Role of prostaglandin-mediated cyclic AMP formation in protein kinase C-dependent secretion of atrial natriuretic peptide in rat cardiomyocytes

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The role of endogenous prostaglandin production in phorbol diester-induced myocardial atrial natriuretic peptide (ANP) secretion was investigated in cultured spontaneously beating ventricular rat cardiomyocytes. Incubation of cells with 4 β -phorbol 12-myristate 13-acetate (PMA; 0.1 μ M) led to a rapid response in ANP release, a response accompanied by increases in cellular prostacyclin (PGI₂) production, cyclic AMP (cAMP) formation and spontaneous contraction frequency. Although PMA-induced ANP secretion exhibited the pharmacological profile of a protein kinase C (PKC)-mediated event, the response was abolished in the presence of the cyclo-oxygenase inhibitors indomethacin (10 μ M) and diclofenac (1 μ M), indicating that endogenous prostaglandin production is responsible for PMA-induced ANP secretion in this system. Confirming this, PMA-induced ANP secretion was strongly correlated with endogenous formation of 6-oxo-prostaglandin F_{1 α} ($r = 0.93$, $P < 0.0005$, $n = 11$), and exogenously applied PGI₂, prostaglandin E₂ (PGE₂) or prostaglandin F_{2 α} (PGF_{2 α}) elicited simultaneous increases in cAMP formation, contraction frequency and ANP secretion in these cells. Furthermore, PMA-induced cAMP formation was

abolished in the presence of either diclofenac or indomethacin, whereas the cAMP-elevating agent forskolin (0.1 μ M) mimicked the secretory and chronotropic effect of PMA in these cells. A role for cAMP in PMA-induced ANP secretion was also apparent insofar as PMA-induced ANP release was substantially decreased in the presence of the R_p-diastereomer of 3',5'-cyclic adenosine monophosphorothioate (R_p-cAMPS; 10 μ M), whereas the cAMP-mimetic agent dibutyryl cAMP (10 μ M) provoked a rapid increase in ANP secretion in this system. Finally, the Ca²⁺-channel antagonist nifedipine (0.1 μ M) severely decreased PGI₂-, PGE₂- and PMA-induced ANP secretion without affecting PGF_{2 α} -induced peptide release, suggesting that PGI₂ and/or PGE₂, but not PGF_{2 α} , are the prostanoids involved in PMA-induced ANP release. Taken together, these results suggest that PKC activation induces ANP secretion in spontaneously beating rat ventricular cardiomyocytes via an autocrine pathway involving increased PGI₂ and/or PGE₂ formation, a response leading to the activation of a myocardial adenylate cyclase and, subsequently, to that of a nifedipine-sensitive Ca²⁺ channel.

INTRODUCTION

Mammalian cardiomyocytes synthesize and secrete atrial natriuretic peptide (ANP), a vasorelaxant factor displaying natriuretic and diuretic properties (Inagami, 1989). Although atrial stretch is generally considered to be the prime physiological stimulus inducing ANP release (Dietz, 1984; Lang et al., 1985), little is known about the intracellular signalling mechanisms leading to the secretion of this peptide. Perfused heart, isolated atria and/or cultured cardiomyocytes secrete ANP upon stimulation with protein kinase C (PKC), activating phorbol diesters (Ruskoaho et al., 1985), Ca²⁺ ionophores (Bloch et al., 1988), L-type voltage-operated Ca²⁺-channel activators (Matsubara et al., 1988) or cyclic AMP (cAMP) derivatives (Schiebinger and Santora, 1989). Furthermore, PKC activation, cAMP formation and Ca²⁺ influx are synergistically involved in this process (for review, see Ruskoaho et al., 1991).

Interestingly, the mammalian myocardium also secretes an array of cyclo-oxygenase products (van Bilsen et al., 1989), notably prostacyclin (PGI₂), prostaglandin E₂ (PGE₂) and prostaglandin F_{2 α} (PGF_{2 α}), potent auto- and para-crine modulators of biological function noted for their secretagogue,

Ca²⁺-mobilizing and/or adenylate cyclase-stimulating effects (Virgolini et al., 1992; Vassaux et al., 1992). It has recently been shown that stimulation of spontaneously beating cultured rat ventricular cardiomyocytes with PKC-activating phorbol diesters leads to the activation of a myocardial phospholipase A₂, an event leading to subsequent PGI₂, PGE₂ and PGF_{2 α} synthesis in this tissue (Church et al., 1993). In view of reports suggesting that both PGI₂ and PGE₂ activate myocardial adenylate cyclase (Metsä-Ketelä, 1981), that cAMP regulates cardiac L-type Ca²⁺ current (Trautwein and Heschler, 1990) and that phorbol diesters increase Ca²⁺ current in neonatal-rat heart myocytes (Lacerda et al., 1988; Dösemeci et al., 1988), it is possible that PKC mediates myocardial ANP secretion via a prostaglandin-dependent autocrine pathway involving the activation of adenylate cyclase and/or of a myocardial Ca²⁺ channel.

We investigated this hypothesis by stimulating spontaneously beating cultured neonatal-rat ventricular cardiomyocytes with PGI₂, PGE₂, PGF_{2 α} and the PKC activator 4 β -phorbol 12-myristate 13-acetate (PMA) and by measuring the effect in terms of ANP release, cAMP formation, increases in the level of cytosolic free Ca²⁺ ([Ca²⁺]_i) and modification of the rate of spontaneous cellular contraction.

Abbreviations used: PKC, protein kinase C; PMA, 4 β -phorbol 12-myristate 13-acetate; PGI₂, prostacyclin; PGE₂, prostaglandin E₂; PGF_{2 α} , prostaglandin F_{2 α} ; 6-oxo-PGF_{1 α} , 6-oxo-prostaglandin F_{1 α} ; cAMP, cyclic AMP; R_p-cAMPS, R_p-diastereomer of 3',5'-cyclic adenosine monophosphorothioate; ANP, atrial natriuretic peptide; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; IBMX, 3-isobutyl-1-methylxanthine.

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MATERIALS AND METHODS

Materials

[γ - 32 P]ATP, 6-oxo-[3 H]prostaglandin $F_{1\alpha}$ (6-oxo-PGF $_{1\alpha}$) and radioimmunoassay kits for the determination of cAMP were obtained from Amersham International (Amersham, Bucks., U.K.). Rat ANP [3- 125 I]Tyr 125] (99–126)-peptide was purchased from Novabiochem (Basel, Switzerland). ATP, histone III-S, 1,2-diolein, phosphatidylserine, γ -globulin, PGI $_2$, PMA, 4 β -phorbol 12-monoacetate, 4 α -phorbol, staurosporine, quinacrine, indomethacin, diclofenac, $N^6,2'$ - O -dibutyl cAMP and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St. Louis, MO, U.S.A.). Forskolin was from Calbiochem (San Diego, CA, U.S.A.). PGE $_2$, PGF $_{2\alpha}$ and 6-oxo-PGF $_{1\alpha}$ were generously given by Upjohn (Kalamazoo, MI, U.S.A.), and the PKC inhibitor CGP 41251 was kindly given by Ciba-Geigy (Basel, Switzerland). Calphostin C, chelerythrine and the R_p -diastereomer of 3',5'-cyclic adenosine monophosphorothioate (R_p -cAMPS) were from LC Laboratories (Woburn, MA, U.S.A.). Fura-2 acetoxymethyl ester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). McCoy's modified 5A medium, Ca $^{2+}$ and Mg $^{2+}$ -free Hanks balanced salt solution, foetal-calf serum, trypsin, DNAase I (type II) and insulin–transferrin–selenium medium supplement (ITS; 5 μ g/ml, 5 μ g/ml and 5 ng/ml respectively) were from Gibco (Basel, Switzerland). Anti- α ANP antiserum and α ANP were obtained from Peninsula Labs Europe (Merseyside, U.K.). Anti-6-oxo-PGF $_{1\alpha}$ antiserum was kindly given by Dr. M. J. Dunn (Division of Nephrology, Case Western Reserve University, Cleveland, OH, U.S.A.), and nifedipine was a gift from Bayer A. G. (Wuppertal, Germany).

Cell culture

Spontaneously beating neonatal-rat ventricular cardiomyocyte cultures were obtained from 1–2-day-old Wistar rats by the trypsin/DNAase sequential-digestion method previously described (Church et al., 1994a). Cells were kept in culture in McCoy's modified 5A medium containing 10% foetal-calf serum, 1% ITS, 100 units/ml penicillin, 10 μ g/ml streptomycin and 0.5 μ g/ml fungizone (fungicidal agent), in a water-saturated air/CO $_2$ (19:1) atmosphere at 37 °C. Cells were used on day 3 of culture for all experiments described herein.

Determination of PGI $_2$ and ANP release

Assessment of PGI $_2$ formation and ANP release was carried out in six-well tissue-culture plates containing spontaneously contracting cardiomyocyte monolayers as previously described (Church et al., 1994b). Pharmacological agents used in these studies were dissolved in water, dimethyl sulphoxide or mixtures thereof, such that the final concentration of dimethyl sulphoxide did not exceed 0.5%. When tested for 6-oxo-PGF $_{1\alpha}$ or ANP release-inhibiting or -stimulating properties, 1% concentrations of dimethyl sulphoxide induced no response in these preparations (results not shown). The detection limit for ANP determinations was 5 pg/ml of incubation medium. Non-specific binding was 4.2 $\pm$ 0.5% of total radioactivity (n = 5). The intra- and inter-assay coefficients of variation were estimated at 5% and 7% respectively (n = 8). The ED $_{50}$ for ANP tracer displacement occurred at 48 $\pm$ 5 pg (n = 5). Parallelism of the assay was determined by constructing standard curves in the presence of additional α ANP. Mean displacement of points above and below the ED $_{50}$ was +17.6 $\pm$ 3.2 pg/tube in the presence of an additional 20 pg of α ANP/tube (n = 6), and +38.7 $\pm$ 2.4 pg/tube

in the presence of an additional 40 pg of α ANP/tube (n = 6). Cross-reactivity of the antiserum to 6-oxo-PGF $_{1\alpha}$ has been previously described in detail (Braconi et al., 1992).

cAMP determination

For cAMP determinations, six-well tissue-culture plates containing spontaneously contracting cardiomyocyte monolayers were washed with 2 $\times$ 2 ml of Krebs–Ringer buffer and incubated with various pharmacological agents at 37 °C for 20 min in the presence of 0.5 mM IBMX. The reaction was stopped by adding 100 μ l of trichloroacetic acid (12%, w/v) to the cell media and freezing the resulting lysates at –80 °C. The samples were then neutralized by extractions with 3 $\times$ 3 vol. of water-saturated diethyl ether and dried down under reduced pressure. The resulting dry cell homogenates were then resuspended in assay buffer (Tris/HCl, pH 7.5) and assayed for cAMP content as previously described (Eistetter et al., 1991).

[Ca $^{2+}$] $_i$ determination

[Ca $^{2+}$] $_i$ determinations were carried out in monolayers of spontaneously contracting ventricular cardiomyocytes prepared on glass slides by using the fluorescent probe fura-2/AM, adapting a method described elsewhere (Capponi et al., 1985). Briefly, the monolayers were washed twice with a modified Krebs–Ringer buffer (136 mM NaCl, 1.8 mM KCl, 1.2 mM KH $_2$ PO $_4$, 1.2 mM MgSO $_4$, 5 mM NaHCO $_3$, 1.2 mM CaCl $_2$, 0.21 mM EGTA, 20 mM Hepes, 5.5 mM glucose and 1% BSA at pH 7.4), covered with 400 μ l of modified Krebs–Ringer buffer containing 2 μ M fura-2 and 0.5% BSA, and incubated for 20 min at 37 °C. At the end of the loading period, the slides were washed twice with modified Krebs–Ringer buffer, inserted into glass cuvettes containing 3 ml of the same buffer, and placed in the thermostatically regulated holder of an LS-3 Perkin–Elmer fluorescence spectrophotometer, stirred continuously with a magnetic stirrer. Fluorescence of fura-2-loaded monolayers was measured by using an excitation wavelength of 340 nm and an emission wavelength of 505 nm. Calibration of the signal was carried out with ionomycin and MnCl $_2$ in the presence of excess of Ca $^{2+}$. The K_d of fura-2 for Ca $^{2+}$ was assumed to be 224 nM (Gryniewicz et al., 1985). Mean diastolic and systolic [Ca $^{2+}$] $_i$ were determined by averaging the appropriate (resting and contracting) [Ca $^{2+}$] $_i$ values for each tracing over a 20 s interval preceding stimulation with the various pharmacological agents.

Determination of the frequency of cell contraction

The frequency of spontaneous cellular contraction was measured by assessing the number of spontaneous Ca $^{2+}$ transients per unit time observed in contracting fura-2-loaded cells (see above). Alternatively, cardiomyocytes were incubated at 37 °C with various pharmacological agents for the indicated times, removed from the incubator, and contraction frequency was immediately determined under a microscope. The two methods gave similar results, basal contraction frequency being estimated at 36.7 $\pm$ 1.1 beats/min by Ca $^{2+}$ fluorimetry (n = 15) and at 42 $\pm$ 2.8 beats/min by visual determination (n = 6).

Statistical analysis

The Student–Fisher unpaired bilateral t test and/or ANOVA using the Scheffe F-test criterion for unbalanced groups was used where applicable. A value of P < 0.05 was accepted as statistically significant. Results represent the means $\pm$ S.E.M. of at least three

separate experiments performed in duplicate or triplicate determinations.

RESULTS

Phorbol-diester-induced ANP release

Incubation of spontaneously beating neonatal-rat cardiomyocytes with PMA ($0.1 \mu\text{M}$) led to a marked increase in cellular ANP secretion. The effect of PMA was maximal at 15 min ($+1.3 \pm 0.25 \text{ ng/mg}$ of cell protein; $n = 8$), exhibited an EC_{50} of $1.5 \pm 0.7 \text{ nM}$ ($n = 8$; see Figure 1), and was accompanied by an increase in the rate of spontaneous cellular contraction (from 39.1 ± 1.8 to 51.3 ± 2.4 beats/min at 15 min; $n = 6$). Furthermore, PMA-induced ANP release was accompanied by a simultaneous increase in cellular 6-oxo-PGF $_{1\alpha}$ production (Figure 1), a response that was detected within 10 min of applying $0.1 \mu\text{M}$ PMA to the cell monolayers (results not shown) and that exhibited an EC_{50} indistinguishable from that observed for PMA-induced ANP secretion ($2 \pm 0.5 \text{ nM}$; $n = 6$).

PMA-induced ANP secretion displayed the full pharmacological profile of a PKC-mediated event. Both 4 β -phorbol 12-monoacetate ($1 \mu\text{M}$) and 4 α -phorbol ($1 \mu\text{M}$) failed to induce either ANP release or chronotropic effects in these cells ($n = 3$;

Figure 1 PMA-induced ANP secretion and 6-oxo-PGF $_{1\alpha}$ production in rat cardiomyocytes

Cultured cardiomyocytes were incubated at 37°C with different concentrations of PMA, and ANP secretion and 6-oxo-PGF $_{1\alpha}$ production were determined as described in the Materials and methods section. Results represent means $\pm$ S.E.M. for 6–8 experiments carried out in triplicate determinations: * $P < 0.05$ versus control.

Table 1 Inhibition of PMA-induced ANP secretion and 6-oxo-PGF $_{1\alpha}$ production by staurosporine, CGP 41251, calphostin C or chelerythrine, and PKC down-regulation

Cultured cardiomyocytes were incubated at 37°C with PMA ($0.1 \mu\text{M}$) in the absence or presence of staurosporine ($1 \mu\text{M}$), CGP 41251 ($1 \mu\text{M}$), calphostin C ($1 \mu\text{M}$) and chelerythrine ($1 \mu\text{M}$), and ANP release and 6-oxo-PGF $_{1\alpha}$ formation were determined as described in the Materials and methods section. Alternatively, cultured cells were pretreated with $0.1 \mu\text{M}$ phorbol diester for 24 h, washed twice in Krebs–Ringer buffer and further stimulated with $0.1 \mu\text{M}$ PMA for 30 min. Results, expressed as percentage inhibition of PMA-induced ANP release and 6-oxo-PGF $_{1\alpha}$ production in untreated (control) cells, represent means $\pm$ S.E.M. for the indicated numbers of experiments performed in triplicate determinations. Neither PKC inhibition or PKC down-regulation had an effect on basal ANP secretion or 6-oxo-PGF $_{1\alpha}$ production (results not shown).

Response	Inhibition of PMA-induced cellular response (%) by:				
	Staurosporine ($1 \mu\text{M}$)	CGP 41251 ($1 \mu\text{M}$)	Calphostin C ($1 \mu\text{M}$)	Chelerythrine ($1 \mu\text{M}$)	24 h PMA ($0.1 \mu\text{M}$)
ANP release	81.3 ± 6.3 ($n = 9$)	74.1 ± 7.3 ($n = 4$)	52.6 ± 10.1 ($n = 6$)	72.8 ± 14.5 ($n = 3$)	89.1 ± 7.3 ($n = 4$)
6-Oxo-PGF $_{1\alpha}$ production	91.5 ± 2.5 ($n = 9$)	84.1 ± 10.6 ($n = 4$)	90.5 ± 3.8 ($n = 6$)	91.3 ± 4.4 ($n = 3$)	95.5 ± 4.4 ($n = 4$)

Table 2 Effect of phospholipase A $_2$ and cyclo-oxygenase inhibition on PMA-induced ANP secretion and 6-oxo-PGF $_{1\alpha}$ production

Cultured cardiomyocytes were incubated at 37°C with PMA ($0.1 \mu\text{M}$) in the absence or presence of quinacrine ($10 \mu\text{M}$), indomethacin ($10 \mu\text{M}$) and diclofenac ($1 \mu\text{M}$), and 6-oxo-PGF $_{1\alpha}$ formation and ANP release were determined as described in the Materials and methods section. Alternatively, the PKC-inhibiting properties of these agents were tested in $100 \mu\text{l}$ samples of purified myocardial PKC as described by Braconi et al. (1992). Results represent means $\pm$ S.E.M. for the indicated numbers of experiments carried out in triplicate determinations: † $P < 0.05$ versus basal PGI $_2$ production; * $P < 0.05$ versus corresponding control.

Inhibitor	6-Oxo-PGF $_{1\alpha}$ formation (pg/mg of protein)		ANP release (ng/mg of protein)		^{32}P incorporation into histone III-S (c.p.m./10 min)
	Control	PMA	Control	PMA	
None	89 ± 8.3 ($n = 20$)	$358 \pm 60.3^*$ ($n = 20$)	2.4 ± 0.07 ($n = 20$)	$3.8 \pm 0.17^*$ ($n = 20$)	13481 ± 1912 ($n = 6$)
Quinacrine ($10 \mu\text{M}$)	66 ± 7.3 ($n = 8$)	75 ± 12.4 ($n = 8$)	2.2 ± 0.19 ($n = 8$)	2.6 ± 0.23 ($n = 8$)	14145 ± 3014 ($n = 3$)
Indomethacin ($10 \mu\text{M}$)	$36 \pm 10.1^\dagger$ ($n = 10$)	58 ± 17.5 ($n = 10$)	2.4 ± 0.11 ($n = 10$)	2.6 ± 0.15 ($n = 10$)	14676 ± 3243 ($n = 3$)
Diclofenac ($1 \mu\text{M}$)	94 ± 15.4 ($n = 6$)	88 ± 12.2 ($n = 6$)	2.1 ± 0.27 ($n = 6$)	2.4 ± 0.31 ($n = 6$)	11001 ± 1388 ($n = 3$)

Figure 2 Effect of prostaglandins on ANP secretion

Cultured cardiomyocytes were incubated at 37 °C for 30 min with increasing concentrations of freshly prepared PGI₂ (●), PGE₂ (○) or PGF_{2α} (■), and ANP release was determined as described in the Materials and methods section. Results represent means ± S.E.M. for 4–6 experiments performed in triplicate determinations: **P* < 0.05 versus control. Contraction frequency was 42.1 ± 2.2 beats/min in control cells (*n* = 6) and 103.4 ± 9.7, 128.5 ± 10.1 and 57.3 ± 3.2 beats/min in cells incubated for 15 min with 1 μM PGI₂ (*n* = 6, *P* < 0.05), 0.1 μM PGE₂ (*n* = 4, *P* < 0.05) or 0.1 μM PGF_{2α} (*n* = 3, *P* < 0.05) respectively.

results not shown). Conversely, PMA-induced ANP release was substantially decreased in the presence of 1 μM concentrations of staurosporine, CGP 41251, calphostin C and chelerythrine (52.6–81.3 % inhibition; see Table 1), these last three compounds being noted for their highly specific PKC-inhibiting properties (Meyer et al., 1989; Herbert et al., 1990; Kobayashi et al., 1989) and having no effect on basal ANP secretion in this system. Similarly, PMA-induced ANP release was also inhibited in cells in which PKC had been previously down-regulated (Church et al., 1993). As shown in Table 1, pretreatment of cells for 24 h with phorbol diester severely impaired PMA-induced ANP secretion (89.1 % inhibition; *n* = 4), a result indicative of the role of PKC in this response.

The profile of PMA-induced ANP secretion described above is identical with that of PMA-induced 6-oxo-PGF_{1α} formation in this same system (Church et al., 1993). As shown in Table 1, PMA-induced 6-oxo-PGF_{1α} production was also abolished in the presence of 1 μM staurosporine, CGP 41251, calphostin C and chelerythrine (84.1–91.5 % inhibition), and was further abolished in PKC-down-regulated cells (95.5 % inhibition; *n* = 4). Indeed,

Figure 3 Effect of prostaglandins on cAMP formation

Cultured cardiomyocytes were incubated at 37 °C for 20 min with increasing concentrations of freshly prepared prostacyclin PGI₂ (●) or with 0.1 μM PGE₂ or 0.1 μM PGF_{2α} and assayed for cAMP formation as described in the Materials and methods section. Values represent means ± S.E.M. for 4–6 separate experiments carried out in duplicate determinations: **P* < 0.05 versus control. cAMP formation was 18.4 ± 1.4 pmol/mg of protein in control cells (*n* = 6) and 165.3 ± 22.7 and 50.6 ± 10.8 pmol/mg of protein in cells incubated for 20 min with 0.1 μM PGE₂ (*n* = 6, *P* < 0.05) or 0.1 μM PGF_{2α} (*n* = 4, *P* < 0.05) respectively.

the data presented in Figure 1 and Table 1 indicate that there exists a high degree of correlation between PMA-induced 6-oxo-PGF_{1α} formation and ANP release in these cultures (*r* = 0.93, *P* < 0.0005, *n* = 11).

Role of lipid mediators in phorbol-diester-induced ANP secretion

It has previously been shown that PKC-dependent prostaglandin formation is at the basis of angiotensin II-induced ANP secretion in cardiomyocytes (Church et al., 1994a), suggesting that endogenous prostaglandin formation also underlies PMA-induced ANP release in this system. Consistent with this, the PLA₂ inhibitor quinacrine (10 μM) decreased both PMA-induced 6-oxo-PGF_{1α} production and ANP secretion (Table 2), as did the cyclo-oxygenase inhibitors indomethacin (10 μM) and diclofenac (1 μM). These inhibitory effects were not induced by the non-specific inhibition of PKC itself, as quinacrine, indomethacin or diclofenac did not inhibit purified myocardial PKC at these concentrations (Table 2).

Incubation of cultured cardiomyocytes with 0.1 μM PMA leads to the formation of PGI₂, PGE₂ and most likely PGF_{2α}

Table 3 Effect of PMA on cAMP formation and inhibition by staurosporine, indomethacin and diclofenac

Cultured cardiomyocytes were incubated for 20 min with or without 0.1 μM PMA in the absence or presence of staurosporine (1 μM), indomethacin (10 μM) and diclofenac (1 μM) and assayed for cAMP formation as described in the Materials and methods section. Results, expressed in pmol/mg of cell protein, represent the means ± S.E.M. for the indicated numbers of experiments carried out in duplicate determinations: **P* < 0.05 versus control.

Treatment	cAMP production (pmol/mg of protein)				
	None	Staurosporine (1 μM)	Quinacrine (10 μM)	Indomethacin (10 μM)	Diclofenac (1 μM)
Control	17.1 ± 0.85 (<i>n</i> = 6)	15.5 ± 1.34 (<i>n</i> = 3)	12.7 ± 0.92* (<i>n</i> = 4)	18.9 ± 1.79 (<i>n</i> = 4)	17.4 ± 1.98 (<i>n</i> = 4)
PMA (0.1 μM)	30.7 ± 1.16* (<i>n</i> = 6)	17.6 ± 1.95 (<i>n</i> = 3)	14.7 ± 1.44 (<i>n</i> = 4)	19.9 ± 1.86 (<i>n</i> = 4)	17.0 ± 0.66 (<i>n</i> = 4)

Figure 4 Effect of cAMP analogues on ANP secretion

Cultured cardiomyocytes were incubated at 37 °C for 30 min with increasing concentrations of dibutyl cAMP (dB-cAMP; ○), R_p -cAMPS (●) or R_p -cAMPS in the presence of 0.1 μ M PMA (■), and ANP release was determined as described in the Materials and methods section. Values represent means $\pm$ S.E.M. for 3–4 separate experiments carried out in triplicate determinations: * P < 0.05 versus control (Ctrl).

(Church et al., 1993), prostaglandins that promote ANP secretion in this system (Church et al., 1994a). As shown in Figure 2, prostaglandin-induced ANP release displayed the rank order of potency $\text{PGF}_{2\alpha} > \text{PGE}_2 > \text{PGI}_2$ (EC_{50} = 12 nM, 32 nM and 240 nM respectively), responses that for PGI_2 and PGE_2 were accompanied by substantial increases in the rate of spontaneous cellular contraction (see legend to Figure 2). As compared with PGE_2 and PGI_2 , $\text{PGF}_{2\alpha}$ elicited a smaller chronotropic effect (from 42.1 ± 2.2 to 57.3 ± 3.2 beats/min; n = 3), yet induced the largest response in terms of ANP secretion.

Role of cAMP in PMA- and prostaglandin-induced ANP secretion

The augmentation of the rate of spontaneous cellular contraction accompanying PMA- and prostaglandin-induced ANP secretion

suggests that these compounds promote ANP release via an increase in cAMP, a known modulator of cardiomyocyte contraction frequency. As shown in Figure 3, PGI_2 induced a 4-fold increase in cardiomyocyte cAMP formation, a response exhibiting an EC_{50} of 0.2 ± 0.04 μ M (n = 4) and that was not observed in the absence of 0.5 mM IBMX (n = 3). PGE_2 displayed similar cAMP-elevating properties (see legend to Figure 3), as did 0.1 μ M forskolin, a compound that simultaneously increased myocyte contraction frequency (from 54.1 ± 2.3 to 110.4 ± 12.1 beats/min; n = 6) and ANP secretion (from 1.8 ± 0.21 to 2.7 ± 0.23 ng/mg of protein; n = 5; results not shown). In this context, the simultaneous determination of cAMP formation and ANP release could not be carried out, as 0.5 mM IBMX induced a marked change in cell morphology, abolishing spontaneous cellular contraction within 5 min of application and inhibiting basal ANP secretion (from 1.9 ± 0.21 to 1.4 ± 0.20 ng/mg of protein; n = 3; results not shown). Although the reason for this is unclear, a high dose of forskolin (10 μ M) induced an identical response (n = 3; results not shown), suggesting that large or prolonged elevations in cAMP production are toxic to cultured cells. Overall, 0.1 μ M $\text{PGF}_{2\alpha}$ induced the smallest increase in prostaglandin-induced cAMP formation in these cultures (see legend to Figure 3).

Interestingly, 0.1 μ M PMA also displayed cAMP-stimulating effects in these cells (Table 3). As for PGI_2 -induced cAMP formation, PMA-induced cAMP production was not observed in the absence of 0.5 mM IBMX, and, as for PMA-induced ANP release, the response was inhibited by 1 μ M staurosporine (72% inhibition), 10 μ M indomethacin (87% inhibition) or 1 μ M diclofenac (95% inhibition; see Table 3). Although PMA-induced cAMP formation was further inhibited by 10 μ M quinacrine (74% inhibition; n = 4), this compound decreased basal cAMP production by 39% (P < 0.05), suggesting that quinacrine also inhibits a cardiac adenylate cyclase. Finally, the response was found to be specific for the 4 β -phorbol diester, as 1 μ M 4 α -phorbol had no effect on cAMP production in these cells [control 17.8 ± 1.5 pmol/mg of protein; 4 α -phorbol (1 μ M), 18.1 ± 1.4 pmol/mg of protein; n = 3; results not shown].

The hypothesis that cAMP formation is responsible for PMA-induced ANP secretion was ultimately tested by incubating cardiomyocytes with 0.1 μ M PMA in the presence of R_p -cAMPS,

Figure 5 Effect of PMA, PGI_2 and PGE_2 on $[\text{Ca}^{2+}]_i$ transients in spontaneously beating cardiomyocytes

$[\text{Ca}^{2+}]_i$ determinations were carried out by using the fluorescent probe fura-2 as described in the Materials and methods section. Panels represent segments of continuous tracings collected over a 15 min interval (left-hand side of each tracing shows recording from t = 0 to t = 30 s; right-hand side shows recording from t = 15 min to t = 15 min 30 s; segments are separated by a //). When compared with control cells (a), cells incubated with 0.1 μ M PMA (b), 1 μ M PGI_2 (c) or 0.1 μ M PGE_2 (d) exhibited a marked increase in the frequency of spontaneous Ca^{2+} transients at 15 min. Tracings are representative of results obtained in 4–5 separate primary cultures.

a membrane-permeant phosphodiesterase-resistant cAMP analogue displaying cAMP-antagonizing properties (Botelho et al., 1988). As shown in Figure 4, 10 μM R_p -cAMPs had no effect on basal ANP secretion, yet decreased PMA-induced ANP release by 65% ($\pm 16.9\%$; $n = 4$). Conversely, a 10 μM concentration of the cAMP-mimetic agent dibutyryl cAMP exhibited an opposite effect, augmenting cardiomyocyte ANP secretion within 15 min of being applied to cell monolayers ($+0.5 \pm 0.10$ ng/mg of cell protein; $n = 4$; see Figure 4). Taken together, and in view of the fact that 10 μM R_p -cAMPs displayed no measurable effect on purified myocardial PKC activity on its own (control, 12866 ± 2155 c.p.m./10 min; 10 μM R_p -cAMPs, 12932 ± 2097 c.p.m./10 min; $n = 3$) and failed to affect PMA-induced 6-oxo-PGF_{1 α} formation ($+290 \pm 51$ and $+269 \pm 46$ pg/mg of protein in the absence and presence of 10 μM R_p -cAMPs respectively; $n = 3$; results not shown), these results suggest that prostaglandin-dependent cAMP formation is at least partly responsible for PMA-induced ANP secretion in spontaneously beating cultured cardiomyocytes.

Role of $[\text{Ca}^{2+}]_i$ in PMA- and prostaglandin-induced ANP secretion

The influence of $[\text{Ca}^{2+}]_i$ on ANP secretion was evaluated by comparing the effects of PMA, PGI₂, PGE₂ and PGF_{2 α} on cellular Ca^{2+} levels. As shown in Figure 5, Ca^{2+} fluorimetry using the Ca^{2+} -sensitive fluorescent probe fura-2 indicated that cultured cardiomyocytes were subject to spontaneously occurring fluctuations in $[\text{Ca}^{2+}]_i$ occurring at a frequency of 34.2 ± 1.3 transients/min ($n = 10$) and varying between 149 ± 7.8 nM (basal diastolic, $n = 10$) and 252 ± 6.7 nM (basal systolic, $n = 10$). Indeed, cultured cardiomyocytes behaved like a syncytium, such that each spontaneous contraction was accompanied by a single Ca^{2+} transient or 'spike' displaying a mean amplitude of 104 ± 5.7 nM at the beginning of each data-collection interval ($n = 10$; see left-hand side of Figure 5a). After continuous exposure to u.v. excitation, the mean amplitude of these transients decreased to 89 ± 1.9 nM at 15 min ($n = 10$, $P < 0.05$ versus initial controls; see right-hand side of Figure 5a).

As illustrated in Figure 5(b), a 0.1 μM concentration of PMA had no immediate effect on cardiomyocyte $[\text{Ca}^{2+}]_i$. However, a 15 min exposure of fura-2-loaded monolayers to 0.1 μM PMA led to a marked decrease in the amplitude of spontaneously occurring Ca^{2+} transients (to 57 ± 3.9 nM; $n = 10$, $P < 0.05$ versus untreated controls), a response that was accompanied by a significant increase in the rate of Ca^{2+} transient occurrence (from 35.0 ± 1.7 to 44.2 ± 0.5 transients/min; $n = 6$, $P < 0.05$). As shown in Figures 5(c) and 5(d), exposure of cells to either 1 μM PGI₂ or 0.1 μM PGE₂ induced a similar response, insofar as both PGI₂ and PGE₂ had no immediate effect on cardiomyocyte $[\text{Ca}^{2+}]_i$, yet also increased the frequency of Ca^{2+} -transient occurrence at 15 min [from 29.2 ± 4.1 to 44.0 ± 2.9 transients/min ($n = 5$, $P < 0.05$) and from 30.6 ± 3.0 to 42.5 ± 2.1 transients/min ($n = 7$, $P < 0.05$) respectively]. Unlike 0.1 μM PMA, however, neither 1 μM PGI₂ nor 0.1 μM PGE₂ displayed a negative effect on the amplitude of spontaneously occurring Ca^{2+} transients at 15 min [76 ± 3.6 nM ($n = 5$) and 106.2 ± 10.2 nM ($n = 8$) respectively]. Indeed, when compared with controls, 0.1 μM PGE₂ induced a slight (but insignificant) increase in Ca^{2+} transient amplitude at 15 min at 4 out of 8 experiments.

Although PMA, PGI₂ and PGE₂ had no immediate effect on cardiomyocyte $[\text{Ca}^{2+}]_i$, and incubation of cells with these agents resulted in 0–3 min tracings identical with those obtained with vehicle (Figure 6a), PGF_{2 α} induced a markedly different effect on cellular $[\text{Ca}^{2+}]_i$. As shown in Figure 6(b), 0.1 μM PGF_{2 α} induced

Figure 6 Effect of PGF_{2 α} on $[\text{Ca}^{2+}]_i$ in spontaneously beating cardiomyocytes in the presence and absence of nifedipine

$[\text{Ca}^{2+}]_i$ determinations were carried out by using the fluorescent probe fura-2 as described in the Materials and methods section. Tracings recording the effects of vehicle (a), and of 0.1 μM PGF_{2 α} in the absence (b) or the presence (c) of nifedipine, are representative of results obtained in 4–5 separate primary cultures. PGF_{2 α} was dissolved in methyl chloride, dried down under N₂ and resuspended in Krebs–Ringer buffer containing 2% BSA by vigorous vortex-mixing.

an immediate increase in mean basal diastolic $[\text{Ca}^{2+}]_i$ (from 147.2 ± 9.6 to 192.3 ± 11.1 nM; $n = 10$), a response that reached a peak within 20 s of applying PGF_{2 α} to the cells and that was followed by a rapid return to initial diastolic $[\text{Ca}^{2+}]_i$ levels. Interestingly, the response was unaffected by either 0.1 μM nifedipine (Figure 6c) or 2 mM EGTA (results not shown), suggesting that, unlike PGI₂ and PGE₂, PGF_{2 α} induces the release of Ca^{2+} from intracellular stores and/or temporarily compromises Ca^{2+} transport into the sarcoplasmic reticulum in this preparation. Not surprisingly, this difference could also be demonstrated at the level of ANP secretion. As shown in Table 4, nifedipine (0.1 μM) partially or fully inhibited PMA-, PGI₂- and PGE₂-induced ANP release without affecting PGF_{2 α} -induced peptide secretion, suggesting that PGI₂ and/or PGE₂, but not PGF_{2 α} , are the autocrine mediators underlying PMA-induced ANP release in these cells. Interestingly, nifedipine had no effect on PMA-induced 6-oxo-PGF_{1 α} formation in the same cells

Table 4 Effect of nifedipine on PMA- and prostaglandin-induced ANP secretion

Cultured cells were incubated for 30 min with 0.1 μ M PMA, 1 μ M PGI₂, 0.1 μ M PGE₂ or 0.1 μ M PGF_{2 α} in the absence or presence of 0.1 μ M nifedipine. ANP secretion was determined as described in the Materials and methods section. Results, expressed in ng/mg of cell protein, represent means $\pm$ S.E.M. for the indicated numbers of experiments performed in triplicate determinations: * P < 0.05 versus basal ANP secretion; † P < 0.05 versus effect of nifedipine alone.

Treatment	ANP release (ng/mg of protein)				
	None	PMA (0.1 μ M)	PGI ₂ (1 μ M)	PGE ₂ (0.1 μ M)	PGF _{2α} (0.1 μ M)
None	2.2 $\pm$ 0.09 (n = 4)	3.5 $\pm$ 0.22* (n = 4)	2.8 $\pm$ 0.13* (n = 4)	2.9 $\pm$ 0.18* (n = 4)	3.4 $\pm$ 0.34* (n = 4)
Nifedipine (0.1 μ M)	2.0 $\pm$ 0.17 (n = 3)	2.7 $\pm$ 0.29 (n = 3)	2.2 $\pm$ 0.34 (n = 3)	2.3 $\pm$ 0.32 (n = 3)	3.2 $\pm$ 0.30† (n = 3)

(+312 $\pm$ 72 and +284 $\pm$ 59 pg/mg of protein in the absence and presence of 0.1 μ M nifedipine respectively; n = 3, results not shown), further illustrating that prostaglandin-mediated Ca²⁺ influx occurs prior to ANP release.

DISCUSSION

The present results suggest that PMA-induced ANP release is a PKC-dependent prostaglandin-mediated autocrine process involving a prostanoid receptor functionally coupled to adenylate cyclase. This is supported by evidence that incubation of cardiomyocytes with PMA augments prostaglandin generation, cAMP formation, contraction frequency and ANP release, and that both PMA-induced cAMP production and ANP secretion are abolished in the presence of the cyclo-oxygenase inhibitors diclofenac and indomethacin. Indeed, PMA-induced prostaglandin formation and ANP release exhibit strikingly similar kinetic and pharmacological profiles. In addition, application of exogenous PGI₂, PGE₂ and PGF_{2 α} induces marked increases in cAMP formation, contraction frequency and ANP release in these cultures, whereas both dibutyl cAMP and forskolin promote ANP secretion in these cells. Finally, PGI₂ and PGE₂ appear to be the lipid mediators underlying this autocrine effect, as PGF_{2 α} promotes a marked increase in [Ca²⁺]_i in these cultures, whereas PGI₂, PGE₂ and PMA do not. Conversely, PMA-, PGI₂- and PGE₂-induced ANP secretion are partially or fully abolished by 0.1 μ M nifedipine, whereas PGF_{2 α} -induced peptide release is unaffected by this compound.

These findings are consistent with previous reports indicating that PKC activation leads to rapid PGI₂ and PGE₂ formation in cultured rat cardiomyocytes (Braconi et al., 1992; Church et al., 1993, 1994a), and are also in agreement with the finding that PMA, PGE₂ and PGF_{2 α} induce positive chronotropic effects and/or ANP secretion either in the rat myocardium or in cultured rat cardiomyocytes (Metsä-Ketelä, 1981; Dösemeci et al., 1988; Gardner and Schultz, 1990; Ruskoaho et al., 1991). Moreover, our results confirm that PGI₂ also induces ANP secretion in this tissue (Church et al., 1994a), and, more interestingly, suggest that prostaglandin-mediated activation of the cAMP effector system and subsequent Ca²⁺-channel activation is at the basis of PKC-mediated ANP release in these cells. Substantiating this conclusion, PMA-induced ANP secretion was decreased in the presence of cyclo-oxygenase inhibitors, R_p-cAMPS and nifedipine, whereas others have shown that PMA, PGI₂ and cAMP activate voltage-gated Ca²⁺ channels in cultured cardiomyocytes (Lacerda et al., 1988; Trautwein and Heschler, 1990; Allouatti et al., 1991), that PGI₂ induces increased cAMP formation in myocardial tissue (Metsä-Ketelä,

1981), and that Ca²⁺-channel activation leads to ANP release in these cells (Matsubara et al., 1988).

We have previously suggested that PKC-mediated phospholipase A₂ activation is responsible for PMA-induced prostaglandin formation in cultured cardiomyocytes (Church et al., 1993). Although it is likely that other phospholipases are also involved in this process (D. J. Church and U. Lang, unpublished work), the present results imply that prostaglandin synthesis leads to Ca²⁺ influx, an event known to promote prostaglandin formation in other systems. Interestingly, both nifedipine and R_p-cAMPS failed to affect PMA-induced 6-oxo-PGF_{1 α} production in cultured cardiomyocytes, further demonstrating that cAMP-sensitive Ca²⁺-influx-dependent ANP release lies downstream of PKC-mediated prostaglandin formation in these cells. Interestingly, this also suggests that myocardial prostaglandin synthesis is relatively insensitive to modifications of [Ca²⁺]_i, a conclusion in agreement with the finding that the Ca²⁺ ionophore A23187 is a remarkably poor activator of prostaglandin synthesis in these cultures (Braconi et al., 1992). Taken together, it can be surmised that, in systems where [Ca²⁺]_i levels are constantly oscillating, Ca²⁺ would be a difficult-to-control messenger for the activation of cellular prostaglandin formation.

Both PGI₂ and PGE₂ are prostanoids noted for their adenylate cyclase-stimulating effects, a property carried out via the activation of specific cell-surface receptors (respectively termed IP and EP receptors: see Coleman et al., 1984; Vasseaux et al., 1992; Virgolini et al., 1992). In this context, the nature of the prostaglandin receptor involved in PMA-induced ANP release remains to be fully elucidated. The rank order of potency of prostaglandin-induced ANP secretion (PGF_{2 α} > PGE₂ > PGI₂) suggests that a PGF_{2 α} receptor is at the basis of the response (Coleman et al., 1984). However, although PGF_{2 α} induced moderate increases in cAMP formation and contraction frequency in these cells, it rapidly increased basal diastolic [Ca²⁺]_i levels, whereas PMA failed to do so. This suggests that PGF_{2 α} is not the eicosanoid mediating the secretory effect of PMA in this system, a conclusion in agreement with the fact that only trace amounts of PGF_{2 α} are present in 3-day-old cardiomyocyte cultures (Church et al., 1993). Indeed, nifedipine decreased PMA-, PGI₂- and PGE₂-induced ANP secretion without affecting PGF_{2 α} -induced ANP release, confirming that PGF_{2 α} -induced ANP secretion takes place via a Ca²⁺-channel-independent pathway (Kovacic-Milivojevic et al., 1991), more likely via a mechanism involving either the mobilization of Ca²⁺ from intracellular stores and/or the temporary compromise of Ca²⁺ transport towards the exterior or into the sarcoplasm reticulum.

The concentration of exogenous PGI₂ necessary to induce ANP secretion was approx. 1000-fold higher than the amount of

6-oxo-PGF_{1 α} produced by PMA-stimulated cardiomyocytes, confirming our previous report indicating that PGE₂ is a more likely candidate for prostaglandin-dependent PKC-mediated ANP release (Church et al., 1994a). In partial agreement with this, 0.1 μ M concentrations of PGE₂ suffice for stimulating ANP secretion in cultured cardiomyocytes (EC₅₀ = 32 $\pm$ 5 nM; *n* = 4). Nevertheless, PGE₂ concentrations in the incubation media of PMA-stimulated cardiomyocytes only reach these levels after maximal ANP secretion has already taken place (ambient PGE₂ concentration is $\sim$ 1 nM following incubation with 0.1 μ M PMA for 1 h; see Church et al., 1993). In this context, PGE₂ is highly lipophilic, and it is likely that autocrine production leads to local concentrations higher than 1 nM at the level of the cardiomyocyte itself.

Consistent with the view that PGI₂ is a poor candidate for mediating PMA-induced ANP secretion, the relatively high EC₅₀ of PGI₂-induced cAMP formation and ANP release suggests that this eicosanoid is not acting through a myocardial IP receptor. Although PGI₂ is an unstable compound, and caution should be exercised in interpreting results, the rank order of potency for prostaglandin-induced cAMP formation (PGE₂ > PGI₂ > PGF_{2 α}) suggests that a myocardial EP receptor is at the basis of PMA-induced ANP secretion in these cells. Arguing against this point, however, 0.1 μ M PMA induced a 32% decrease in cellular Ca²⁺ treatment amplitude, whereas both PGE₂ and PGI₂ failed to do so. In this context, it is likely that PMA also exerts prostaglandin-independent [Ca²⁺]_i-modifying properties, such that the negative effect of PMA on cellular Ca²⁺-transient amplitude cannot be mimicked with exogenously applied PGE₂ or PGI₂, a hypothesis reinforced by reports indicating that phorbol diesters decrease Ca²⁺ liberation from the cardiac sarcoplasmic reticulum, whereas cAMP-elevating agents display an opposite effect (Rogers et al., 1990). In summary, it appears that cardiomyocytes contain multiple prostaglandin receptors and that PGE₂ and/or PGI₂ mediate PMA-induced ANP secretion via the EP- and/or IP-receptor-induced activation of adenylate cyclase.

PMA-induced ANP release was substantially decreased in the presence of R_p-cAMPS, pointing to a role for cAMP in PKC-mediated ANP secretion. In this regard, PMA-induced cAMP formation was abolished in the presence of indomethacin and was not observed in the absence of IBMX, suggesting that cAMP production is due to prostaglandin formation and not to phosphodiesterase inhibition (Houslay, 1991). Nevertheless, the effect of cAMP on myocardial ANP release remains to be fully investigated (Ruskoaho, 1992). cAMP induces positive chronotropic effects in this tissue, a response that is strongly correlated with ANP release (Bilder et al., 1989). Consistent with this view, isoprenaline, calcitonin-gene-related peptide and dibutyl cAMP have been shown to induce ANP release and/or pro-ANP gene transcription in beating rat heart or cardiomyocytes (Wong et al., 1988; Claycomb, 1988; Schiebinger and Santora, 1989), and we found that dibutyl cAMP and forskolin augmented ANP secretion in the preparations used in the present study. Nevertheless, it has previously been reported that forskolin has only a slight effect on ANP release in isolated atria (Ruskoaho et al., 1991), or even inhibits ANP secretion altogether (Shields and Glembotski, 1989). In this light, the finding that forskolin exhibits concentration-dependent stimulatory and inhibitory properties appears to explain some of the discrepancies found in the current literature. Our results clearly show that ANP secretion is augmented in all experiments in which an increase in the rate of cellular contraction is observed. As a high dose of forskolin (10 μ M) inhibits spontaneous cellular contraction and ANP secretion, it appears that moderate increases in cAMP lead to

increased ANP release, whereas high or prolonged increases in cAMP are toxic to cultured cells. A similar toxic effect is likely to explain reports showing that IBMX inhibits agonist-induced ANP secretion in spontaneously beating cultured cardiomyocytes (Kovacic-Milivojevic et al., 1991), as this compound clearly inhibited spontaneous cellular contraction in our study. Interestingly, we have recently shown that nifedipine-sensitive Ca²⁺ influx is an absolute requirement for both forskolin- and dibutyl cAMP-induced ANP secretion in these cultures (Church et al., 1994b), illustrating that the effects of cAMP on ANP release are indeed mediated by the activation of a myocardial Ca²⁺ channel.

Clearly, further studies need to be undertaken in order to ascertain whether PKC-dependent prostaglandin-induced activation of myocardial adenylate cyclase is of importance in agonist-induced ANP secretion. In view of the fact that the vasoactive peptide angiotensin II displays a marked chronotropic effect in these cultures (Kem et al., 1991), augmenting both prostaglandin production and ANP release in the process (Church et al., 1994a), prostaglandin-mediated cAMP formation may be an important component in understanding the myocardium-stimulating properties of this and similar peptides.

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