presently in the process of determining if autophosphorylation plays any regulatory role in CK1α-mediated receptor phosphorylation. Studies are also underway to determine the mechanism of membrane association and the receptor substrate specificity of CK1α.

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