

Identification of a Novel Receptor Kinase That Phosphorylates a Phospholipase C-linked Muscarinic Receptor*

(Received for publication, September 22, 1995, and in revised form, November 13, 1995)

Andrew B. Tobin‡, Barbara Keys, and Stefan R. Nahorski

From Leicester University, Department of Cell Physiology and Pharmacology, P.O. Box 138, Medical Sciences Building, University Road, Leicester, LE1 9HN, United Kingdom

Phosphorylation of G-protein-linked receptors is thought to play a central role in receptor regulation and desensitization. Unlike the case of the extensively studied β -adrenergic receptor/adenylate cyclase pathway, in which receptor-specific phosphorylation is known to be mediated by β -adrenergic receptor kinase (β -ARK), the kinases responsible for phosphorylation of phospholipase C-linked receptors have yet to be identified, although a role for β -ARK has been implicated. This study describes the purification of a novel 40-kDa receptor kinase from porcine cerebellum that is able to phosphorylate the phospholipase C-linked m3-muscarinic receptor in an agonist-dependent manner. The assay for kinase activity was based on the ability of the kinase to phosphorylate a bacterial fusion protein, Ex-m3, containing amino acids Ser³⁴⁵-Leu⁴⁶³ of the third intracellular loop of the m3-muscarinic receptor. Purification of the muscarinic receptor kinase from a high speed supernatant fraction of porcine cerebellum was achieved using the following steps: (i) 30–60% ammonium sulfate cut and successive chromatography on (ii) butyl-Sepharose (iii) Resource Q, (iv) Resource S, and (v) heparin-Sepharose. The purified protein kinase represented an ~18,600-fold purification and was a single polypeptide with a molecular weight of ~40 kDa. Based on the chromatographic mobility, molecular weight, and kinase inhibitor studies, the kinase, designated MRK, was shown to be distinct from previously characterized second messenger regulated protein kinases, β -ARK, and other members of the G-protein-linked receptor kinase family. It therefore represents a new class of receptor kinase.

Many cell surface neurotransmitter and hormone receptors respond to agonist occupation by activation of phospholipase C. The subsequent hydrolysis of the lipid substrate phosphatidylinositol 4,5-bisphosphate releases the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃)¹ and diacylglycerol (reviewed in Ref. 1). The second messenger response to a number of phospholipase C-linked receptors, including the m3-muscarinic receptor, is often complex, consisting of a burst of Ins(1,4,5)P₃ production reaching a peak within the first few

seconds of receptor stimulation followed by a lower sustained phase of Ins(1,4,5)P₃ generation that is maintained for tens of minutes to hours (2–4). Similar patterns of Ins(1,4,5)P₃ generation are seen in response to agonist occupation of receptors for GRH (5), angiotensin (6), bombesin, and CCK (7).

Little is known of the molecular mechanisms underlying these complex second messenger responses, although recent evidence suggests that some phospholipase C-linked receptors undergo a rapid partial desensitization that results in decreased phospholipase C activity within seconds of agonist occupation (3, 4, 7, 8). Consistent with this notion are studies from our laboratory demonstrating that the early peak phase of Ins(1,4,5)P₃ production in response to m3-muscarinic receptor stimulation can be desensitized by a short pre-exposure to agonist, whereas the sustained phase of Ins(1,4,5)P₃ production is resistant to desensitization (3).

One possible mechanism regulating the receptor/phospholipase C pathway is receptor phosphorylation. The involvement of β -adrenergic receptor phosphorylation in the desensitization of the β -adrenergic/adenylate cyclase system has been well documented (reviewed in Ref. 10). In this case the agonist-occupied form of the β -adrenergic receptor is phosphorylated by two kinases, protein kinase A and a receptor-specific kinase termed β -adrenergic receptor kinase (β -ARK). The process of receptor phosphorylation results in uncoupling of the β -adrenergic receptor from the G_s-protein (10). It is now clear that in addition to β -adrenergic receptors other G-protein linked receptors also exist as phosphoproteins. In particular, phospholipase C/G_{q/11}-coupled CCK (11), m3-muscarinic (12), α_{1B} -adrenergic (13), platelet-activating factor (14), thrombin (15, 16), and neurokinin-2 receptors (17) have all been shown to exist as phosphoproteins in intact cells, and the level of phosphorylation is enhanced following agonist stimulation. The receptor-specific kinase(s) responsible for these phosphorylation events have yet to be fully characterized, although some authors have suggested that β -ARK may act as a general G-protein-linked receptor kinase with a broad substrate specificity (18). Certainly, β -ARK has been implicated in the phosphorylation of CCK receptors in pancreatic acinar cells (19) and recombinant thrombin receptors expressed in *Xenopus* oocytes and fibroblasts (15). Furthermore, substance P and m3-muscarinic receptors have been shown to act as *in vitro* substrates for β -ARK (18, 20).

The two isoforms of β -ARK (β -ARK-1 and -2) are members of a family of protein kinases that includes rhodopsin kinase, IT-11, GRK-5, and GRK-6, which are related on the basis of primary amino acid sequence homology and are collectively termed the G-protein-linked receptor kinase family (GRK) (reviewed in Ref. 21). The existence of the GRK family suggests that G-protein-linked receptor phosphorylation may be mediated by more than one receptor-specific kinase. However, the cellular substrates for IT-11, GRK-5, and GRK-6 are unknown

* This work has been funded by a Programme Grant from the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 0116-2522922; Fax: 0116-2523996.

¹ The abbreviations used are: Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; β -ARK, β -adrenergic receptor kinase; CHO, Chinese hamster ovary; MRK, muscarinic receptor kinase; GRK, G-protein linked receptor kinase; H-89, (N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinoline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

despite *in vitro* evidence that these kinases are able to phosphorylate a number of G-protein-linked receptors (22, 23).

The possibility that a receptor kinase(s) other than β -ARK may be involved in the phosphorylation of G-protein-linked receptors has been suggested by recent studies from our laboratory on the phospholipase C-linked m3-muscarinic receptor (12). These studies have demonstrated that recombinant human m3-muscarinic receptors expressed in CHO cells (CHO-m3 cells) undergo agonist-mediated phosphorylation on serine. The time course for receptor phosphorylation is very rapid, occurring within seconds of agonist addition, and correlates with the rapid but partial desensitization of the phosphoinositide response seen within the first 20 s of receptor stimulation (8, 9, 12). Initial characterization demonstrated the kinase to be distinct from the known second messenger-regulated protein kinases, cAMP-dependent protein kinase, cGMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, and protein kinase C (12). Further characterization in a broken cell preparation revealed that a membrane-associated kinase was able to phosphorylate the m3-muscarinic receptor and that this kinase was not affected by heparin or zinc at concentrations that inhibit β -ARK (24). These findings indicated that m3-muscarinic receptor phosphorylation was mediated by a novel receptor kinase.

Described here is the purification from porcine cerebellum of a novel 40-kDa protein kinase that is able to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner. The purification was based on the ability of the muscarinic receptor kinase to phosphorylate a bacterial fusion protein encoding a region of the third intracellular loop of the m3-muscarinic receptor (Ex-m3).

EXPERIMENTAL PROCEDURES

Preparation of the Bacterial Fusion Protein Ex-m3—Preparation of the bacterial expression vector pEx-m3 has previously been described (12). Briefly, a region of the human m3-muscarinic receptor cDNA encoding the third intracellular loop between Ser³⁴⁵ and Leu⁴⁶³, inclusive, was subcloned into the bacterial expression plasmid pGEX-2T (Pharmacia Biotech Inc.). This produced the plasmid pEx-m3 where the region encoding amino acids Ser³⁴⁵–Leu⁴⁶³ of the m3-muscarinic receptor was downstream of, and in frame with, the coding sequence for glutathione S-transferase contained in pGEX-2T. Induction of pEx-m3-transformed *Escherichia coli* (DH5 α) with isopropyl-1-thio- β -D-galactopyranoside (IPTG, 1 mM) resulted in the production of a fusion protein of ~43.5 kDa (glutathione S-transferase = 27.5 kDa, Ser³⁴⁵–Leu⁴⁶³ ~ 16 kDa). The fusion protein was purified over a glutathione-Sepharose affinity matrix (Pharmacia) before being used in the assay described below.

Construction of Truncated Bacterial Fusion Proteins—In order to characterize the substrate specificity of muscarinic receptor kinase preparations a series of truncated forms of Ex-m3 were synthesized. For a summary of the fusion proteins see Fig. 8A.

pEx345–427 was constructed using the following PCR primers: 5' primer, CCCGATCCCTGGAGAAGCTCCGCC; 3' primer, CCGAAT-TCAAGCTTGGAGAAGCTTTT. These primers were used to amplify a region of the human m3-muscarinic receptor cDNA that encodes amino acids Ser³⁴⁵–Leu⁴²⁷. The primers were designed to allow in-frame cloning into pGEX-2T via a *Bam*HI site at the 5' end and an *Eco*RI site at the 3' end.

pEx376–463 was synthesized using the same strategy, but in this case the 5' PCR primer was CCCGATCCACCATCTCACTCCACC, and the 3' primer was CCCGAATTCAGAGTGGCTTCTTGAAG. This amplified a region of human m3-muscarinic cDNA that encoded amino acids Thr³⁷⁶–Leu⁴⁶³, which was then cloned into pGEX-2T.

p Δ Hind was constructed by digestion of pEx-m3 with the restriction enzyme *Hind*III. This removed a section of cDNA from the muscarinic region of pEx-m3 that encoded for amino acids Leu³⁷¹–Lys⁴²⁶, inclusive.

p Δ H-V was constructed by ligating two PCR reaction products into pGEX-2T in a three-way ligation where pGEX-2T was digested with *Bam*HI (5') and *Eco*RI (3'), PCR reaction product 1 was digested with *Bam*HI (5') and *Apa*I (3'), and PCR reaction product 2 was digested with *Apa*I (5') and *Eco*RI (3'). PCR primers used were (for product 1) 5' primer CCCGATCCCTGGAGAAGCTCCGCC and 3' primer GCTGG-

GCCCCGGAAGCTTGAGCAC and (for product 2) 5' primer CAGGGG-CCCGAGGAGAGCTGGGG and 3' primer CCCGAATTCAGAGTG-GCTTCTTGAAG. The resulting construct encoded a truncated form of Ex-m3 where amino acids His³⁷⁴–Val³⁹¹, inclusive, were deleted.

The above constructs were used to transform *E. coli* (DH5 α). Induction and purification of fusion proteins was the same as that described for Ex-m3.

Assay for Muscarinic Receptor Kinase Activity—Samples from chromatography fractions (10 μ l) or from cell or tissue preparations were incubated with purified Ex-m3 (3.5 μ g) in kinase buffer (20 mM Tris-HCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, pH 7.4) containing 50 μ M [γ -³²P]ATP (0.4–1.0 cpm/fmol) for 10 min at 37 °C (final volume, 110 μ l). The reaction was terminated by the addition of 1 ml of ice-cold TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.4). Glutathione-Sepharose (20 μ l, Pharmacia) was added and collected by centrifugation (13,000 \times *g* for 10 s), washed twice with 1 ml of TE buffer. Bound fusion protein was dissociated by boiling in 2 \times SDS-PAGE sample buffer (20 μ l). Proteins were resolved by 12% SDS-PAGE. In order to ensure the equal recovery of fusion proteins and to confirm the relative positions of fusion proteins, gels were stained with Coomassie Blue. This was particularly important in experiments using truncated fusion proteins, where the extent of phosphorylation was compared as a method of determining substrate specificity of the kinase preparations. Gels were then dried and autoradiographs obtained, and/or bands corresponding to the fusion protein were excised and counted.

Cell Culture—CHO cell cultures stably transfected with human m3-muscarinic receptor cDNA (CHO-m3 cells, a kind gift from Dr. N. J. Buckley, Department of Pharmacology, University College, London) contained ~2100 fmol of receptor/mg of protein. These cells were routinely maintained in α -minimal essential medium supplemented with penicillin (100 IU/ml), streptomycin (100 μ g/ml), fungizone (2.5 μ g/ml), and fetal calf serum (10% v/v).

Preparation of Crude Cytosolic or Membrane Fractions from CHO-m3 Cells—Initial muscarinic receptor kinase preparations were obtained from cytosolic and membrane fractions of CHO-m3 cells. The cytosolic preparation was obtained by harvesting 10 (175-cm²) flasks of CHO-m3 cells, which were resuspended in 2 ml of kinase buffer plus protease inhibitors (1 mM PMSF, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 100 μ g/ml benzamide, 100 μ g/ml iodoacetamide). The cells were allowed to swell for 10–15 min and were then homogenized by a 10-s pulse in a tissue homogenizer (Polytron). A high speed supernatant fraction was then obtained by centrifugation for 30 min at 300,000 \times *g*, and proteins were adjusted to 1–5 mg/ml.

Membranes were prepared from CHO-m3 cells by homogenization of the cells as above but this time in 15 ml of TE buffer plus protease inhibitors. Membranes were collected by centrifugation at 15,000 \times *g* for 10 min and resuspended in kinase buffer at 1 mg protein/ml.

10 μ l of either the membrane or cytosolic preparations were used in the assay for kinase activity.

Purification of Muscarinic Receptor Kinase from Porcine Cerebellum—Cerebella from 15 young pigs (~180 g of tissue) were homogenized in 1 liter of TE buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 100 μ g/ml benzamide, 100 μ g/ml iodoacetamide). All subsequent buffers contained this complement of protease inhibitors, and the preparation was carried out at 4 °C. The homogenate was centrifuged at 12,000 \times *g* for 45 min, and a high speed supernatant fraction (S200 fraction) was obtained by further centrifugation at 200,000 \times *g* for 60 min. The protein pellet from a 30–60% ammonium sulfate cut of the S200 fraction was resuspended in TE buffer (120 ml) and dialyzed against TE buffer containing 1 M (NH₄)₂SO₄. This preparation was loaded onto a 70-ml butyl-Sepharose fast flow column (16 \times 320 mm) equilibrated with TE buffer containing 1 M (NH₄)₂SO₄. Proteins were eluted with a linear gradient of 1–0 M (NH₄)₂SO₄ over 10 bed volumes (flow rate = 1 ml/min). Fractions (12 ml) were collected, and the peak activity, which eluted at ~0.54 M (NH₄)₂SO₄, was pooled and dialyzed against TE buffer.

The sample was then passed through a 6-ml Resource Q (Pharmacia) column. This resolved muscarinic receptor kinase activity from casein kinase II that during the course of this study was found to also phosphorylate the Ex-m3 fusion protein (see "Results" and "Discussion"). The flow-through from the Resource Q column was applied to a 1-ml Resource S (Pharmacia) column. The Resource S column was then eluted using a linear gradient of 0–0.5 M NaCl over 20 bed volumes (flow rate = 1 ml/min). 1-ml fractions were collected. The kinase activity eluted as a single peak at ~0.32 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 0.32–2.0

M NaCl. The kinase activity eluted as a single peak at ~ 0.87 M NaCl.

Partial Purification of Muscarinic Receptor Kinase Activity from Particulate and Soluble Fractions of CHO-m3 Cells—Ten confluent (175-cm^2) flasks of CHO-m3 cells were harvested and resuspended in 10 ml of TE buffer containing protease inhibitors (as above). Cells were allowed to swell for 10–15 min and then were homogenized using a 10-s pulse in a Polytron. Cell debris was removed by centrifugation (3 min at $500 \times g$). Membrane and cytosolic fractions were then prepared by centrifugation at $15,000 \times g$ for 10 min. The supernatant was passed through a 1-ml Resource S column equilibrated with TE buffer, and the column was eluted using a linear gradient from 0 to 0.5 M NaCl. 1-ml fractions were collected. Kinase activity eluted from the Resource S column at the same position as that observed in the brain preparation, i.e. ~ 0.32 M NaCl.

Membranes from the above preparation were either used directly to test for muscarinic receptor kinase activity or extracted with 15 ml of 1.5 M KCl/TE buffer for 3 h at 4°C . The extract was dialyzed against TE buffer and run through a 1-ml Resource S column. The column was eluted as described above. The peak kinase activity eluted at ~ 0.32 M NaCl.

Phosphorylation of m3-Muscarinic Receptors in Membrane Preparations from CHO-m3 Cells—Crude CHO-m3 cell membranes were prepared as described above and resuspended in kinase buffer at 1 mg of protein/ml. 50 μl 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 MgCl_2 , 1 mM EGTA, 2 mM dithiothreitol, 100 μM [$\gamma\text{-}^{32}\text{P}$] ATP (1–4 cpm/fmol of ATP) \pm 1 mM carbachol and \pm 10 μM atropine. 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 $13,000 \times g$ for 30 s. The supernatant was removed by aspiration, and membranes were solubilized with 1 ml of solubilization buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 500 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 antiserum (number 332) as described previously (12).

In experiments to test the ability of the fusion protein Ex-m3 to inhibit the m3-muscarinic receptor phosphorylation, Ex-m3 (3.5 μg) or a molar equivalent of glutathione *S*-transferase was added to the reaction mixture. At the end of the reaction Ex-m3 (3.5 μg) was added to control tubes, thereby ensuring that all tubes contained equal amounts of Ex-m3, since the fusion protein will compete with the receptor for the antibody in the immunoprecipitation. The reaction was then stopped in the way described above, and m3-muscarinic receptors were then solubilized and immunoprecipitated.

In experiments where purified muscarinic receptor kinase was tested for its ability to phosphorylate the intact m3-muscarinic receptor, an aliquot of the kinase preparation (10 μl , ~ 2.5 pmol) was added to the reaction mixture. To control tubes a buffer blank was added (this gave a final NaCl concentration of 87 mM). The reaction was then started and terminated as described above.

Muscarinic Receptor Kinase Autophosphorylation—An aliquot of purified muscarinic receptor kinase (20 μl) was added to a reaction mix containing 20 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 1 mM EGTA, 2 mM dithiothreitol, 130 mM NaCl, 100 μM [$\gamma\text{-}^{32}\text{P}$] ATP (1–4 cpm/fmol of ATP) (total volume, 135 μl). After 30 min at 37°C the reaction was terminated by the addition of 15 μl of ice-cold 100% trichloroacetic acid. The precipitated protein pellet was resuspended in 20 μl of $2 \times$ SDS-PAGE loading buffer and resolved on a 12% SDS-PAGE gel.

Miscellaneous Procedures—Silver stain was performed using a Bio-Rad silver stain kit. Determination of the relative intensities of phosphorylated bands was carried out using a Bio-Rad model GS 670 densitometer.

RESULTS

Assay for Muscarinic Receptor Kinase Activity—Previous studies from our laboratory have demonstrated that the m3-muscarinic receptor expressed in CHO cells (CHO-m3 cells) was phosphorylated in an agonist-dependent manner by a kinase distinct from the G-protein-linked receptor kinases characterized to date (12, 24). Hence, in order to isolate the novel m3-muscarinic receptor kinase an assay for kinase activity was developed.

The bacterial fusion protein Ex-m3, containing a region of the third intracellular loop of the human m3-muscarinic receptor (Ser³⁴⁵–Leu⁴⁶³) acted as a substrate for a kinase in cytosolic extracts from CHO-m3 cells (Fig. 1). There was no phosphoryl-

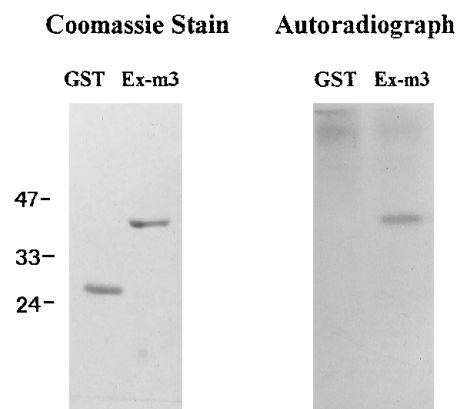


FIG. 1. Phosphorylation of Ex-m3 by a cytosolic extract from CHO-m3 cells. A high speed supernatant extract from CHO-m3 cells (10–50 μg of protein) was tested for kinase activity capable of phosphorylating the recombinant bacterial proteins, Ex-m3 (3.5 μg) and glutathione *S*-transferase (2.0 μg , GST). Shown is a Coomassie Blue stain of the purified bacterial proteins indicating their relative positions on a 12% SDS-PAGE gel and an autoradiograph showing the phosphorylated products. Indicated are the positions of prestained molecular weight standards. The results are representative of at least five experiments.

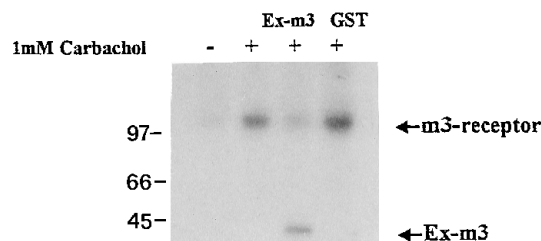


FIG. 2. Inhibition of agonist-driven phosphorylation of m3-muscarinic receptors in membranes from CHO-m3 cells. Membranes from CHO-m3 cells (50 μg of protein, ~ 0.1 pmol of m3-receptor) were challenged with or without 1 mM carbachol in the presence of 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP (1–4 cpm/fmol of ATP) for 10 min at 32°C . The receptors were then solubilized, immunoprecipitated, and resolved by 8% SDS-PAGE. The effect of Ex-m3 (3.5 μg) or a molar equivalent of glutathione *S*-transferase (2.0 μg , GST) on agonist-driven m3-muscarinic receptor phosphorylation was determined. Note: in the lane where Ex-m3 was added, the fusion protein as well as the receptor was immunoprecipitated. Indicated are the positions of prestained molecular weight standards. The results are representative of two experiments.

ation of the bacterially expressed glutathione *S*-transferase (Fig. 1). Furthermore, following digestion of phosphorylated Ex-m3 fusion protein with thrombin, a process that releases the m3-muscarinic receptor region, the ^{32}P label was associated only with the m3-muscarinic region and not the glutathione *S*-transferase portion of the fusion protein (data not shown). A similar kinase activity was also identified in membrane preparations from CHO-m3 cells, but the kinase activity was ~ 30 -fold lower than that observed in cytosolic extracts. Studies described below indicate that the kinase associated with the membrane fraction is likely to be the same as the cytosolic kinase.

We have previously demonstrated agonist-driven m3-muscarinic receptor phosphorylation in membranes from CHO-m3 cells, suggesting that the muscarinic receptor kinase is, at least in part, associated with the plasma membrane (24). In order to confirm that the Ex-m3 fusion protein was acting as a pseudosubstrate for the m3-muscarinic receptor kinase, experiments were conducted to investigate the ability of Ex-m3 to block agonist sensitive muscarinic receptor phosphorylation in membranes from CHO-m3 cells. Fig. 2 shows that Ex-m3 was able to inhibit agonist-induced m3-muscarinic receptor phosphorylation in CHO-m3 membranes, whereas glutathione *S*-

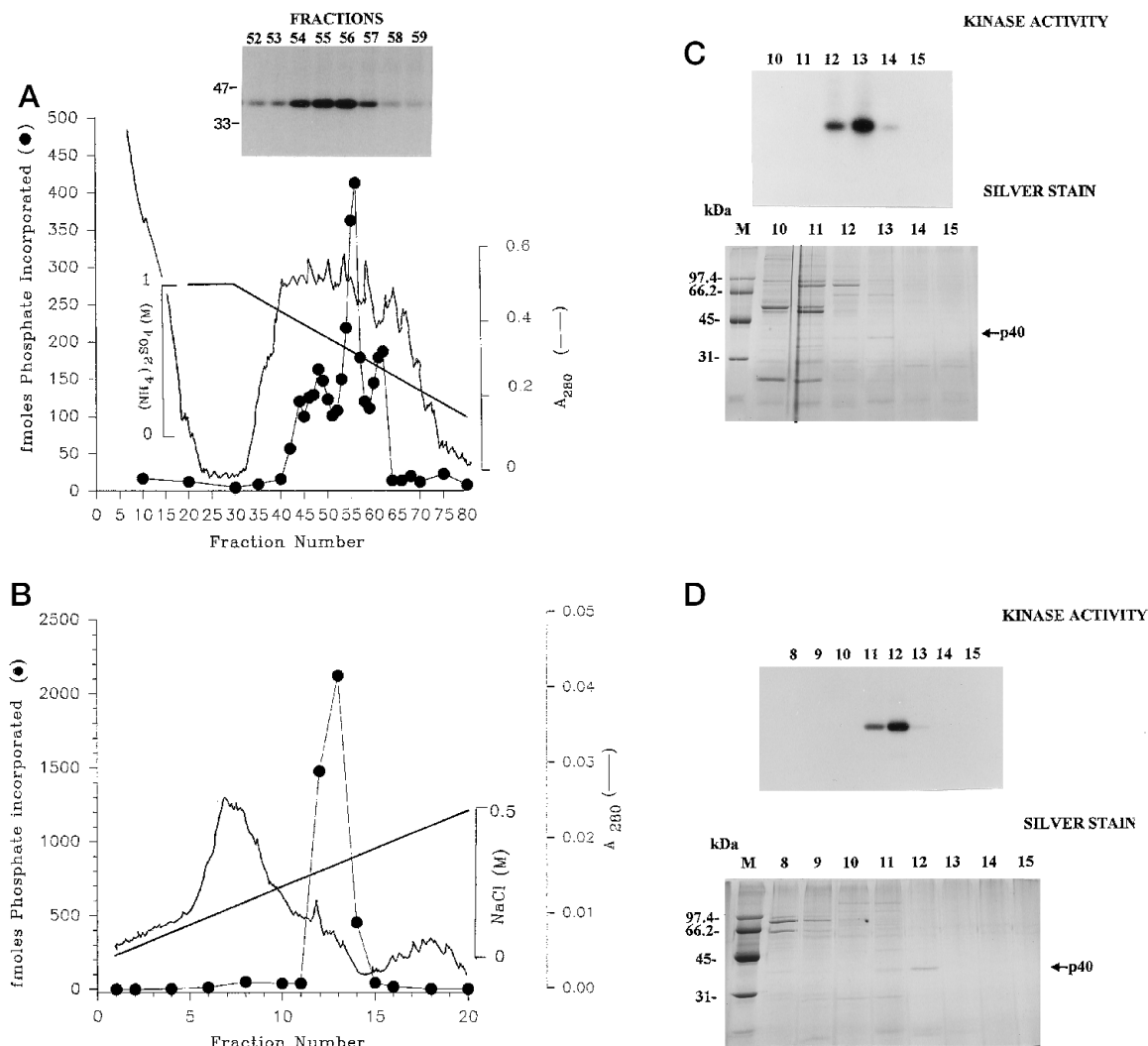


FIG. 3. Purification of MRK from porcine cerebellum. 30–60% ammonium sulfate cut of a high speed supernatant preparation from 15 pig cerebella was dialyzed against TE buffer and applied to a 70-ml butyl-Sepharose column. *A*, the elution profile of kinase activity and proteins from the butyl-Sepharose column. The autoradiograph shows the peak phosphorylation of Ex-m3 fusion protein. The peak fractions were pooled, dialyzed against TE buffer, and passed through a 6-ml Resource S column before being resolved on a Resource S column. *B*, the elution profile of kinase activity and proteins from the Resource S column. *C*, an autoradiograph showing phosphorylation of Ex-m3 by fractions from the Resource S elution against a silver stain of 20- μ l aliquots of each of the fractions. Shown is the position of a 40-kDa polypeptide, the elution of which correlates with the kinase activity. The peak fractions from the Resource S elution were pooled and applied to a 1-ml heparin-Sepharose column. *D*, autoradiograph showing Ex-m3 kinase activity of fractions eluting from the heparin-Sepharose column against a silver stain of 90- μ l trichloroacetic acid-precipitated aliquots of each of the fractions. The position of a 40-kDa polypeptide that co-elutes with the kinase activity is shown. The results shown are from a single purification protocol that was repeated at least four times.

transferase had no effect. Note also, that in addition to blocking m3-muscarinic receptor phosphorylation, the Ex-m3 fusion protein was itself phosphorylated (Fig. 2).

Purification of the Muscarinic Receptor Kinase (MRK) from Porcine Cerebellum—During the course of developing the purification strategy it was found that casein kinase II, which has a ubiquitous distribution, was able to phosphorylate the Ex-m3 fusion protein (see “Discussion”). This complicated initial tissue distribution studies that showed very little Ex-m3 fusion protein phosphorylation by kinases in cytosolic extracts from peripheral tissues (liver, lung, kidney, and heart) but a robust phosphorylation of the Ex-m3 fusion protein by extracts obtained from a number of brain regions (striatum, brain stem, cerebellum, cerebral cortex, and hippocampus). Further studies were conducted where muscarinic receptor kinase activity was resolved from casein kinase II by cationic chromatography over a Resource S column. Following partial purification of high speed supernatant fractions from rat cerebellum and cerebral cortex, the muscarinic receptor kinase activity was found

to be enriched in the cerebellum ~10-fold. It was therefore decided to purify the muscarinic receptor kinase from a cytosolic extract of the porcine cerebellum.

The chromatography steps involved in purification of the muscarinic receptor kinase are illustrated in Fig. 3 and summarized in Table I. Cerebella from 15 pigs (~180 g) were homogenized in 1 liter of TE buffer before centrifugation to prepare a cytosolic S200 fraction (volume, 750 ml; protein, 5.2 g). Following a 30–60% ammonium sulfate cut and dialysis against TE buffer containing 1 M $(\text{NH}_4)_2\text{SO}_4$ the sample (0.94 g in 120 ml) was fractionated on a 70-ml butyl-Sepharose column. A number of minor peaks of kinase activity eluted in the gradient, but the main kinase activity eluted as a single peak at ~0.54 M $(\text{NH}_4)_2\text{SO}_4$ (Fig. 3A).

The sample was then run through a 6-ml Resource Q anion exchange column. Casein kinase II, which co-purifies with the muscarinic receptor kinase activity up to this stage, binds to this column, whereas muscarinic receptor kinase with its alkali pI passes through the column. The flow-through from the Re-

TABLE I
Summary of the purification of MRK from porcine cerebellum

15 porcine cerebella (~180 g) were homogenized in 1 liter of TE buffer. A high speed supernatant was obtained, designated the S200 fraction, and this was considered the start material. The steps in the purification protocol are described under "Experimental Procedures." The results shown are from a representative purification run that was repeated at least four times.

Step	Volume	Protein	Specific activity	Purification	Yield
	ml	mg	pmol phosphate/ μ g/10 min	-fold	%
S200	750	5,200	0.34		100
30–60% ammonium sulphate	120	940	2.28	6.7	121
Butyl-Sepharose	72	83.5	6.81	20	32
Resource S	3	0.3	819.0	2,408	13.8
Heparin-Sepharose	1	0.01	6,340	18,647	3.5

source Q column was applied to a 1-ml Resource S cation exchange column. Ex-m3 phosphorylation activity eluted from this column as a single peak at ~0.32 M NaCl (Fig. 3B). Fig. 3C shows the elution profile of kinase activity and the silver stain of 20- μ l aliquots of each of the fractions. The kinase activity co-elutes with a protein of ~40 kDa. In the final chromatography step, utilizing a 1-ml heparin-Sepharose column, the elution profile of kinase activity again correlated precisely with the elution of the 40-kDa protein (Fig. 3D). Furthermore, silver staining indicated that fraction 12 from the heparin-Sepharose elution, containing the peak of kinase activity, contains only the 40-kDa polypeptide. The kinase was, therefore, considered homogeneous and designated MRK (muscarinic receptor kinase).

Using β -ARK-specific antibodies (a kind gift from Dr. R. J. Lefkowitz, Howard Hughes Medical Institute, Duke University, Durham, NC) it was established that β -ARK co-purified with the muscarinic receptor kinase on the butyl-Sepharose column. β -ARK, reported previously to have an alkali pI (25), bound to the Resource S column and eluted at ~0.175 M salt (data not shown). It was therefore possible to resolve β -ARK from MRK (eluting at ~0.32 M NaCl) on the Resource S column. Furthermore, β -ARK immunoreactivity corresponded to fractions 6, 7, and 8 on the Resource S elution; however, no Ex-m3 phosphorylation activity was observed in these fractions, demonstrating that β -ARK was not able to phosphorylate the Ex-m3 fusion protein (see Fig. 3B).

Muscarinic Receptor Kinase Autophosphorylation—Autophosphorylation is a property common to many protein kinases. An experiment was therefore designed to determine if purified MRK was able to undergo autophosphorylation. Fig. 4 shows that the 40-kDa protein present in the heparin-Sepharose fraction 12 was able to autophosphorylate.

Phosphorylation of m3-Muscarinic Receptors in Membranes from CHO-m3 Cells—As described above, membranes derived from CHO-m3 cells contain an endogenous kinase that is able to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner (24). In order to determine if the kinase purified from porcine cerebellum was able to phosphorylate the m3-muscarinic receptor the purified kinase preparation was added to membranes freshly prepared from CHO-m3 cells. Fig. 5 shows that the addition of agonist, in the absence of exogenous kinase, stimulates m3-muscarinic receptor phosphorylation in CHO-m3 membranes, confirming the presence of an endogenous m3-muscarinic receptor kinase activity. The addition of purified MRK (~2.5 pmol) had little effect on basal levels of m3-muscarinic receptor phosphorylation but increased the agonist-driven phosphorylation by 2–3-fold over that observed in the absence of added kinase (Fig. 5). The increase in agonist-sensitive phosphorylation in the presence of purified MRK was completely reversed by the muscarinic antagonist atropine (Fig. 5).

In control experiments using phosphorylated and nonphosphorylated Ex-m3 (phosphorylated using MRK) the m3-musca-

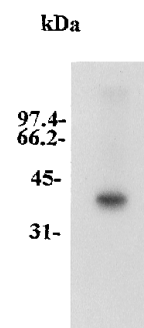


FIG. 4. **Autophosphorylation of the muscarinic receptor kinase.** An aliquot (20 μ l, ~20 ng of kinase) of the heparin-Sepharose-purified kinase preparation (MRK) was incubated in kinase buffer containing 100 μ M [γ - 32 P]ATP (1–4 cpm/fmol of ATP) for 30 min at 37 °C. The reaction was stopped by trichloroacetic acid precipitation of the proteins. The protein pellet was dissolved in SDS-loading buffer and resolved on a 12% SDS-PAGE gel. Indicated are the positions of prestained molecular mass standards. The results are representative of three experiments.

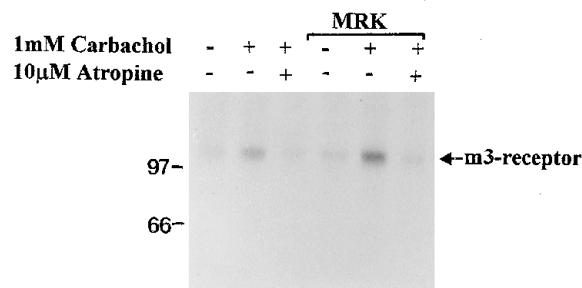


FIG. 5. **Phosphorylation of the m3-muscarinic receptor in CHO-m3 cells.** Membranes from CHO-m3 cells (50 μ g of protein, 0.1 pmol of receptor) made up in kinase buffer containing 100 μ M [γ - 32 P]ATP (1–4 cpm/fmol of ATP) were incubated in the presence or absence of 1 mM carbachol and with or without the antagonist atropine for 10 min at 32 °C. To these preparations either a buffer control or purified brain MRK (10 μ l, ~2.5 pmol) was added. The reaction was stopped by adding solubilization buffer. The receptors were then immunoprecipitated and resolved on an 8% SDS-PAGE gel. The autoradiograph shown is representative of three experiments. Indicated are the positions of prestained molecular weight standards.

rinic receptor antiserum 332 was able to immunoprecipitate both forms of Ex-m3 equally well (data not shown). Thus, despite being raised against the region of the third intracellular loop that contains the phosphoacceptor sites for MRK, the binding of the m3-muscarinic receptor antiserum appears not to be affected by phosphorylation of these sites.

Characterization of the Purified Muscarinic Receptor Ki-

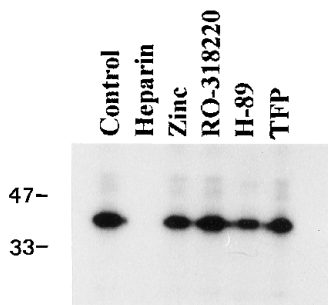


FIG. 6. **Effect of protein kinase inhibitors on muscarinic receptor kinase activity.** The effect of heparin (1 $\mu\text{g}/\text{ml}$), zinc chloride (100 μM), RO-318220 (0.1 μM), H-89 (5 μM), and trifluoperazine (50 μM) on MRK (10 μl of Resource S-purified kinase) phosphorylation of Ex-m3 (3.5 μg) was tested. Indicated are the positions of prestained molecular weight standards. The results are representative of three experiments.

nase—Purified MRK was not affected by the potent protein kinase C inhibitor RO-318220 (0.1 μM ; Ref. 26) nor the calcium/calmodulin inhibitor trifluoperazine (50 μM ; Ref. 27) (Fig. 6). The protein kinase inhibitor H-89 (5 μM ; Ref. 28), which was used at a concentration 10-fold over the IC_{50} for inhibition of cGMP-dependent protein kinase and 100-fold over the IC_{50} for cAMP-dependent protein kinase had only a small inhibitory effect on MRK activity (Fig. 6). Furthermore, zinc chloride at 100 μM , which is a concentration previously shown to inhibit purified β -ARK by >90% (25), had no effect on MRK-mediated phosphorylation of Ex-m3. However, heparin at 1 $\mu\text{g}/\text{ml}$, a concentration reported to inhibit purified β -ARK (29), completely inhibited MRK activity (Fig. 6).

Phosphoamino acid analysis demonstrated that purified MRK phosphorylated Ex-m3 at serine (data not shown).

Kinetic analysis of MRK-mediated phosphorylation of Ex-m3 revealed a V_{max} of 1.15 ± 0.3 nmol phosphate incorporated per min per mg of kinase and a K_m of 0.4 ± 0.11 μM ($n = 4$; Fig. 7).

Comparison of Purified Brain MRK and the Endogenous Muscarinic Receptor Kinase Activity Contained in CHO-m3 Cells—The question of whether the muscarinic receptor kinase purified from porcine cerebellum was the same as that contained in CHO-m3 cells was addressed by designing a range of bacterial fusion proteins that were truncated versions of Ex-m3 (Fig. 8, A and B). The ability of purified porcine MRK and both membrane and cytosolic kinase preparations from CHO-m3 cells to phosphorylate these fusion proteins was tested.

Fig. 8C shows the pattern of phosphorylation of the various fusion proteins by purified brain MRK. Glutathione *S*-transferase was not phosphorylated, whereas Ex-m3 was the best substrate. ΔHind , Ex345–427, and $\Delta\text{H-V}$ were phosphorylated by 23, 44, and 29% the level of Ex-m3, respectively, whereas Ex376–463 was the poorest substrate, showing only 2% of the phosphorylation seen for Ex-m3 (as determined by densitometric analysis).

A muscarinic receptor kinase activity with properties indistinguishable from brain MRK could be isolated from CHO-m3 cell cytoplasm. A high speed supernatant fraction of CHO-m3 cells was passed through a Resource S column from which a kinase able to phosphorylate Ex-m3 was eluted as a single peak at ~ 0.32 M NaCl. This is the same point at which brain MRK elutes from the Resource S column. Testing the partially purified CHO-m3 cell cytosolic kinase preparation against the fusion protein substrates, it was clear that in addition to possessing chromatographic properties equivalent to brain MRK, the CHO-m3 cytosolic kinase also phosphorylated the complement of fusion proteins with the same specificity as that seen for the brain kinase (Fig. 8D). This suggests that the brain MRK and the kinase responsible for Ex-m3 phosphorylation in CHO-m3

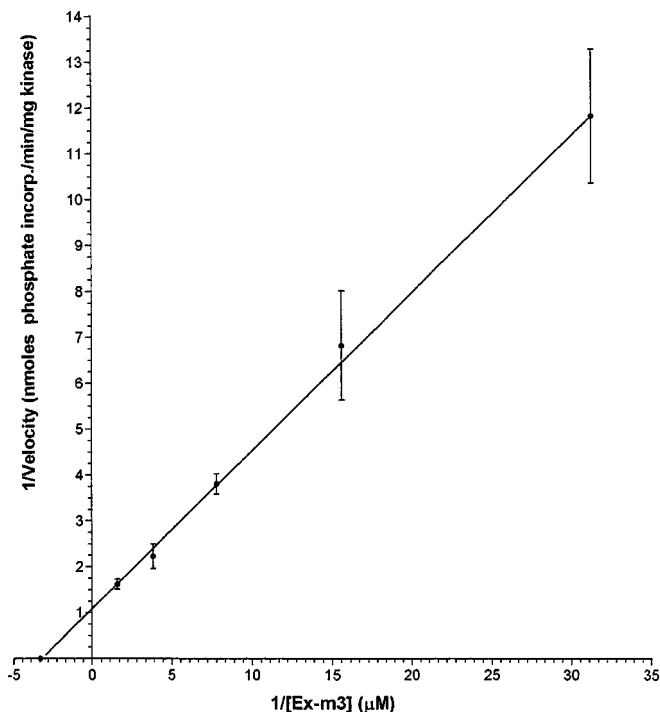


FIG. 7. **Kinetic analysis of MRK phosphorylation of Ex-m3.** Various concentrations of Ex-m3 (0.6–0.03 μM) were incubated with ~ 50 ng of Resource S-purified MRK in kinase buffer containing 50 μM [γ - ^{32}P]ATP (0.4–1.0 cpm/fmol of ATP) for 15 min. The reaction was terminated, and fusion proteins were isolated as described in the text. Ex-m3 fusion protein was then resolved by 12% SDS-PAGE, the gel was stained, and fusion proteins were excised and counted. $V_{\text{max}} = 1.15 \pm 0.3$ nmol of phosphate incorporated per min per mg of kinase, and $K_m = 0.4 \pm 0.11$ μM ($n = 4$). The results shown are the mean \pm S.E. of four experiments.

cell cytoplasm are likely to be homologous or very closely related.

Crude membrane preparations of CHO-m3 cells also possess the ability to phosphorylate the Ex-m3 fusion protein (Fig. 8E). However, the substrate specificity of this kinase was quite different from that seen for the CHO-m3 cytosolic kinase and for the brain MRK. In order to test whether the difference in substrate specificity may be due to the lipid environment, CHO-m3 membranes were washed with 1.5 M KCl for 3 h. This procedure removed agonist-mediated m3-muscarinic receptor kinase activity from the membrane, indicating that the kinase was not an integral membrane protein (data not shown). Following high salt treatment, membranes no longer contain the ability to phosphorylate the Ex-m3 fusion protein. The extracted proteins were resolved on a Resource S column. On elution of this column a single peak of kinase activity was detected at ~ 0.32 M NaCl. This kinase activity showed a similar preference for fusion protein substrates as observed for brain kinase and CHO-m3 cytosolic kinase (Fig. 8F). Therefore, on the basis of substrate specificity and chromatographic behavior it appears that the muscarinic receptor kinase activity extracted from CHO-m3 membranes is similar or identical to that observed in CHO-m3 cell cytosol and to that of brain MRK.

DISCUSSION

The present study describes the purification from porcine cerebellum of a 40-kDa protein kinase that phosphorylates the agonist-occupied form of the phospholipase C-linked m3-muscarinic receptor. Based on its molecular weight, protein kinase inhibitor studies, and chromatographic mobilities, the kinase purified here, termed MRK, was shown to be distinct from second messenger-regulated protein kinases, β -ARK and other

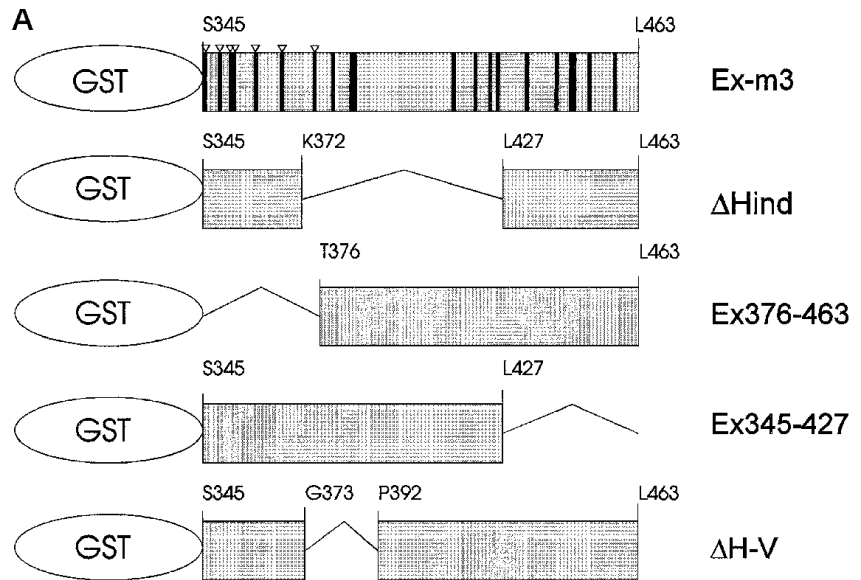
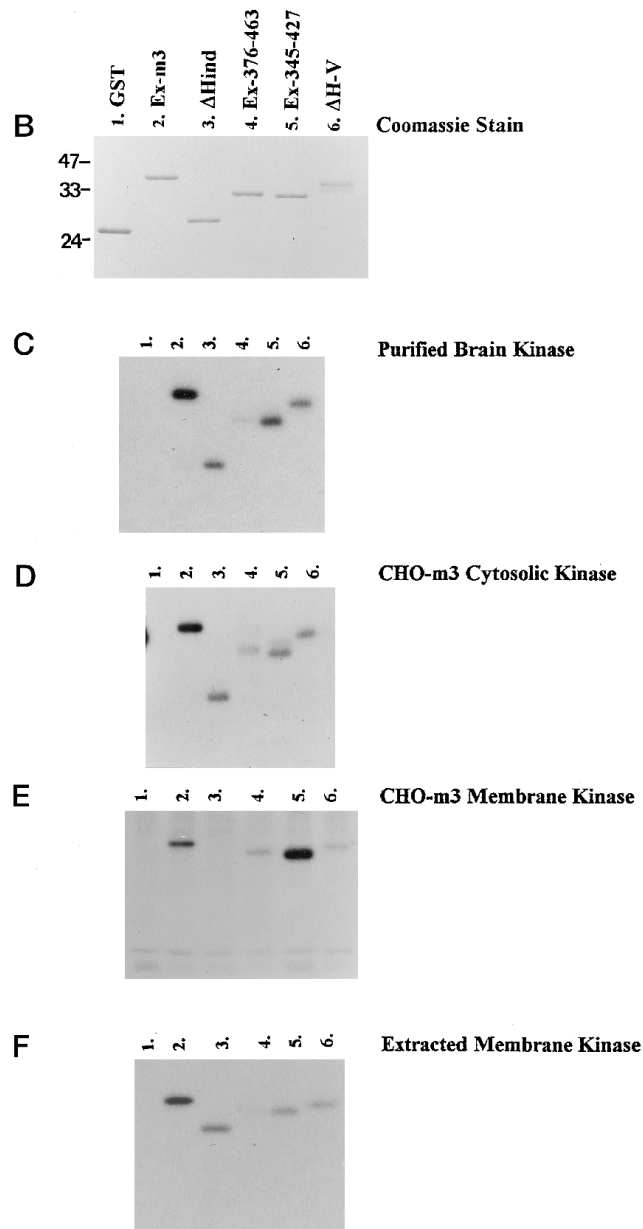


FIG. 8. Phosphorylation of truncated fusion proteins by extracts from CHO-m3 cells and purified porcine brain MRK. *A*, diagrammatic representation of the fusion proteins. The filled bars in Ex-m3 denote the position of serine residues and ∇ denotes the serine residues that are strong candidates for the phosphoacceptor site(s) for MRK (see "Discussion"). *B*, Coomassie Blue stain showing the relative positions of equimolar amounts ($\sim 0.8 \mu\text{M}$) of the fusion proteins on a 12% SDS-PAGE gel. *C*, autoradiograph showing the phosphorylation of the fusion proteins by purified porcine brain MRK. *D*, autoradiograph showing the phosphorylation of the fusion proteins by partially purified CHO-m3 cytosolic kinase. *E*, autoradiograph showing the phosphorylation of the fusion proteins by membranes from CHO-m3 cells. *F*, autoradiograph showing the phosphorylation of the fusion proteins by a partially purified kinase extracted from CHO-m3 membranes by a high salt wash. For each of the above experiments the fusion proteins were visualized on the gel by Coomassie Blue staining to confirm equal loading and to confirm the relative mobilities of the fusion proteins on the gel. Indicated are the positions of prestained molecular weight standards. The results shown are representative of two experiments.



members of the GRK family and therefore represents a new class of receptor kinase.

Purification of MRK was based on an assay in which the bacterial fusion protein (Ex-m3) containing amino acids Ser³⁴⁵–Leu⁴⁶³ of the third intracellular loop of the human m3-muscarinic receptor was phosphorylated by the same kinase as that responsible for m3-muscarinic receptor phosphorylation. Evidence that Ex-m3 acted as a pseudosubstrate for the m3-muscarinic receptor kinase came from studies on CHO-m3 cell membranes. Previously we described agonist-sensitive phosphorylation of m3-muscarinic receptors in membrane preparations from CHO-m3 cells indicating that the muscarinic receptor kinase was, at least in part, associated with the plasma membrane (24). In this study we demonstrate the ability of Ex-m3 to inhibit agonist-mediated phosphorylation of intact m3-muscarinic receptors in membrane preparations. Furthermore, in these experiments the Ex-m3 fusion protein was itself phosphorylated, suggesting that Ex-m3 blocked the action of the m3-muscarinic receptor kinase by acting as a pseudosubstrate.

During the course of this study it was found that Ex-m3 was also a substrate for casein kinase II. Indeed, using the Ex-m3 phosphorylation assay the three subunits of casein kinase II were purified to homogeneity and identified by amino acid sequencing.² Although casein kinase II was able to weakly phosphorylate the m3-muscarinic receptor in membrane preparations, this was not agonist-dependent.² These data clearly suggest that casein kinase II is not involved in agonist-mediated phosphorylation of the m3-muscarinic receptor in intact cells.

Tissue distribution studies revealed muscarinic receptor kinase activity to be rich in cortex and cerebellum, although the activity in cerebellum was ~10-fold greater. It was, therefore, decided to purify this activity from a cytosolic fraction of porcine cerebellum. The elution profile of Ex-m3 phosphorylation activity in the final two column steps in the purification protocol precisely correlated with the elution of a single polypeptide at ~40 kDa. Fraction 12 from the heparin-Sepharose column (the final column step) contained the peak of kinase activity, and the only detectable protein present, as determined by silver stain, was the 40-kDa polypeptide. The kinase preparation was therefore considered to be homogeneous at this stage, and this represented ~18,600-fold purification. The fact that the 40-kDa polypeptide contained kinase activity was established by demonstrating that in common with many protein kinases (*e.g.* Refs. 25 and 30) the 40-kDa protein was able to undergo autophosphorylation.

Reconstitution of the 40-kDa protein kinase with membranes prepared from CHO-m3 cells was used to determine if the purified brain kinase was able to phosphorylate the intact m3-muscarinic receptor. Previous studies from our laboratory have established the presence of an endogenous kinase able to mediate agonist sensitive phosphorylation of m3-muscarinic receptors in membranes from CHO-m3 cells (24). These findings were confirmed in the present study, where, in the absence of exogenous kinase, agonist-sensitive phosphorylation of the m3-muscarinic receptor was observed in membranes prepared from CHO-m3 cells. However, the addition of the purified brain kinase preparation, although not significantly effecting basal phosphorylation, increased agonist-mediated phosphorylation of the m3-muscarinic receptor by 2–3-fold over that seen in the absence of exogenous kinase. This effect was completely reversed by the muscarinic antagonist atropine. These results confirm that the purified 40-kDa protein kinase was an m3-

muscarinic receptor kinase. The kinase was therefore termed MRK (muscarinic receptor kinase).

The ability of the purified brain MRK to drive agonist-dependent phosphorylation of the m3-muscarinic receptor was one of two important criteria we set for establishing that the kinase purified by our method was the kinase responsible for m3-muscarinic receptor phosphorylation in intact cells. The other criterion was to establish that MRK was homologous or closely related to the kinase found in CHO-m3 cells. Since the only system in which m3-muscarinic receptor phosphorylation in intact cells has been demonstrated to date is in CHO-m3 cells, we reasoned that these cells must contain the relevant kinase and therefore would provide an important "positive control" for comparison with a purified kinase. Muscarinic receptor kinase activity could be detected in both membrane and cytosolic fractions from CHO-m3 cells. The kinase present in the cytosol of CHO-m3 cells possessed identical chromatographic properties as the purified brain MRK and showed the same substrate preference for truncated fusion proteins as MRK. However, the kinase associated with the membranes of CHO-m3 cells appeared to have a different substrate specificity than that of MRK. To test whether this was due to the membrane environment of the kinase, proteins were stripped from the plasma membrane using a high salt buffer. Ex-m3 phosphorylation activity contained within this extract could be resolved on a Resource S column and showed identical chromatographic properties and substrate specificity as that of CHO-m3 cytosolic kinase and brain MRK. It seems likely, therefore, that purified brain MRK and the muscarinic receptor kinase activity extracted from CHO-m3 membranes and present in CHO-m3 cell cytosol are either homologous or very closely related.

Recent studies have indicated that in addition to phosphorylating adenylate cyclase-linked receptors (*e.g.* Ref. 31) β -ARK is also able to phosphorylate the agonist-occupied forms of a number of phospholipase C-linked receptors. For example, heparin inhibition studies have suggested that phospholipase C-linked CCK receptors are phosphorylated, at least in part, by a " β -ARK-like" kinase in pancreatic acinar cells (19). Co-expression of thrombin receptors with β -ARK-2 in *Xenopus* oocytes blocks thrombin-mediated inositol phosphate/calcium signaling (15). Furthermore, in reconstituted systems purified β -ARK has been demonstrated to phosphorylate the partially purified substance P receptor (18) and recombinant m3-muscarinic receptors contained in urea-treated SF9 cell membranes (20). There seems little doubt, therefore, that β -ARK has a relatively broad substrate specificity and has the potential to phosphorylate a number of phospholipase C-linked receptors including m3-muscarinic receptors.

The kinase identified in this study, however, is clearly distinct from β -ARK and offers an alternative mechanism for agonist-mediated m3-muscarinic receptor phosphorylation. First, the molecular mass of MRK (~40 kDa) is significantly less than that of β -ARK 1 and 2 (~80 kDa) and also less than the molecular masses of other members of the GRK family that fall within the range of 53–67.7 kDa (see Ref. 32). Furthermore, immunoblot studies using an anti- β -ARK antiserum revealed that β -ARK was resolved from MRK at the Resource S ion exchange chromatography step. These data also demonstrated that β -ARK is unable to phosphorylate the Ex-m3 fusion protein.

Protein kinase inhibitor studies further distinguished MRK from β -ARK and previously characterized second messenger-regulated protein kinases. The protein kinase inhibitors RO-318220 (protein kinase C; Ref. 26), trifluoperazine (Ca²⁺/calmodulin-dependent protein kinase; Ref. 27), and H-89 (cGMP-

² A. B. Tobin and S. R. Nahorski, unpublished observation.

dependent protein kinase and cAMP-dependent protein kinase; Ref. 28) at concentrations at least 10-fold above their reported IC_{50} values had little effect on MRK activity. Furthermore, zinc at a concentration reported to inhibit purified β -ARK activity by >90% (25) had no inhibitory effect on MRK activity. In contrast, heparin at 1 μ g/ml completely blocked MRK activity. Heparin has been reported to be a relatively potent but nonselective protein kinase inhibitor acting on a number of kinases including members of the GRK family: β -ARK (29), GRK5, and GRK6 (23), in addition to casein kinase II (33) and low density lipoprotein receptor kinase (34). The finding that heparin is also a potent inhibitor of MRK raises a question about the suitability of this reagent in studies aimed at characterization of protein kinases responsible for G-protein-linked receptor phosphorylation (e.g. Ref. 19).

Interestingly, analysis of the kinetic parameters of MRK phosphorylation of Ex-m3 demonstrate that Ex-m3 is a good substrate for MRK with a K_m of 0.4 μ M and V_{max} of 1.15 nmol of phosphate incorporated/min/mg. The K_m for MRK phosphorylation of Ex-m3 is similar to that of β -ARK phosphorylation of the purified β -adrenoreceptor ($K_m = 0.25 \mu$ M; Ref. 25) and suggests that the K_m that MRK shows for Ex-m3 may be approaching that for the intact m3-muscarinic receptor. Unfortunately, purified m3-muscarinic receptors are not presently available to test the kinetic properties of MRK against the intact receptor substrate.

The mechanism of activation of MRK is at present unclear. The presence of MRK in the cytoplasmic and membrane fractions of CHO-m3 cells suggests that a translocation process where MRK migrates to a membrane site on agonist stimulation in a manner similar to that described for β -ARK (35) may be in operation. Initial studies have demonstrated that there is an increase in MRK activity in the membrane fraction following agonist stimulation;² however, without antibodies to MRK it is difficult to discern between translocation of the kinase and an increase in the kinase activity. Dose response curves constructed for m3-muscarinic receptor phosphorylation in intact CHO-m3 cells have, however, suggested that the mechanism of m3-muscarinic receptor phosphorylation is dependent on a small amplification step downstream of receptor activation (36). Experiments are currently in progress to further elucidate this amplification process and to investigate the role translocation may play.

It is interesting to note that the cerebellum is enriched in MRK since this tissue is known to contain only a small population of m3-muscarinic receptors (for review see Ref. 37). This suggests that MRK may have a broader substrate specificity than just the m3-muscarinic receptor. In support of this assertion are recent studies from our laboratory showing that the m1-muscarinic receptor is phosphorylated in an agonist-dependent manner by a kinase with similar properties to MRK (38). Further studies using a range of recombinant G-protein-linked receptors are presently underway to investigate the substrate specificity of MRK. Particular attention is being directed toward the metabotropic glutamate 1 α receptor, which is abundantly expressed in Purkinje cells of the cerebellum and has recently been reported to undergo agonist-driven phosphorylation in transfected BHK cells, although in this system a role for protein kinase C has been implicated (39).

The use of truncated fusion proteins of Ex-m3 suggested that at least one site of MRK phosphorylation is contained in the region of the third intracellular loop of the m3-muscarinic receptor between Ser³⁴⁵ and Thr³⁷⁶, since truncation of this region in Ex-m3 resulted in a fusion protein (Ex376–463) that was very poorly phosphorylated. This region contains seven potential serine phosphoacceptor sites, denoted with ∇ in Fig.

8A, which include the SASS motif identified recently as being important for internalization of the m3-muscarinic receptor (40). The possible involvement of phosphorylation in internalization of the receptor would conform with the generalized view that G-protein-linked receptor phosphorylation is associated with a diminution of receptor responsiveness. Agonist-sensitive phosphorylation of the phospholipase C-linked α_{1B} -adrenergic (13), thrombin (15, 16), platelet-activating factor (14), and neurokinin-2 receptors (17) are all associated with desensitization of receptor responses. In the case of the m3-muscarinic receptor, agonist-induced inositol phosphate responses undergo partial desensitization within seconds of agonist addition (4, 9). The rapid onset of this desensitization event correlates with the rapid time course of m3-muscarinic receptor phosphorylation (12), suggesting that the two processes may be linked (8). We are presently in the process of determining the exact MRK phosphorylation sites on the m3-muscarinic receptor with a view to making point mutations that will render the receptor unable to be phosphorylated. Such a receptor will be an invaluable tool in dissecting the role this novel receptor kinase plays in regulation of transmembrane signaling.

Acknowledgments—We thank Dr. G. B. Willars and Dr. J. L. Blank for many fruitful discussions.

REFERENCES

- Berridge, M. J. (1993) *Nature* **361**, 315–325
- Lambert, D. G., and Nahorski, S. R. (1990) *Biochem. J.* **265**, 555–562
- Tobin, A. B., Lambert, D. G., and Nahorski, S. R. (1992) *Mol. Pharmacol.* **42**, 1042–1048
- Fisher, S. K., Slowiejko, D. M., and McEwen, E. L. (1994) *Neurochem. Res.* **19**, 549–554
- Morgan, R. O., Chang, J. P., and Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1166–1171
- Balla, T., Baukal, A. J., Guillemette, G., and Catt, K. J. (1988) *J. Biol. Chem.* **263**, 4083–4091
- Menniti, F. S., Takemura H., Oliver, K. G., and Putney, J. W. (1991) *Mol. Pharmacol.* **40**, 727–733
- Wojcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1993) *Trends Pharmacol. Sci.* **14**, 279–285
- Wojcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1994) *J. Neurochem.* **63**, 177–185
- Lohse, M. J. (1993) *Biochim. Biophys. Acta* **1179**, 171–188
- Cluettelberg, U. G., Gates, L. K., Gorelick, F. S., and Miller L. J. (1991) *J. Biol. Chem.* **266**, 2403–2408
- Tobin, A. B., and Nahorski, S. R. (1993) *J. Biol. Chem.* **268**, 9817–9823
- Lattion, A.-L., Diviani, D., and Cotecchia, S. (1994) *J. Biol. Chem.* **269**, 22887–22893
- Ali, H., Richardson, R. M., Tomhave, E. D., DuBose, R. A., Haribabau, B., and Synderman, R. (1994) *J. Biol. Chem.* **269**, 24557–24563
- Ishii, K., Chen, J., Ishii, M., Koch, W. J., Freedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (1994) *J. Biol. Chem.* **269**, 1125–1130
- Vouret-Craviari, V., Grall, D., Chambard, J.-C., Rasmussen, U. B., Pouyssegur, J., and van Obberghen-Schilling, E. (1995) *J. Biol. Chem.* **270**, 8367–8372
- Alblas, J., van Etten, I., Khanum, A., and Moolenaar, W. H. (1995) *J. Biol. Chem.* **270**, 8944–8951
- Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krauss, J. E., Caron, M. G., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 91961–9164
- Gates, L. K., Ulrich, C. D., and Miller, L. (1993) *Am. J. Physiol.* **264**, G840–G847
- Debburman, S. K., Kunapuli, P., Benovic, J. L., and Hosey, M. (1995) *Mol. Pharmacol.* **47**, 224–233
- Lefkowitz, R. J. (1993) *Cell* **74**, 409–412
- Kunapuli, P., and Benovic, J. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5588–5592
- Loudon, R. P., and Benovic, J. L. (1994) *J. Biol. Chem.* **269**, 22691–22697
- Tobin, A. B., Keys, B., and Nahorski, S. R. (1993) *FEBS Letts.* **335**, 353–357
- Benovic, J. L., Mayor, F., Jr., Staniszewski, C., Lefkowitz, R. J., and Caron, M. G. (1987) *J. Biol. Chem.* **262**, 9026–9032
- Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D., and Wilkinson, S. E. (1989) *FEBS Letts.* **259**, 61–63
- Hoffmann, E. K., Simonsen, L. O., and Lambert, I. H. (1984) *Membr. Biochem.* **78**, 211–222
- Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) *J. Biol. Chem.* **265**, 5267–5272
- Benovic, J. L., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264**, 6707–6710
- Kunapuli, P., Gurevich, V. V., and Benovic, J. L. (1994) *J. Biol. Chem.* **269**, 10209–10212
- Richardson, R. M., Kim, C., Benovic, J. L., and Hosey, M. M. (1993) *J. Biol.*

- Chem.* **268**, 13650–13656
32. Inglese, J., Freedman, N. J., Koch, W. J., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 23737–23738
33. Hathaway, G. M., Lubben, T. H., and Traugh, J. A. (1980) *J. Biol. Chem.* **255**, 8038–8041
34. Kishimoto, A., Brown, M. S., Slaughter, C. A., and Goldstein, J. L. (1987) *J. Biol. Chem.* **262**, 1344–1351
35. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. K., Casey, P. L., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) *Science* **257**, 1264–1267
36. Tobin, A. B., Willars, G. B., Burford, N. T., and Nahorski, S. R. (1995) *Br. J. Pharmacol.* **116**, 1723–1728
37. Caulfield, M. P. (1993) *Pharmacol. & Ther.* **58**, 319–379
38. Waugh, M. G., Burford, N. T., Nahorski, S. R., and Tobin, A. B. (1995) *Br. J. Pharmacol.* **114**, 143P
39. Alaluf, S., Mulvihill, E. R., and McIlhinney, R. A. J. (1995) *FEBS Letts.* **367**, 301–305
40. Moro, O., Lameh, J., and Sadee, W. (1993) *J. Biol. Chem.* **268**, 6862–6865