Beta Adrenergic Receptor Stimulated Prostacyclin Synthesis in Rabbit Coronary Endothelial Cells Is Mediated by Selective Activation of Phospholipase D: Inhibition by Adenosine 3′5′-Cyclic Monophosphate

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ABSTRACT

Activation of beta adrenergic receptors in the isolated rabbit heart by catecholamines stimulates prostacyclin (PGI2) synthesis, which is inhibited by adenosine 3′5′-cyclic monophosphate (cAMP). The purpose of this study was to determine if activation of beta adrenergic receptors in cultured coronary endothelial cells (CEC) of rabbit heart with isoproterenol (ISOP) stimulates PGI2 synthesis and if cAMP inhibits the synthesis of this prostanoid and to investigate the underlying mechanism. Incubation of CEC with ISOP increased production of cAMP and PGI2, measured as immunoreactive cAMP and 6-keto-prostaglandin F1α, (6-keto-PGF1α), respectively. Forskolin, an activator of adenylyl cyclase, increased cAMP accumulation and inhibited ISOP-stimulated 6-keto-PGF1α synthesis. 8-(4-chlorophenyl-thio) cAMP also inhibited ISOP-induced 6-keto-PGF1α production. However, miconazole, an inhibitor of adenylyl cyclase, reduced cAMP accumulation and enhanced ISOP-stimulated 6-keto-PGF1α synthesis in CEC. ISOP-induced 6-keto-PGF1α synthesis was attenuated by C2-ceramide, an inhibitor of phospholipase D (PLD) by propranolol, a beta-AR antagonist that also inhibits phosphatidate phosphohydrolase and by the diacylglycerol lipase inhibitor, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC 80267). Acetylcholine (ACh) induced 6-keto-PGF1α synthesis was also inhibited by these agents. Both ISOP and ACh increased PLD activity, which was inhibited by C2-ceramide but not by RHC 80267 or propranolol. ACh but not ISOP increased phospholipase A2 activity in CEC. ISOP- but not ACh-induced increase in PLD activity was attenuated by forskolin and 8-(4-chlorophenyl-thio)-adenosine 3′5′-cyclic monophosphate and augmented by miconazole. These data suggest that beta adrenergic receptors activation promotes PGI2 synthesis in the CEC by selective activation of PLD and that cAMP decreases PLD synthesis by decreasing PLD activity. Moreover, beta adrenergic receptors activated PLD appears to be distinct from that stimulated by ACh.

ABBRÉVIATIONS: AA, arachidonic acid; ACh, acetylcholine; AR, adrenergic receptor; cAMP, adenosine 3′5′-cyclic monophosphate; CEC, coronary endothelial cells; cpt-cAMP, 8-(4-chlorophenyl-thio)-cAMP; DAG, diacylglycerol; D-609, tricyclodecan-9-yl xanthogenate; K; HELSS, e-6(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; IBMX, 3-isobutyl-1-methyl-xanthine; ISOP, isoproterenol; PG, prostaglandins; PGI2 prostacyclin; PLA2, phospholipase A2; PLD, phospholipase D; PPH, phosphatidate phosphohydrolase; RHC 80267, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane; KHB, Krebs-Henseleit buffer; PEt, phosphatidylethanol.
by ISOP (Ruan et al., 1996), platelets (Gerrard et al., 1977), Madin Darby Canine Kidney (MDCK) cells (Hassid, 1983) and collecting tubular cells (Teitelbaum et al., 1986). In contrast, cAMP and its analogs or forskolin increase PG synthesis in human decidua and amnion cells (Warrick et al., 1985) and human adherent synovial cells (Baker et al., 1985) but do not alter PG synthesis in vascular endothelial cells (Brotherton and Hoak, 1982; Whorton et al., 1985).

In the heart, cAMP, or agents that increase cAMP accumulation also do not alter basal production of PGI2 but inhibit that elicited by stimulation of beta-AR with ISOP (Williams and Malik, 1989). These findings, together with the demonstration that agents that reduce cAMP levels increase ISOP-induced PGI2 synthesis, suggest that cAMP acts as an inhibitory modulator of beta-AR-stimulated PGI2 synthesis in the heart (Williams and Malik, 1989). PGs are synthesized in several cell types, including ventricular myocytes (Ahumada et al., 1980; Bolton et al., 1980), and coronary vascular endothelial cells (Gerritsen and Cheli, 1983; Revtyak et al., 1988). However, the site(s) of beta-AR-stimulated PGI2 synthesis and the mechanism by which cAMP inhibits PGI2 synthesis in the heart is not known. This study was undertaken to determine if activation of beta-AR with ISOP stimulates PGI2 synthesis and cAMP generated during beta-AR activation inhibits PGI2 production in CEC of rabbit heart. To elucidate the mechanism of modulation of PGI2 synthesis by cAMP, we examined the contribution of different phospholipases to PGI2 production and modification of their activity by alterations in cAMP levels in response to beta-AR activation in CEC.

Methods
Preparation of Coronary Endothelial Cells
CEC were isolated from rabbit coronary vessels by modification of the method previously described (Nees et al., 1981). Male New Zealand White Rabbits (1.5–1.8 kg; Myrtle's Rabbitry, Thompson's Station, TN) were anesthetized with sodium pentobarbital (20 mg/kg) and the heart was rapidly removed and perfused via the aorta according to the method of Langendorff at 20 ml/min (37°C) with KHB containing in mM: 118.1 NaCl, 3.0 KCl, 1.8 CaCl2, 1.2 MgSO4, 1.0 KH2PO4, 27.3 NaHCO3, 10.0 glucose and 2.5 pyruvic acid, pH 7.4 and saturated with 95% O2, 5% CO2. After 10 min, the perfusion solution was changed to KHB without Ca++. For an additional 10 min to stop beating of the heart. Then 5 ml Hanks’ balanced salt solution without calcium and containing collagenase (type D; Boehringer Mannheim, Indianapolis, IN), trypsin inhibitor (type I-S; Sigma Chemical Co., St. Louis, MO) and bovine serum albumin (1.5 mg/ml each) was injected into the coronary arteries. Meanwhile the heart was completely immersed in Hanks’ balanced salt solution containing 20% sucrose for 10 min. Endothelial cells dissociated from the vessel wall were flushed out of the heart by perfusion with 50 ml KHB without Ca++. The cell suspension that overlaid the sucrose was collected and centrifuged (500 x g for 5 min). Cell pellets were washed with M199 culture medium and centrifuged again. The cells were resuspended in M199 containing 20% fetal bovine serum, seeded into 100-mm cell culture dishes and incubated at 37°C in an atmosphere of 5% CO2 for 90 min. Then the incubation medium was replaced with fresh culture medium and thereafter was changed every 2 days. More than 95% of the cells obtained by this method were CEC. These cells have been well characterized by previous investigators (Nees et al., 1981; Gerristen and Cheli, 1983). Primary cultured cells were passed to 24-well plates for experiments to determine PGI2 synthesis and cAMP accumulation or grown in 60-mm culture dishes for PLD measurement. First passage cells were used for all experiments. Three batches of cells were prepared from each heart and three to four hearts were used for each experimental group. Nine to 12 wells of cells were used for each experiment.

Experimental Protocols
Series 1. The first series of experiments was performed to measure synthesis of 6-keto-PGF1α and accumulation of cAMP induced by ISOP in CEC. CEC were washed twice with 1 ml of balanced salt solution containing in mM: NaCl 116, KCl 5.4, MgCl2·6H2O 1.2, NaH2PO4·H2O 1.2, CaCl2·2H2O 1.8, glucose 5.5, and HEPES 25 (pH 7.4) and then incubated with 1 ml of balanced salt solution at 37°C. For cAMP measurements, IBMX (10 μM) was added to minimize cAMP degradation by phosphodiesterases. After 10 min equilibration, ISOP at various concentrations or the vehicle was added into the wells and incubation was continued for 10 min. The incubation medium was collected for measurement of 6-keto-PGF1α by radioimmunoassay, and the cells were digested in 1 ml of sodium hydroxide (NaOH; 1 M) for protein determination. For cAMP determination, the reaction was terminated by adding 1 ml of sodium acetate (50 mM, pH 4.2); cAMP was measured by radioimmunoassay.

In another group of experiments, the effect of propranolol and prazosin on ISOP-stimulated 6-keto-PGF1α and cAMP production was determined. For 6-keto-PGF1α measurement, CEC were incubated at 37°C with propranolol (1 μM), prazosin (1 μM) or their respective vehicles. After 10 min equilibration, ISOP at various concentrations was added and incubation was continued for 10 min. The incubation medium was removed for determination of 6-keto-PGF1α and cAMP accumulation was measured in the cells by radioimmunoassay.

Series 2. This series of experiments was conducted to investigate the effects of an adenylyl cyclase activator, forskolin (Seamon et al., 1981), and inhibitor, miconazole (Watson et al., 1991), and cAMP analogue, cpt-cAMP on ISOP-induced PGI2 and cAMP accumulation. CEC were washed twice with balanced salt solution and incubated at 37°C with forskolin (1 μM), miconazole (10 μM), cpt-cAMP (0.1 μM) or their respective vehicles. In experiments for cAMP measurement, IBMX (10 μM) was added to the incubation buffer. After 10 min, ISOP at various concentrations was added and the cells incubated for another 10 min. The incubation medium was removed for 6-keto-PGF1α determination. cAMP was measured in the cells by radioimmunoassay.

Series 3. This series of experiments was conducted to determine the contribution of different lipases to ISOP-induced 6-keto-PGF1α synthesis in CEC. ACh, which has been reported to stimulate 6-keto-PGF1α production was determined. The CEC were preincubated with propranolol (50 μM), a beta-AR blocker known to inhibit PPH (Pappu and Hauser, 1983), RHC 80267 (10 μM), a DAG lipase inhibitor (Sutherland and Amin, 1982), HELSS (10 μM), a PLA2 inhibitor (Hazen et al., 1991), D-609 (100 μM), a phospholipase C inhibitor (Schutze et al., 1992), for 30 min or C2-ceramide (10 μM), a PLD inhibitor (Gomez-Munoz et al., 1995), for 4 hr, or their respective vehicles. The cells were twice washed twice with BSS and replaced with fresh BSS containing the same concentration of agents as above. After 10 min equilibration, ISOP (10 μM) or ACh (3 μM) was added, and the cells were incubated for another 10 min. The incubation buffer was separated for 6-keto-PGF1α measurement and cells were digested with 1 ml NaOH for protein assay.

In an additional group of experiments, the effect of the above agents on AA-induced 6-keto-PGF1α production was determined. The CEC were preincubated with forskolin (1 μM) or cpt-cAMP (0.1 μM) for 10 min, or RHC 80267 (10 μM), prazosin (50 μM) for 30 min or C2-ceramide (10 μM) for 4 hr. The cells were washed twice with BSS and fresh BSS containing above agents was added. The cells were challenged with AA (1 μM) for 10 min at 37°C and the buffer removed for 6-keto-PGF1α measurement.
Series 4. This series of experiments was performed to determine the effect of ISOP on PLD activity in CEC, measured by PET production, in the absence and presence of forskolin, cpt-cAMP, miconazole, RHC 80267 or C2-ceramide or their vehicle. PLD activity was measured by a modification of the method previously described (Liu et al., 1992). Subconfluent cells were incubated for 16 hr in culture medium containing [3H]oleic acid (1 \( \mu \text{Ci/ml}; 15 \text{Ci/mmole}). Labeled CEC were incubated in culture medium containing forskolin (1 \( \mu \text{M}) or cpt-cAMP (0.1 \( \mu \text{M}) for 10 min, or RHC 80267 (10 \( \mu \text{M}) for 30 min or C2-ceramide (10 \( \mu \text{M}) for 4 hr at 37°C. The cells were then washed twice with BSS and fresh serum free culture medium containing the above agents and 200 mM ethanol was added. This concentration of ethanol did not affect CEC morphology. A similar concentration of ethanol has also been used by other investigators (Natarajan et al., 1993; Schmidt et al., 1995). After 10 min, CEC were stimulated with ISOP (10 \( \mu \text{M}) for an additional 10 min. The reaction was terminated by adding 1 ml of ice-cold methanol/HCl (2:1; 9:1 v/v). The cells were scraped from the culture dish and washed one more time with 0.25 M HCl. The cells and wash solutions were transferred to 15-ml centrifuge tubes and sonicated for 1 min in an ice bath. One ml of chloroform/form was added to each tube, which was mixed and centrifuged at 1000 \( \times \text{g} \) for 3 hr at 4°C. The top aqueous layer was discarded, and 0.8 ml from the bottom chloroform layer was transferred to a glass tube. A 40-\( \mu \text{l} \) aliquot was removed to estimate radioactivity in the total lipid fraction. The remaining chloroform phase was evaporated under a nitrogen stream and the residue resuspended in 50 \( \mu \text{l} \) of methanol/chloroform (1:9) containing 10 \( \mu \text{g} \) of nonradiolabeled PEt standard (Biomol, Plymouth Meeting, PA). Samples were spotted onto a channelled silica gel thin layer chromatography plate (Analtech, Newark, DE). The plate was developed with chloroform/acetone/methanol/acetic acid/H2O (100:40:25:20:10). Lipids were visualized with iodine and identified by comigration with standards. Lanes containing PEt were moistened with water and scraped into scintillation vials containing 5 ml of scintillation fluid. Radioactivity was measured by scintillation spectroscopy. [3H] PET production was expressed as the fraction of total [3H] lipids. The effect of forskolin, cpt-cAMP, miconazole, RHC 80267 or C2-ceramide on the action of ACh on PLD activity was measured as described above.

Series 5. This series of experiments was performed to determine the effect of ISOP and ACh on PLA2 activity (de Carvalho et al., 1995). CEC grown in 100-mm dishes were washed twice with BSS and stimulated with ISOP (10 \( \mu \text{M}) ACh (3 \( \mu \text{M}) or vehicle for 10 min at 37°C. The cells were scraped and sonicated in lysis buffer (pH 7.4) containing 340 mM sucrose, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 \( \mu \text{g} \)/ml leupeptin, 20 \( \mu \text{g} \)/ml aprotinin and 20 \( \mu \text{g} \)/ml soybean trypsin inhibitor. The concentration of protein in the lysate was determined by Lowry’s assay and adjusted to 1 mg/ml. PLA2 activity was measured using phosphatidylcholine, 1-a-1-palmitoyl-2-arachidonoyl [14C] (57 \( \text{mCi/mmole}) as substrate (approx. 50 000 cpm/assay tube), cosonicated with 9 \( \mu \text{g} \) oleic acid, 1 mg/ml bovine serum albumin, 150 mM NaCl, 5 mM CaCl2 and 25 mM HEPES, pH 7.4. Cells lysate containing 25 \( \mu \text{g} \) of protein was added, and the reaction mixture was incubated for 30 min at 37°C. The reaction was terminated with 2.5 ml of Dole’s reagent [2-propanol/heptane/0.5 molar \( \text{H2SO4} (20:5:1)], to which 1 ml of heptane and 1 ml of water containing 20 \( \mu \text{g} \) of nonradiolabeled AA as carrier was added. The contents of the tube were mixed and the heptane layer was applied to a Sep-Pak 3cc silica column (Waters, Milford, MA) for separation of radiolabeled fatty acid. Each column was further washed with 1 ml heptane, and the elutes were air dried and radioactivity measured by liquid scintillation spectroscopy.

Radioimmunoassay of 6-Keto-PGF1a

6-keto-PGF1a, the stable product of PG12 hydrolysis, was measured by radioimmunoassay as described previously (Shaffer and Malik, 1982). Samples, 100 \( \mu \text{l} \), were mixed with 50 \( \mu \text{l} \) of [3H]6-keto-PGF1a (3500–4000 cpm; 5.6 TBq/mmol) plus an appropriate concentration of antibody, obtained from Dr. Charles Leffler (Department of Physiology, University of Tennessee, Memphis, TN). The tracer and antibody were prepared in a buffer consisting of (g/liter) 1.0 Na\textsubscript{2}CO\textsubscript{3}, 9.0 NaCl, 6.8 K\textsubscript{2}PO\textsubscript{4}, 26.1 K\textsubscript{3}HPO\textsubscript{4}, and 2 gelatin. Standards (1–1000 pg) were prepared in KH\textsubscript{2}O. After vortexing, the tubes were incubated overnight at 4°C. Bound and free tracer were separated by adding 1 ml of dextran-coated charcoal to each tube. After centrifugation, the supernatant was decanted into 5 ml of scintillation cocktail, and radioactivity was measured by liquid scintillation spectroscopy. Cross-reactivity of the 6-keto-PGF1a antibody was <1% with thromboxane B\textsubscript{2} and 13,14-dihydro-15-keto-PGF\textsubscript{2}a and <0.5% with PGF\textsubscript{2}a and PGE\textsubscript{2}a. The minimum detection level of the radioimmunoassay for 6-keto-PGF1a was 1.95 pg.

Radioimmunoassay of cAMP

cAMP was determined by radioimmunoassay by modification of the method previously described (Bruckner et al., 1985). The cells were frozen at −20°C, then thawed and boiled for 3 min. The samples were centrifuged at 1000 \( \times \text{g} \) for 5 min, and 0.5 ml of the supernatants were acetylated by addition of triethylamine and acetic anhydride (3:2) to increase the sensitivity of the assay. Briefly, 50 \( \mu \text{l} \) of acetylated sample or standards were added to 50 \( \mu \text{l} \) of [125I]cAMP (<3000 cpm, <65 TBq/mmol; prepared by the procedure described by Steiner et al., 1972)) and 200 \( \mu \text{l} \) of antibody (Biomedical Technologies Inc., Stoughton, MA) were added. Both [125I]cAMP and antibody were diluted in 50 mM sodium acetate buffer (pH 6.2) containing 5 mg/ml bovine serum albumin. The mixture was vortexed and incubated at 4°C overnight. Phosphate buffer (150 \( \mu \text{M}) containing 1% γ-globulin and 2 ml of 25% polyethylene glycol was added to the tubes, which were then vortexed and centrifuged at 1700 \( \times \text{g} \) for 30 min. The supernatant was removed, and the radioactivity determined in a gamma counter (model 4200, Micromedic Systems, Horsham, PA).

Drugs

The drugs used in this study that were purchased are the following: ISOP, arachidonic acid, miconazole, IBMX, cpt-cAMP, ACh, phenylmethlysulfonfluoride and propranolol from Sigma; forskolin from Research Biochemicals International (Natick, MA), leupeptin and aprotinin from Calbiochem-Novabiochem (San Diego, CA); HELSS, D-609, RHC 80267 and C2-ceramide from Biomol Research Laboratories, Inc. (Plymouth meeting, MA). [5,8,9,11,12,14,15-3H(N)]:6-keto-PGF\textsubscript{1a} from Du Pont Corp. (Boston, MA). [3H]Oleic acid and phosphatidylcholine, 1-a-1-palmitoyl-2-arachidonoyl [14C] were purchased from American Radiolabeled Chemicals (St. Louis, MO). ISOP was dissolved in 1.0 M HCl at a concentration of 100 mM. The stock solution was diluted in BSS 10 min before the experiment. AA (sodium salt) was dissolved in distilled water at a concentration of 100 mM, and vials were stored under nitrogen gas. Miconazole, RHC 80267, C2-ceramide and IBMX were initially dissolved in dimethylsulfoxide at a concentration of 10 mM, and further dilutions were made in BSS. All stock solutions were stored at −20°C. cpt-cAMP were dissolved in distilled water before the experiment.

Data Analysis

The results are expressed as means ± S.E. The data were analyzed by one-way analysis of variance. The unpaired Student’s t test was applied to determine the difference between two groups and the Newman-Keuls’ A Posterior Test to determine the difference between multiple groups. The null hypothesis was rejected at P < .05. Basal 6-keto-PGF\textsubscript{1a} levels are expressed as nanograms of immunoreactive 6-keto-PGF\textsubscript{1a} per mg of protein. Basal 6-keto-PGF\textsubscript{1a} production was variable among different batches of CEC. However, ISOP- and ACh-induced increase in 6-keto-PGF\textsubscript{1a} production although smaller than in the intact heart (Weis and Malik, 1985) was consistent within the same batch of cells. Therefore, the increase in 6-keto-PGF\textsubscript{1a} production elicited by ISOP and by ACh are expressed as percentage above basal level. Basal cAMP level is expressed as...
picomoles per milligram of protein in CEC. PLD and PLA₂ activity was expressed as the fold increase from basal.

Results

Effect of ISOP on 6-keto-PGF₁α synthesis and cAMP accumulation in the presence and absence of propranolol and prazosin. ISOP produced a consistent concentration-dependent increase in the synthesis of 6-keto-PGF₁α (fig. 1A) and cAMP (fig. 1B) in CEC. The effect of ISOP on cAMP accumulation was surprisingly well correlated with the rise in 6-keto-PGF₁α synthesis in CEC. This increase in 6-keto-PGF₁α and cAMP accumulation was abolished by the beta-AR antagonist propranolol (1 μM), but not by the alpha-AR antagonist prazosin (1 μM; data not shown).

Effect of forskolin, cpt-cAMP and miconazole on ISOP-induced 6-keto-PGF₁α production and cAMP accumulation. Forskolin, an activator of the catalytic subunit of adenylyl cyclase, increased cAMP accumulation (fig. 2B) that was not altered by propranolol (1 μM) (data not shown). Forskolin also enhanced cAMP accumulation and reduced 6-keto-PGF₁α production elicited by ISOP (fig. 2A and B); forskolin did not alter basal 6-keto-PGF₁α synthesis (P > .05) (fig. 2A). The nonhydrolyzable cAMP analogue cpt-cAMP, inhibited ISOP-induced 6-keto-PGF₁α without any effect on the basal 6-keto-PGF₁α production (P > .05; fig. 2A). Miconazole, an adenylyl cyclase inhibitor, produced a concentration-dependent potentiation in the ISOP-induced increase in 6-keto-PGF₁α production without altering basal 6-keto-PGF₁α synthesis (fig. 3A). The ISOP-induced increase in cAMP accumulation was attenuated by the same concentration of miconazole (fig. 3B).

Effect of PLD, PPH and DAG lipase inhibitors on ISOP- and ACh-induced 6-keto-PGF₁α synthesis and PLD activity. Both C₂-ceramide, an inhibitor of PLD activation, and the DAG lipase inhibitor RHC 80267 attenuated ISOP- (fig. 4A) and ACh- (fig. 5A) stimulated formation of 6-keto-PGF₁α in CEC. Propranolol, a beta-AR antagonist that also inhibits PPH activity, abolished ISOP-induced 6-keto-PGF₁α synthesis (fig. 4A) and attenuated ACh-induced 6-keto-PGF₁α synthesis (fig. 5A). Both ISOP and ACh induced activation of PLD was inhibited by C₂-ceramide but not by RHC 80267 (figs. 4B and 5B). Propranolol abolished activation of PLD elicited by ISOP (fig. 4B) but did not alter ACh-induced increase in PLD activity (fig. 5B). Neither C₂-ceramide nor RHC 80267 or propranolol altered basal 6-keto-PGF₁α production and PLD activity (P > .05).

Effect of HELSS on ISOP and ACh induced 6-keto-PGF₁α production and PLA₂ and PLD activity. HELSS, a PLA₂ inhibitor, reduced 6-keto-PGF₁α synthesis elicited by ACh but not by ISOP (fig. 6A). However, HELSS did not affect the increase in PLD activity elicited by ACh or ISOP (fig. 6B) but inhibited ACh-induced increase in PLA₂ activity from 1.66 ± 0.13 to 1.24 ± 0.08 fold/above basal (n = 6; P > .05). ACh increased both PLA₂ and PLD activity in CEC; the
increase in PLA2 activity was much greater than that in PLD activity (fig. 7). ISOP, which increased PLD activity, failed to alter PLA2 activity under identical experimental conditions (fig. 7). D-609 (20 μM), a phospholipase C inhibitor, failed to alter ISOP- (10 μM), and ACh- (3 μM) stimulated 6-keto-PGF1α synthesis (data not shown).

Effects of forskolin, cpt-cAMP and miconazole on ISOP- and ACh-stimulated PLD activation. The increase in PLD activity elicited by ISOP was reduced by forskolin and cpt-cAMP and enhanced by miconazole. Basal PLD activity was not altered by these agents (P > .05) (fig. 8A). Forskolin, cpt-cAMP or miconazole did not alter ACh induced increase in PLD activity (fig. 8B).

Effects of forskolin, cpt-cAMP, C2-ceramide and RHC 80267 on AA-induced 6-keto-PGF1α formation. The conversion of exogenous AA to 6-keto-PGF1α in CEC was not altered by forskolin, cpt-cAMP, C2-ceramide, RHC 80267 or propranolol (fig. 9).

Discussion

Catecholamines are known to increase levels of cAMP via activation of beta-AR (Kopecky et al., 1965) and cAMP mediates some cardiac actions of catecholamines (Drummond and Severson, 1979). Surprisingly, however, PGI2 synthesis elicited by adrenergic transmitter that is mediated through activation of beta-AR (Shaffer and Malik, 1982), is inhibited by cAMP in the isolated rabbit heart (Williams and Malik, 1989). Our study indicates that activation of beta-AR with ISOP in CEC of rabbit heart promotes PGI2 synthesis, measured as its stable hydrolysis product 6-keto-PGF1α, via selective activation of PLD and not PLA2. Moreover, cAMP generated during beta-AR activation interestingly inhibits 6-keto-PGF1α synthesis by attenuating PLD activity. These conclusions are based on our findings that ISOP, a selective beta-AR agonist, increased the synthesis of 6-keto-PGF1α.

The increase in ISOP-induced 6-keto-PGF1α was associated with increased cAMP accumulation and these effects of ISOP were inhibited by the beta-AR antagonist propranolol, but not by the alpha-AR antagonist prazosin. Furthermore, propranolol did not alter forskolin-induced rise in cAMP. Our finding that an inhibitor of adenylyl cyclase, miconazole (Watson et al., 1991), reduced cAMP accumulation and enhanced 6-keto-PGF1α synthesis elicited by ISOP, suggests that cAMP generated during beta-AR activation attenuates 6-keto-PGF1α synthesis. Supporting this conclusion is our
finding that cAMP inhibited ISOP-induced 6-keto-PGF₁α production. Moreover, forskolin, an activator of adenylyl cyclase that increased cAMP accumulation, also attenuated 6-keto-PGF₁α production elicited by ISOP. The effect of cAMP to inhibit β-AR stimulated 6-keto-PGF₁α synthesis in CEC appears to be selective because forskolin or cAMP do not alter 6-keto-PGF₁α synthesis elicited by muscarinic receptor activation with ACh in CEC (Kan et al., 1996), and α₁-AR activation with adrenergic receptor agonists in aortic smooth muscle cells (Nebigil and Malik, 1990).

Although in CEC changes in cAMP modulated 6-keto-PGF₁α production elicited by ISOP, the basal synthesis of this prostanoid was not altered. However, in some other cell types, cAMP or its analogs and agents that alter cAMP have been reported to either decrease (Gerrad et al., 1977; Hassid, 1983; Teitelbaum et al., 1986), or increase PG synthesis (Warrick et al., 1985; Baker et al., 1985). The inhibitory effect of cAMP on PG synthesis in some cells has been attributed to a decrease in the cyclooxygenase activity (Malmsten et al., 1976), decrease in the availability of AA to cyclooxygenase (Adler et al., 1981; Lim et al., 1983; Teitelbaum et al., 1986), or to a decrease in AA release and its metabolism (Hassid, 1983; Nielson et al., 1992). However, AA-induced increase in PG synthesis was not altered by cAMP in gastric mucosal cells (Hiraishi et al., 1986, 1989). The reason for these differences in the action of cAMP on basal PG synthesis is not known.
PLA2 (Billah and Lapetina, 1981). Phospholipase D promotes phosphatidic acid, which in turn can serve as a substrate for PLC and generate DAG and phosphocholine (Schutze et al., 1979). However, phosphatidylcholine can also serve as a substrate for activation of PLA2, PLC and/or PLD. PLA2 hydrolyzed by PPH to DAG, which in turn is metabolized to AA produced via beta-AR. Supporting this view is our demonstration that ACh, but not ISOP, increased PLA2 activity in CEC. Beta-AR-stimulated 6-keto-PGF1α synthesis in CEC also does not appear to involve PLC, because the phosphatidylinositol-specific PLC inhibitor, D-609 (Schutze et al., 1992), failed to alter ISOP-induced 6-keto-PGF1α synthesis. U-73122, a phosphatidylinositol specific inhibitor (Bleasdale et al., 1990) could not be used because it exhibited cytotoxicity in CEC.

An important finding in our study is that a PLD inhibitor, C2-ceramide (Gomez-Munoz et al., 1995), attenuated ISOP-induced 6-keto-PGF1α synthesis. Supporting involvement of PLD in our finding that DAG lipase inhibitor RHC 80267 (Sutherland and Amin, 1982) also attenuated ISOP-induced 6-keto-PGF1α production. Moreover, ISOP, which did not alter PLA2 activity, markedly increased PLD activity in CEC. These observations, together with the demonstration that heart has high PLD activity (Lindmar and Loffehölz, 1992), strongly suggest that beta-AR-stimulated 6-keto-PGF1α production in CEC is mediated via selective activation of PLD. However, ACh, which increased PLA2 activity, also enhanced PLD activity. Although ACh stimulates PGI2 synthesis in CEC via activation of cytosolic PLA2 (Kan et al., 1996), it appears PLD also contributes to ACh-induced PGI2 synthesis. Supporting this view are our findings that inhibitors of PLD (C2-ceramide), PPH (propranolol) (Pappu and Hauser, 1983) and DAG lipase (RHC 80267) also attenuated ACh-induced 6-keto-PGF1α in CEC. The effect of propranolol and RHC 80267 to inhibit 6-keto-PGF1α production elicited by ISOP or ACh was not due to decrease in PLD activity, because these agents did not alter ISOP or ACh-induced increase in activity of the enzyme. Although the effect of propranolol to block ISOP-induced 6-keto-PGF1α synthesis was most likely due to its beta-AR blocking activity, it may also decrease PPH activity (Pappu and Hauser, 1983), in CEC as
indicated by its effect to reduce ACh-induced 6-keto-PGF$_1$$_{18}$ synthesis.

The mechanism by which C$_2$-ceramide inhibits PLD activity is not known. C$_2$-ceramide has been reported to activate a specific protein kinase and phosphatase (Liu et al., 1994; Hannun, 1994). It is also possible that C$_2$-ceramide could inhibit the activity of PLD by interfering with the translocation to the membrane of small GTP binding proteins (Abous-alham et al., 1997) proposed to be involved in PLD activation (Singer et al., 1991; Bowman et al., 1993; Massenburg et al., 1994; Malcolm et al., 1994; Siddiqi et al., 1995; Brown et al., 1995; Jiang et al., 1995). However, a long exposure (4 hr or longer) was required to inhibit PLD activity (Gomez-Munoz et al., 1995). It is possible that the C$_2$-ceramide might reduce the availability of phosphatidyicholine to PLD by competing with phosphocholine synthesis and promoting formation of sphingomyelin. In our study the effect of C$_2$-ceramide, propranolol and RHC 80267 on ISOP- or ACh-induced 6-keto-PGF$_1$$_{18}$ synthesis was not due to a decrease in cyclooxygenase activity, because these agents did not alter the conversion of exogenous AA to 6-keto-PGF$_1$$_{18}$.

The demonstration that in CEC 1) beta-AR stimulated 6-keto-PGF$_1$$_{18}$ synthesis is due to activation of PLD and 2) the inhibitory effect of cAMP on beta-AR stimulated 6-keto-PGF$_1$$_{18}$ synthesis is not due to its action on cyclooxygenase activity, raises the possibility that cAMP might act by inhibiting PLD activity. Supporting this possibility are our observations that both forskolin and cpt-cAMP inhibited and micronazole, an inhibitor of adenyl cyclase, enhanced ISOP-induced increase in PLD activity in CEC. Dibutryl cAMP or agents that elevate cAMP levels have also been reported to inhibit the increase in PLD activity elicited by formyl-methionyl-leucylphenylalanine in neutrophils (Tyagi et al., 1991). Our finding that cpt-cAMP or forskolin did not alter ACh-induced increase in PLD activity suggests that the type of PLD linked to muscarinic receptor is distinct from that coupled to beta-AR in CEC. However, elevation of cAMP has been reported to enhance thrombin-stimulated PLD in endothelial cells (Garcia et al., 1992). Protein kinase A activation was also shown to increase vasopressin-stimulated PLD activity in rat hepatocytes but had no effect on phorbol ester- or calcium-induced increase in PLD activity (Gustavsson et al., 1994). Similarly, agents that alter CAMP levels had no effect on phorbol ester-stimulated increase in activation of PLD in neutrophils (Agwu et al., 1991; Tyagi et al., 1991). PLD activity in many mammalian cells have been shown to differ in pH optimum, requirement for divalent cations, subcellular localization and requirement for phosphatidylinositol 4,5 bisphosphate and degree of activation by small G-proteins including ARFs, Rho A and B, Rac, Cdc42 and Ras, protein kinase C or a tyrosine kinase (Singer et al., 1991; Bowman et al., 1993; Massenburg et al., 1994; Malcolm et al., 1994; Siddiqi et al., 1995; Brown et al., 1995; Jiang et al., 1995; Conricode et al., 1992; Singer et al., 1996; Natarajan et al., 1993). Therefore, it is possible that one or more of these factors might differentially regulate various subtypes of PLD in response to various agents. The mechanism by which beta-AR stimulation promotes PLD activation and cAMP inhibits PLD activity is not known and could involve one or more of the above signaling molecules directly or indirectly. cAMP may also inhibit PLD activity by stimulation of protein kinase A and phosphorylation of PLD or a G protein coupled to PLD. Alternatively, protein kinase A, by phosphorylating and activating a protein phosphatase, may promote dephosphorylation of PLD. The recent identification of the human gene encoding PLD (Hammond et al., 1995), should allow the development of tools required to address these questions.

In conclusion, beta-AR stimulation in the CEC of rabbit heart promotes PGI$_2$ synthesis via selective activation of PLD. cAMP generated during beta-AR stimulation acts as an inhibitory modulator of PGI$_2$ synthesis by decreasing PLD activity. Whether CAMP also affects the activity of phosphatidic acid phosphohydrolase and/or DAG lipase remains to be investigated.

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References


