The Role of Phosphodiesterase in Mediating the Effect of Protein Kinase C on Cyclic AMP Accumulation upon k-Opioid Receptor Stimulation in the Rat Heart

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ABSTRACT
This study determined whether phosphodiesterase (PDE) was activated by protein kinase C (PKC) upon k-receptor stimulation, and if so, to identify the isozyme. We first studied the effects of trans-(+)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide methanesulphonate (U50,488H), a selective k-opioid receptor (OR) agonist, and phorbol-12–myristate-13-acetate (PMA), a PKC activator, on cAMP accumulation and PDE activity in rat ventricular myocytes when PKC and PDE were inhibited by respective inhibitors. Like PMA, U50,488H decreased forskolin-stimulated cAMP accumulation and PDE activity by U50,488H and PMA. The observations suggest that PKC may enhance cAMP degradation through activating PDE upon k-OR stimulation. To identify the isozyme(s) mediating the effect of PKC upon k-OR stimulation, selective inhibitors were used. We found that 10^-6 M Ro-20–1724, a selective cAMP-specific PDE (PDE-IV) inhibitor, abolished the inhibitory effects of U50,488H and PMA, whereas 8-methoxymethyl-3-isobutyl-1-methylxanthine, erythro-9-(2-hydroxy-3-nonyl) adenine, cilostamide, and zaprinast, selective inhibitors of Ca2+/calmodulin-dependent PDE (PDE-I), cGMP-stimulated PDE (PDE-II), cGMP-inhibited PDE (PDE-III), and cGMP-specific PDE (PDE-V), respectively, had no effect. Moreover, rolipram, another selective PDE-IV inhibitor, also dose-dependently attenuated the inhibition on forskolin-stimulated cAMP accumulation and stimulation on PDE activity by U50,488H and PMA. In conclusion, this study has provided evidence for the first time that PKC and PDE-IV mediate the action of k-OR.

Previous studies in our (Bian et al., 1998) and other (Ventura et al., 1991) laboratories have shown that effects of k-opioid receptor (OR) stimulation in the heart are mediated by protein kinase C (PKC). We have found that staurosporine, a nonspecific PKC inhibitor, dose-dependently attenuates the inhibitory effect of both trans-(+)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide methanesulphonate (U50,488H), a selective k-OR agonist, and phorbol-12–myristate-13-acetate (PMA), a selective PKC activator, on forskolin-stimulated accumulation of cAMP, suggesting that PKC mediates the action of k-OR stimulation. The mechanisms are, however, not fully understood. Previous studies have shown that PKC either stimulates (Bode and Brunton, 1988; Michael and Webley, 1991; Tettsuka et al., 1995) or inhibits (Le Goas et al., 1991; Yingling et al., 1994) phosphodiesterase (PDE) in different tissues. In the heart, Schluter et al. (1995) reported that the PKC, activated by parathyroid hormone, stimulates PDE. It is likely that PDE may mediate the action of PKC on cAMP accumulation upon k-OR stimulation in the heart.

More than 20 isoenzymes have been identified and are classified into five subfamilies (Beavo and Reifsnyder, 1990). They are differentially distributed. Growing evidence has shown that cAMP-specific PDE (PDE-IV) is one of the most important PDE isoenzymes in regulating cAMP breakdown in the ventricular tissue (Prigent et al., 1984, Dubois et al., 1990). However, PKC also has been shown to enhance the Ca2+/calmodulin-dependent PDE (PDE-I) activity in the hypertrophic cardiomyopathic hamster heart (Yu et al., 1996). ABBREVIATIONS: OR, opioid receptor; PKC, protein kinase C; U50,488H, trans-(+)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide methanesulphonate; PMA, phorbol-12-myristate-13-acetate; PDE, phosphodiesterase; PDE-IV, cAMP-specific PDE; PDE-I, Ca2+/calmodulin-dependent PDE; PDE-II, cGMP-stimulated PDE; PDE-III, cGMP-inhibited PDE; PDE-V, cGMP-specific PDE; BSM, bisindolylmaleimide I; MIBMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; DMSO, dimethyl sulfoxide; nor-BNI, nor-binaltorphimine; AC, adenylyl cyclase.
and cGMP-stimulated phosphodiesterase (PDE-II) in a liver Golgi-endosomal fraction (Geoffroy et al., 1999).

The purpose of this study was therefore twofold. First, we determined the role of PDE in mediating the action of PKC on cAMP accumulation upon κ-OR stimulation. Second, we screened and identified the isozyme(s) of PDE involved. We determined the forskolin-stimulated cAMP accumulation and the PDE activity in ventricular myocytes upon κ-OR stimulation when PKC or PDE was inhibited with respective inhibitors. For inhibition of PDE, we used a nonselective PDE inhibitor as well as selective PDE-I, II, III, IV, and V inhibitors. The results indicate that PDE-IV mediates the action of PKC on cAMP accumulation upon κ-OR stimulation.

Materials and Methods

Isolation of Ventricular Myocytes. Ventricular myocytes were isolated from the heart of male Sprague-Dawley rats (190–210 g), with a collagenase perfusion method described previously (Dong et al., 1993). Immediately after decapitation, the hearts were rapidly removed from the pericardium and mounted to the Langendorff apparatus for perfusion. Perfusion was performed in a retrograde manner at a constant flow rate (10 ml/min) with oxygenated Joklik modified Eagle’s medium supplemented with 1.25 mM CaCl2 and 10 mM HEPES, pH 7.2, at 37°C for 5 min. This was followed by 5 min with the same medium free of Ca2+. Collagenase was then added to the medium to a concentration of 125 U/ml with 0.1% (w/v) BSA. After 35 to 45 min of perfusion with a medium containing collagenase, the atria were discarded. The ventricular tissue was shaken in the same oxygenated collagenase solution for 5 min at 37°C and cut into small pieces with a pair of scissors after stirring with a glass rod for 5 min.

The procedure separated the ventricular myocytes from each other. The residue was filtered through 250-μm mesh screens, sedimented by centrifugation at 100g for 1 min and resuspended in fresh Joklik solution with 2% BSA. More than 70% of the cells were rod-shaped and impermeable to trypan blue. Ca2+ concentration of the Joklik solution was increased gradually to 1.25 mM in 30 min.

Assay of cAMP. Samples containing 3 × 106 to 6 × 106 freshly isolated ventricular myocytes after Ca2+ loading were incubated in an atmosphere of 5% CO2/95% air for 2 h. One hour and 45 min after treatment with this dose range U50,488H increases cytosolic Ca2+ concentration of the Joklik solution was increased gradually to 1.25 mM in 30 min.

PDE activity was measured at a substrate concentration of 0.25 μM cAMP according to methods of Boudreau and Drummond (1975) and Thompson and Appleman (1971). The assay consists of a two-step isotopic procedure. [3H]cAMP is hydrolyzed to [3H]5'-AMP by phosphodiesterase and then further hydrolyzed to [3H]adenosine by the nucleotidase. The reaction was initiated by adding 50 μl of the crude enzyme to 200 μl of reaction mixture containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 0.25 μM cAMP, and [3H]cAMP. The reaction was carried out at 30°C for 10 min and terminated by placing the tubes in a dry ice-ethanol bath. The tubes were then placed in a boiling water bath for 3 min following by chilling in ice. Following reequilibration to 30°C, 50 μl of a 1.0-nmol/ml solution of 5'-nucleotidase in H2O was added. After a 20-min incubation, the reaction was terminated by the addition of 1.0 ml of the resin slurry (3 H2O:1 resin). The resin binds all charged nucleotides and leaves [3H]adenosine as the only labeled compound to be counted. The tubes were allowed to stand at least 10 min, and centrifuged at 100g for 10 min. Then 0.5 ml was removed for scintillation counting.

Drugs and Chemicals. U50,488H, PMA, type I collagenase, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO); EHNA from Research Biochemicals International (Natick, MA); and chelerythrine chloride, BSM, IBMX, MIBMX, cilostamide, Ro-20–1724, rolipram, and zaprinast were from Research Biochemicals International (Natick, MA); and chelerythrine chloride, BSM, IBMX, MIBMX, EHNA, cilostamide, Ro-20–1724, rolipram, zaprinast, and forskolin were dissolved in dimethyl sulfoxide (DMSO) and the rest in distilled water. The final concentration of DMSO was 0.1%, at which DMSO had no effect on cAMP level and PDE activity.

The doses of U50,488H used were 10–6 to 4 × 10–5 M based on previous studies in our (Sheng et al., 1997; Bian et al., 1998; Zhang and Wong, 1998) and other laboratories (Ventura et al., 1991). At this dose range U50,488H increases cytosolic Ca2+ and decreases forskolin-induced cAMP accumulation, and the effects are blocked by nor-binaltorphimine (nor-BNI), indicating the actions are κ-opioid receptor-mediated. The dose of PMA used also was based on previous studies (Ventura et al., 1991; Bian et al., 1998). The concentration of the two PKC antagonists chelerythrine (Kandasamy et al., 1995) and BSM (Chulak et al., 1995; Zhu et al., 1997; Berts et al., 1999) used in this study was 1 μM based on previous studies. The concentrations of PDE antagonists MIBMX (Shimizu et al., 1999), EHNA (Mery et al., 1995), cilostamide (Schwartz et al. 1993), Ro-20–1724 (Hichami et al., 1996), and zaprinast (Liu et al., 1992) used were 10–5, 10–6, 10–5, 10–5, 10–5, and 10–6 M, respectively, which also were chosen according to previous studies. The exception of BSM, which has been shown to produce selective actions at the dose range of 10–5 to 5 × 10–5 M (Toulec et al., 1991; Weissmann et al., 1999), the concentrations of PKC and PDE inhibitors used were slightly higher than the corre-
The most important observations in this study are 1) \(\kappa\)-OR stimulation and activation of PKC decrease forskolin-induced cAMP accumulation and stimulate PDE activity, and the effects are antagonized by inhibition of PKC or PDE; and 2) inhibition of PDE-IV with its selective inhibitors Ro-20–1724, a specific PDE-IV inhibitor, was used. Rolipram at 10\(^{-5}\) M did not further enhance the forskolin-induced cAMP accumulation, U50,488H and PMA were significantly attenuated by 10\(^{-6}\) M chelerythrine and 10\(^{-6}\) M BSM, which themselves had no effect on cAMP (Fig. 1). This is in agreement with our previous study that showed that the inhibitory effect of U50,488H and PMA were dose-dependently attenuated by the nonspecific PKC inhibitor staurosporine (Bian et al., 1998).

Effects of U50,488H and PMA on Forskolin-Induced cAMP Accumulation and PDE Activity in Presence of PDE Inhibitor IBMX. To determine whether PKC acted at PDE upon \(\kappa\)-OR stimulation, the effects of U50,488H and PMA on the forskolin-induced accumulation of cAMP were studied in the presence of IBMX, a nonspecific PDE inhibitor. IBMX at 10\(^{-6}\) M did not further increase the forskolin-induced increase in cAMP accumulation (Fig. 2), indicating that at this concentration it nearly has no effect on PDE. Nor did 10\(^{-6}\) M IBMX alter the effect of either U50,488H or PMA on cAMP (Fig. 2). At 10\(^{-5}\) to \(\sim\)10\(^{-3}\) M, IBMX dose-dependently enhanced forskolin-induced cAMP accumulation, indicating that at this dose range IBMX is effective in inhibiting the action of PDE. At this dose range, IBMX abolished completely the inhibitory effect of 2 \times 10^{-5} M U50,488H and 10\(^{-6}\) M PMA (Fig. 2).

In agreement with the inhibitory effects of U50,488H and PMA on forskolin-stimulated cAMP accumulation, U50,488H at 1 \times 10^{-5} to \sim 4 \times 10^{-5} M (Fig. 3A) and PMA at 10^{-8} to \sim 10^{-6} M (Fig. 3B) dose-dependently stimulated PDE activity, and these effects were abolished by 10\(^{-6}\) M chelerythrine and 10\(^{-3}\) M IBMX (Fig. 3).
1724 and rolipram significantly attenuates the effects of κ-OR stimulation and activation of PKC on cAMP and PDE activities. These observations demonstrate for the first time that PDE-IV mediates the action of PKC on cAMP modulation upon κ-OR stimulation in the rat heart. Our previous study has shown that a nonspecific PKC inhibitor staurosporine significantly attenuates the suppression of cAMP by κ-OR stimulation (Bian et al., 1998). Because staurosporine has been reported to inhibit not only PKC but also tyrosine kinase, cAMP-dependent protein kinase, and Ca²⁺/calmodulin-dependent protein kinase II (Nakano et al., 1987; Yanagihara et al., 1991), the role of PKC in mediating the action of κ-receptor stimulation on cAMP accumulation needs confirmation. In this study, we found that two specific PKC inhibitors, 10⁻⁶ M chelerythrine and BSM, significantly attenuated the inhibitory effects of U50,488H and PMA on forskolin-stimulated cAMP accumulation. Chelerythrine (Kandasamy et al., 1995) and BSM (Toullec et al., 1991; Weissmann et al., 1999) at this concentration have been shown to selectively inhibit PKC activity. The result confirms our previous finding that the inhibitory effect of κ-OR stimulation on cAMP accumulation results at least partly from activation of PKC (Bian et al., 1998).

In this study, we found that the effects of U50,488H and PMA on cAMP accumulation and PDE activity were completely abolished by IBMX. These results indicate that PKC activates PDE upon κ-OR stimulation in the heart. Similar observations have been reported when the heart was stimulated by the parathyroid hormone (Schluter et al., 1995) and an α-adrenergic receptor agonist (Buxton and Brunton, 1985). In the neuroblastoma × glioma NG108–15 cell, D-la₂, D-Leu⁵-enkephalin, a δ-OR agonist, also inhibits cAMP accumulation via activation of PDE (Law and Loh, 1993). The isomer of PDE involved is PDE-IV because we found that the effects of U50,488H and PMA were significantly attenuated by Ro-20–1724 and rolipram, two specific PDE-IV inhibitors, but not by MIBMX, EHNA, cilostamide, and zaprinast, selective inhibitors of PDE-I, PDE-II inhibitor, PDE-III, and PDE-V, respectively. The results indicate clearly that PDE-IV is the isoenzyme that mediates the actions of PKC upon κ-OR stimulation in the heart. This is similar to the situation in renal collecting tubule (Tetsuka et al., 1995).

In this study, we found that IBMX at 10⁻⁵ to 10⁻³ M, but
PKC/PDE pathway also mediates the inhibitory action of inhibition of cAMP in the heart (Zhang and Wong, 1998) at 10⁻⁶ M, further increased the forskolin-induced cAMP accumulation, indicating that only 10⁻⁵ to 10⁻³ M IBMX significantly inhibited the effect of PDE. This is in agreement with the previous observations of Turner et al. (1993). Shahid and Rodger (1989) also found that IBMX at this concentration inhibits cAMP hydrolyzing in the rabbit heart. Similarly, rolipram at 10⁻⁶ to 10⁻⁴ M, but not at 10⁻³ M, increased the forskolin-induced cAMP accumulation, indicating that only 10⁻⁶ to 10⁻⁴ M rolipram inhibited the effect of PDE in agreement with previous findings (Lerner et al., 1986; Turner et al., 1993).

Previous study has found that a positive feedback relationship exists between the synthesis of κ-opioid peptides and κ-OR stimulation in the heart (Ventura and Pintus, 1997). The observations suggest that a large amount of κ-opioid peptides may be produced during myocardial ischemia/reperfusion. So, the high concentrations of κ-OR peptides are not necessarily unreal in our body. In these studies, the effects of the κ-OR agonist at as high as 5 × 10⁻⁷ M are blocked by the selective κ-OR antagonist nor-BNI, indicating that the effects are κ-OR-mediated (Ventura et al., 1991; Sheng et al., 1997; Bian et al., 1998; Zhang and Wong, 1998).

Two pathways have been shown to mediate κ-OR stimulation on cAMP accumulation. A previous study in our laboratory has shown that activation of the phosphoinositol/Ca²⁺ pathway by κ-receptor stimulation with U50,488H leads to inhibition of cAMP in the heart (Zhang and Wong, 1998). This study has clearly provided unequivocal evidence that PKC/PDE pathway also mediates the inhibitory action of κ-OR stimulation on cAMP. In addition to PDE, PKC also has been shown to stimulate AC in the neonatal and adult cardiac myocytes (Strasser et al., 1992; Reupeke et al., 1993). Further study is needed to delineate the role of AC in mediating the action of PKC upon κ-receptor stimulation in the heart. In conclusion, this study has provided evidence for the first time that upon κ-OR stimulation PKC activates PDE-IV, leading eventually to a reduction in cAMP accumulation in the heart.

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References


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