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

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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 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 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) and diacylglycerol, which are responsible for the activation 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 Gβγ 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/Gq/11-coupled CCK (11), m3-muscarinic (12), αβ-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 isotypes of β-ARK (β-ARK-1 and -2) are members of a family of protein kinases that includes rhodopsin kinase, IRK-1, 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.

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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-isooquinoline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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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, including into pGEX-2T via an ApaI (5’)-HindIII (3’), PCR reaction product 1 was digested with pGEX-2T in a three-way ligation where pGEX-2T was digested with BamHI (5’) and EcoRI (3’), PCR reaction product 1 was digested with BamHI (5’) and Apal (3’), and PCR reaction product 2 was digested with pGEX-2T and EcoRI (3’). PCR primers used were (for product 1) 5’ primer CCCGGATCCCTGGAGAATCTGGCC and (for product 2) 5’ primer CAGGGGGCCCGAGGAGGAGCTGGGAGGCC and 3’ primer CCCGATCCCTGGAGAATCTGGCC and 3’ primer GCTGG-CCCGCGAGCTGGAGACAC and (for product 2) 5’ primer CAGGGGGCCCGAGGAGGAGCTGGGAGGCC and 3’ primer CCCGATCCCTGGAGAATCTGGCC and 3’ primer GCTGG-
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-cm²) 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 × g). Membrane and cytosolic fractions were then prepared by centrifugation at 15,000 × 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 centrifuged at 15,000 × 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.

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 MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, 100 μM [γ-32P] ATP (1–4 cpm/μmol of ATP) ≤ 1 μM carbachol and ≥ 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 then stopped by centrifugation at 13,000 × 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 antisera (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 Auto-phosphorylation—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 MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, 130 mM NaCl, 100 μM [γ-32P] ATP (1–4 cpm/μmol 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 × 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 (Ser⁴⁴⁵-Lys⁴⁶³) acted as a substrate for a kinase in cytosolic extracts from CHO-m3 cells (Fig. 1). There was no phosphorylation 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 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-
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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ (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 pl passes through the column. The flow-through from the Resource Q column was then fractionated on a 1-ml heparin-Sepharose column. As shown in Fig. 3D, an autoradiograph showing Ex-m3 kinase activity of fractions eluting from the heparin-Sepharose column against a silver stain of 90-μl 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.

**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 Q 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-μl 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, an autoradiograph showing Ex-m3 kinase activity of fractions eluting from the heparin-Sepharose column against a silver stain of 90-μl 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.
Muscarinic Receptor Kinase

TABLE I
Summary of the purification of MRK from porcine cerebellum

| Step                  | Volume | Protein | Specific activity | Purification | Yield |
|-----------------------|--------|---------|-------------------|--------------|-------|
|                       | ml     | mg      | pmol phosphate/µg 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              | 32           |       |
| Resource S            | 3      | 0.3     | 819.0             | 2,408        |       |
| Heparin-Sepharose     | 1      | 0.01    | 6,340             | 18,647       | 13.8  |

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.

Fig. 4. Autophosphorylation of the muscarinic receptor kinase. An aliquot (20 µl, ~20 ng of kinase) of the heparin-Sepharose-purified kinase preparation (MRK) was incubated in kinase buffer containing 100 µM [γ-32P]ATP (1–4 cpm/mmol 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 (150 µg of protein–1, 0.1 pmol of receptor) made up in kinase buffer containing 100 µM [γ-32P]ATP (1–4 cpm/mmol 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 µl, ~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.

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-µl 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 NaCl (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-muscarinic receptor antisemur 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 antisemur appears not to be affected by phosphorylation of these sites.

Characterization of the Purified Muscarinic Receptor Ki-
nase—Purified MRK was not affected by the potent protein kinase inhibitor 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.

**FIG. 6.** Effect of protein kinase inhibitors on muscarinic receptor kinase activity. The effect of heparin (1 μg/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.

**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-32P]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_{max} = 1.15 ± 0.3 nmol of phosphate incorporated per min per mg of kinase, and K_{m} = 0.4 ± 0.11 μM (n = 4). The results shown are the mean ± S.E. of four experiments.
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 \( \checkmark \) denotes the serine residues that are strong candidates for the phosphoacceptor sites for MRK (see "Discussion"). B, Coomassie Blue stain showing the relative positions of equimolar amounts (~0.8 \( \mu \)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.
Purification of MRK was based on an assay in which the bacterial fusion protein (Ex-m3) containing amino acids Ser^{345}-Leu^{463} 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. Although casein kinase II was able to weakly phosphorylate the m3-muscarinic receptor in membrane preparations, this was not agonist-dependent. 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 ∼18,000-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 affecting 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 adenylyl 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 (Ca^{2+}/calmodulin-dependent protein kinase; Ref. 27), and H-89 (cGMP-
dependent protein kinase and CAMP-dependent protein kinase; Ref. 28) at concentrations at least 10-fold above their reported IC\textsubscript{50} values had little effect on MRK activity. Furthermore, zinc at a concentration reported to inhibit purified \(\beta\)-ARK activity by \(\geq 90\%\) (25) had no inhibitory effect on MRK activity. In contrast, heparin at 1 \(\mu\)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: \(\beta\)-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 (eg. 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 \(\mu\)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 \(\beta\)-ARK phosphorylation of the purified \(\alpha\)-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 \(\beta\)-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; however, without antibodies to MRK it is difficult to discern between translocation of the kinase and an increase in the kinase activity. Dose responses 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 m3-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 1x 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 Ser\textsuperscript{345} and Thr\textsuperscript{376}, since truncation of this region in Ex-m3 resulted in a fusion protein (Ex\textsuperscript{376-463}) that was very poorly phosphorylated. This region contains seven potential serine phosphoacceptor sites, denoted with \(\nabla\) 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 phosphoprotein C-linked \(\alpha\)-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.

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