Protein kinase-dependent and \(Ca^{2+}\)-independent cAMP inhibition of ANP release in beating rabbit atria

Xun Cui, Jin Fu Wen, Jying Yu Jin, Wen Xie Xu, Sung Zoo Kim, Suhn Hee Kim, Ho Sub Lee, and Kyung Woo Cho

Department of Physiology, Institute for Medical Sciences, Jeonbug National University

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There have been reports on the variable modulators for the control of ANP release (23). However, the specific control mechanism for ANP release must be defined. The most prominent activator for atrial secretion of ANP has been shown to be the stretch and/or release of atrial wall (2, 10, 16). However, the intracellular mechanism responsible for the activation of ANP release by mechanical stimulation is unknown.

The potential roles of cyclic nucleotides and \(Ca^{2+}\) in the regulation of ANP release have been subjects of interest. Recently we found that both cGMP and \(Ca^{2+}\) are negative regulators for atrial myocytic release of ANP (4, 14, 17). There are diverse reports on the effects of cAMP in the regulation of ANP secretion. Forskolin, an activator of adenylyl cyclase (AC), has been shown to decrease ANP release from cultured atrial myocytes (12, 20, 30) and perfused rat heart (25); 3-isobutyl-1-methylxanthine (IBMX), a nonselective inhibitor of adenylyl cyclase (BrcAMP) (12, 30), have also been shown to inhibit ANP secretion. In contrast, it has also been shown that cAMP-elevating agents (1, 5, 24) and cell membrane-permeant cAMP analogs (1, 5, 27, 28) increase ANP secretion in cultured cardiac myocytes (5), sliced atria (1), isolated atria (27, 28), and perfused rat heart (24). Hence, the current understanding of cAMP-dependent regulation of ANP secretion is controversial. Further, the mechanism has to be defined.

Diverse effects of \(Ca^{2+}\) on ANP secretion have also been reported. Increases in \(Ca^{2+}\) influx via L-type \(Ca^{2+}\)-channel activation by BAY K 8644 increased ANP secretion in perfused heart (24, 26), isolated beating atria (28, 29), and cultured atrial myocytes (18). Similarly, L-type \(Ca^{2+}\)-channel blockers inhibit ANP secretion in perfused heart (26) and isolate beating atria (28, 29) and atrial myocytes (5, 18). Increased extracellular \(Ca^{2+}\) also results in an increase in ANP secretion in beating atria (15). In contrast, \(Ca^{2+}\) has also been reported to be a negative regulator for ANP secretion in perfused heart (13, 25) and isolated beat-
PROTEIN KINASE-DEPENDENT INHIBITION OF ANP RELEASE

METHODS

Beating, Perfused Rabbit Atrial Preparation

New Zealand White rabbits were used. An isolated perfused atrial preparation was prepared using methods described previously (3, 6) that allow atrial pacing and measurements of changes in atrial volume during contraction (stroke volume), pulse pressure, transmural extracellular fluid (ECF) translocation, cAMP efflux, and ANP secretion. The atrium was perfused with HEPES buffer solution by means of a peristaltic pump (1 ml/min).

Experimental Protocols

The atria were perfused for 60 min to stabilize ANP secretion. [3H]inulin was introduced to the pericardial fluid 20 min before the start of the sample collection (3). The perfusate was collected at 2-min intervals at 4°C for analyses. In one series of experiments, atrial pacing at 0.8, 1, 1.3, 1.6, and 2 Hz was performed consecutively for 2 min at each frequency with repetitive frequency changes, which were spaced by 2 min of 0.8-Hz pacing. Experiments were carried out using three groups of atria. The control cycle (a 12-min period) was followed by infusion of the cAMP-elevating agents forskolin (1 μM; group 1, n = 6; Fig. 1) or IBMX (1 mM; group 2, n = 6; Fig. 2). The effects were evaluated after two cycles (24 min) of administration of the agent. For the time control, vehicle was introduced, and values obtained during the periods corresponding to the control and experimental observations were compared (group 3, n = 6).

In another series of experiments, the atria were paced at 1.3 Hz. Experiments were carried out using 22 groups of atria. The control cycle (a 12-min period) was followed by an infusion of the cAMP-elevating agents forskolin or IBMX for 36 min (forskolin, 1 μM; group 1, n = 14; Figs. 3, 4, and 10; IBMX, 1 mM, group 2, n = 4).

In other groups of experiments, to define the involvement of L-type Ca2+ channels or protein kinases, 36 min of infusion of inhibitors was followed by an infusion of forskolin or vehicle in the presence of the prior agent. The following inhibitors were used: 1) L-type Ca2+ channel inhibitors including nifedipine (1 μM, group 3, n = 9; Fig. 5A and group 4, n = 7, Fig. 5B, and Figs. 6 and 10) and diltiazem (10 μM, group 5, n = 12 and group 6, n = 6, Fig. 10B); 2) protein kinase A (PKA) inhibitors including KT-5720 (3–6 μM, group 7, n = 6, Fig. 10B) and Rp-adenosine 3’,5’-cyclic monophosphorothioate (Rp-cAMPS; 62.5–125 μM, group 8, n = 5; Fig. 10); 3) a specific protein kinase C (PKC-α) inhibitor, Gö-6976 (1 μM, group 9, n = 5; Fig. 10, and group 10, n = 3; Fig. 10B); 4) the Ca2+/calmodulin kinase inhibitor KN-62 (10 μM, group 11, n = 3, Fig. 10B); 5) the nonspecific protein kinase inhibitor staurosporine at 0.01 μM (group 12, n = 5, and group 13, n = 3, Fig. 10B), 0.1 μM (group 14, n = 9, and group 15, n = 4, Fig. 10B), and 1 μM (group 16, n = 7, Fig. 8A; group 17, n = 6, Figs. 8B, 9, 10); and 6) PKC activator phorbol 12-myristate 13-acetate (PMA; 60 nM, group 18, n = 5; Fig. 10B) and its specific inhibitor Gö-6976 (1 μM, group 19, n = 4, and group 20, n = 3; Fig. 10B) and nonspecific inhibitor staurosporine (0.1 μM, group 21, n = 3, Fig. 10B) were also used.

In another group of experiments for the control (Figs. 7 and 10), vehicle only was infused for 72 min. The effects of forskolin in the presence of modulating agent or vehicle were compared with the period before and the third 12-min period after forskolin infusion. For the modulating-agent control, values obtained during the periods corresponding to the control and experimental observations were compared.

Radioimmunoassay of cAMP

Production of cAMP was measured by equilibrated radioimmunoassay (6). Briefly, standards or samples were taken up in a final volume of 100 μl of 50 mM sodium acetate buffer (pH 4.8) containing theophylline (8 mM), and then 100 μl of diluted cAMP antiserum (Calbiochem-Novabiochem, San Diego, CA) and iodinated 2’-O-monomuscullin-adenosine 3’,5’-cyclic monophosphate tyrosyl methyl ester (125I-ScAMP-TME, 10,000 counts/min per 100 μl) were added and incubated for 24 h at 4°C. For the acetylation reaction, 5 μl of a mixture of acetic anhydride and triethylamine (1:2 dilution) was added to the assay tube before antimser and tracer were also added. The bound form was separated from the free form by charcoal suspension. 125I-ScAMP-TME was prepared as described previously (31). Briefly, 2 μg of ScAMP-TME (Sigma, St. Louis, MO) was introduced into a vial containing 100 mM phosphate buffer (pH 7.4), and 1 μCi of 125I-Na (Amersham International, Buckinghamshire, UK) was added. Chloramine-T (0.4 mg/ml) was added to the reaction vial (total reaction volume = 50 μl) and mixed gently, and 1 min later the reaction was terminated with sodium metabsulfit (0.2 mg/ml) and NaI (5 mM). The reaction mixture was immediately applied to a Sephadex G-10 column (1 × 20 cm) previously washed with 10 mM phosphate buffer. 125I-ScAMP-TME was eluted with 10 mM phosphate buffer containing 150 mM NaCl (pH 7.4) and stored at −20°C until use. Immediately before it was used, 125I-ScAMP-TME was repurified by high-performance liquid chromatography on a reversed-phase μBondapak column (Waters Associates, Milford, MA) with a linear gradient (0–60% acetonitrile in 0.1% trifluoroacetic acid) elution. Radioimmunoassay for cAMP was done on the day of experiments, and all samples from one experiment were analyzed in a single assay. Nonspecific binding was <2.0%. The 50% intercept was at 16.50 ± 0.79 fmol/tube (n = 10). The intra- and interassay coefficients of variation were 5.0% (n = 10) and 9.6% (n = 10), respectively. The amount of cAMP efflux was expressed as picomoles CAMP per minute per gram atrial tissue. The molar concentration of cAMP efflux in terms of ECF translocation, which may reflect the concentration of CAMP in the interstitial space fluid (2, 3, 6), was calculated as CAMP efflux (μM) = CAMP (in pmol·min−1·g−1·ECF translocated) in (μl·min−1·g−1).

Preparation of Samples for cAMP Assay

For the preparation of perfusates, 100 μl of the perfusate was treated with trichloroacetic acid (900 μl) to be a final
concentration of 6% for 15 min at room temperature and was centrifuged at 4°C. The supernatant (500 μl) was transferred to a polypropylene tube, extracted with water-saturated ether (1 ml) three times, and then dried using a SpeedVac concentrator (Savant, Farmingdale, NY). The dried samples were resuspended with sodium acetate buffer.

Radioimmunoassay of ANP

Immunoreactive ANP in the perfusate was measured by a specific radioimmunoassay as described previously (3). The secreted amount of immunoreactive ANP was expressed as nanograms of ANP per minute per gram of atrial tissue. The molar concentration of immunoreactive ANP in terms of the ECF translocation reflects the concentration of ANP in the interstitial space of the atrium and, therefore, indicates the rate of myocytic release of ANP into the surrounding paracellular space (2, 3). It was calculated as ANP released (μM) = immunoreactive ANP (in pg·min⁻¹·g⁻¹)/ECF translocated (in μl·min⁻¹·g⁻¹)·3.063 [mol wt of ANP (1–28)]. Most of the ANP secreted is processed ANP (3).

Statistical Analysis

Significant difference was compared using a two-way ANOVA analysis for repeated measures (Figs. 1 and 2, E-G, and Fig. 10A). Significant differences between paired data for a given pacing frequency (Figs. 1 and 2, A-D) and also the data of Figs. 3, 5, 7, and 8 were analyzed by repeated-measures ANOVA followed by Bonferroni’s multiple-comparison test. Student’s t-test for unpaired data (Fig. 10B) was also applied. Correlation coefficients were determined with the use of linear regression analysis. Statistical significance was defined as P < 0.05. The results are given as means ± SE.
RESULTS

Inhibition by cAMP-Elevating Agents of Myocytic ANP Release

Activation of adenylyl cyclase with forskolin inhibits ANP secretion. An increase in pacing frequency resulted in an increase in secretion of ANP concomitantly with translocation of the ECF, which is coincident with an increase in atrial stroke volume (see Fig. 1, A, B, and D). The concentration of ANP in perfusate in terms of the ECF translocation was 0.4–0.6 μM (see Fig. 1C). Because the secretion of ANP is related to the atrial work in beating atria (Ref. 3 and present data), the response of ANP secretion was analyzed as a function of atrial stroke volume (see Fig. 1E). Both secretion of ANP and translocation of the ECF were functions of atrial stroke volume (see Fig. 1, E and F). At higher atrial stroke volumes, the incremental changes in the secretion of ANP and translocation of the ECF showed a peak and fall (see Fig. 1, A and B). The change in ANP secretion was well correlated with translocation of the ECF (see Fig. 1G).

Forskolin inhibited the secretion of ANP (all P < 0.01 at 1.3–2.0 Hz; see Fig. 1A). The concentration of ANP in perfusate in terms of the ECF translocation was significantly decreased by forskolin (all P < 0.01; see Fig. 1C). Translocation of the ECF was increased at low (both P < 0.01 at 0.8 and 1.0 Hz) and decreased at higher (P < 0.01 at 1.6 Hz) atrial rates, respectively, by forskolin (see Fig. 1B). Atrial stroke volume was increased by forskolin (all P < 0.01 at 0.8–1.6 Hz; see Fig. 1D). Forskolin shifted relationships between the
secretion of ANP and atrial stroke volume or translocation of the ECF downward and rightward (both P < 0.01; see Fig. 1, E and G). This means that forskolin inhibits myocytic release of ANP. This is related to the decrease in the concentration of ANP shown in Fig. 1C. Forskolin shifted the relationship between translocation of the ECF and atrial stroke volume downward and rightward (P < 0.01; see Fig. 1F). For the time control, changes in secretion of ANP and translocation of the ECF in response to repetitive changes in pacing frequency were constant and stable. The responses of the parameters were reproducible during the periods corresponding to the control and experimental observations (differences between periods were not significant; n = 6).

Fig. 3. Effects of forskolin (FSK, 1.0 μM) on ANP secretion (A), ECF translocation (B), ANP concentration (C), cAMP efflux (D), cAMP concentration (E), atrial stroke volume (F), and pulse pressure (G) in perfused beating rabbit atria (1.3 Hz; n = 10). Values are means ± SE; *P < 0.01 vs. control period (Cont).

Fig. 4. Forskolin-induced changes in cAMP production and ANP release. A: forskolin (1.0 μM)-induced decrease in ANP concentration is coincident with an increase in cAMP production. Data were derived from Fig. 3. B: relationship between changes in cAMP production and ANP concentration: y = 0.246x + 14.366, R = 0.9669; P < 0.01. Data were derived from Fig. 4A. Relationship was analyzed with mean values of 14 experiments. Values are expressed as Δ% changes over the mean value of 2 periods before administration of forskolin. Fraction no, serial 2-min sample collections after the administration of forskolin.
Inhibitor of phosphodiesterase inhibits ANP secretion. IBMX inhibited the secretion of ANP (both \( P < 0.01 \) at 1.6 and 2.0 Hz; see Fig. 2A). The concentration of ANP was significantly decreased by IBMX (all \( P < 0.01 \) at 0.8–2.0 Hz; see Fig. 2C). Translocation of the ECF was increased at low and decreased at higher atrial rates without significance, respectively, by IBMX (see Fig. 2B). Atrial stroke volume was increased by
IBMX (all \( P < 0.01 \) at 0.8–1.3 Hz; see Fig. 2D). IBMX shifted relationships between the secretion of ANP and atrial stroke volume or translocation of the ECF downward and rightward (both \( P < 0.05 \); see Fig. 2, E and G). This means that IBMX inhibits myocytic release of ANP. IBMX shifted the relationship between translocation of the ECF and atrial stroke volume downward and rightward (\( P < 0.05 \); see Fig. 2F).

**Protein Kinase-Dependent Inhibition by cAMP of Myocytic ANP Release**

Relationship between changes in cAMP production and atrial release of ANP. To define more clearly the relationship between changes in cAMP production and

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**Fig. 6.** Forskolin-induced changes in cAMP production and ANP release in the presence of nifedipine. A: forskolin (1.0 \( \mu \text{M} \))-induced decrease in ANP concentration (release, ■) coincident with an increase in cAMP production (●) in the presence of nifedipine (1.0 \( \mu \text{M} \)). Effects of nifedipine alone on changes in ANP concentration (○) and cAMP production (□). Data were derived from Fig. 5, A and B. B: effects of nifedipine on the relationship between changes in cAMP production and ANP concentration: \( y = -0.327x - 1.427, R = 0.9848; P < 0.01 \). Data were derived from Fig. 6A. Relationship was analyzed with mean values of 9 experiments. Values are expressed as \( \Delta \% \) changes over the mean value of the 2 periods before administration of forskolin.

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**Fig. 7.** Time control for ANP secretion (A), ECF translocation (B), ANP concentration (C), cAMP efflux (D), cAMP concentration (E), and atrial dynamic changes (F and G).
release of ANP, another series of experiments have been done in atria paced at a fixed frequency. Forskolin decreased ANP secretion and myocytic ANP release (ANP concentration) concomitantly with increases in atrial cAMP efflux and dynamics in a time-dependent manner (see Fig. 3). IBMX revealed similar effects (n = 4; data not shown). The effects observed with the atria paced at a fixed frequency of forskolin on ANP secretion and atrial dynamics were similar to those observed with the atria paced at repetitive frequency.
changes. The responses were stable during the third cycle of the administration of forskolin. As shown in Fig. 4, inhibition by forskolin of myocytic ANP release was a function of elevation of cAMP production. Inhibition in atrial myocytic ANP release by forskolin was coincident with an increase in cAMP production (see Fig. 4A). Figure 4B shows an inverse relationship between the changes in cAMP production and ANP concentration.

A minor effect of L-type Ca$^{2+}$-channel blockade on cAMP-induced inhibition in ANP release. To define involvement of L-type Ca$^{2+}$ channels in inhibition of ANP release by forskolin, nifedipine was administered. L-type Ca$^{2+}$-channel blockade with nifedipine significantly increased ANP secretion and myocytic ANP release (see Fig. 5, $Aa$ and $Ac$). No significant change in cAMP production was observed by nifedipine (see Fig. 5, $Ad$ and $Ac$). Nifedipine decreased atrial dynamics (see Fig. 5, $Af$ and $Ag$). In the presence of nifedipine, forskolin inhibited ANP secretion and myocytic ANP release (see Fig. 5, $Ad$ and $Ac$) with concomitant increases in cAMP production (see Fig. 5, $Ad$ and $Ac$). Nifedipine attenuated a minor portion of forskolin-induced inhibition of ANP release (in difference in percent changes over the control, $51.57 \pm 4.34\%$ for nifedipine plus forskolin, $n = 9$, vs. $68.48 \pm 2.56\%$ for forskolin alone, $n = 14$; $P < 0.01$, Fig. 10B; in percent changes of the control, $P > 0.05$, Fig. 10A). In the presence of nifedipine, forskolin significantly increased cAMP production (see Fig. 5, $Ad$ and $Ac$). In the presence of nifedipine, forskolin-induced accentuation of atrial dynamics was not observed (see Fig. 5, $Af$ and $Ag$). Responses in cAMP production and atrial dynamics by forskolin were dissociated by Ca$^{2+}$-channel blockade. Nifedipine-induced changes in ANP release, cAMP production, and atrial dynamics were maintained stably during the period corresponding to forskolin infusion (see Fig. 5, $Ba-Bg$). As shown in Fig. 6A, in the presence of nifedipine, forskolin inhibited myocytic release of ANP concomitantly with an increase in cAMP production. The inverse relationship between cAMP production and ANP concentration was not changed by the treatment with nifedipine (Fig. 6B). The slopes of the relationships were not significantly different between the groups of forskolin and nifedipine plus forskolin ($-0.316 \pm 0.058$ for nifedipine plus forskolin, $n = 9$ vs. $-0.279 \pm 0.050$ for forskolin alone; $n = 14$; $P > 0.05$). Diltiazem (10 $\mu$M), another L-type Ca$^{2+}$-channel inhibitor attenuated slightly but not significantly forskolin-induced inhibition of ANP release (in difference in percent changes, $-57.64 \pm 4.88\%$ for diltiazem plus forskolin, $n = 12$ vs. $-68.48 \pm 2.56\%$ for forskolin alone, $n = 14$; $P = 0.051$, Fig. 10B). For the time control, changes in secretion of ANP, ECF cAMP, and atrial dynamics were constant and stable (see Fig. 7). The differences between control observations and periods corresponding to experimental observations were not significant.

Inhibition of protein kinases blocks cAMP-induced decrease in ANP release. To further dissect the pathway of cAMP-induced inhibition in ANP secretion, the atrium was treated with a nonspecific protein kinase inhibitor, staurosporine. At a dose of 1 $\mu$M but not 0.01 or 0.1 $\mu$M, staurosporine decreased ANP secretion, and the effect was stable and maintained after the third cycle of staurosporine (Fig. 9, vs. third cycle of staurosporine, see METHODS, Fig. 8). No significant change in cAMP production was observed by staurosporine alone. Staurosporine attenuated forskolin-induced inhibition of atrial ANP release in a concentration-dependent manner (Fig. 10). No significant change in forskolin-induced inhibition of ANP release in difference in percent changes, $-45.24 \pm 6.09\%$ for staurosporine plus forskolin, $n = 9$ vs. $-68.48 \pm 2.56\%$ for forskolin, $n = 14$; $P < 0.001$). In the presence of staurosporine (1 $\mu$M), forskolin slightly decreased ANP secretion and the concentration of ANP (see Fig. 8, $Aa$ and $Ac$). The responses were not significantly different from those of the atrium treated with staurosporine alone (see Fig. 8, $Ba-Bg$, and Fig. 10B). Percent changes of the forskolin-induced inhibition of ANP release were significantly attenuated by the staurosporine ($P < 0.05$; Fig. 10A). Forskolin significantly increased cAMP production in the presence of staurosporine (see Fig. 8, $Ad$ and $Ac$). Forskolin-induced increases in atrial dynamics were not observed in the presence of staurosporine (see Fig. 8, $Af$ and $Ag$). In this condition, forskolin rather decreased atrial dynamics. In the presence of staurosporine, forskolin-induced changes in atrial dynamics were dissociated from the increase in cAMP production. As shown in Fig. 9, in the presence of staurosporine, forskolin increased cAMP production without significant changes in ANP release, which indicates a dissociation of the relationship between cAMP production and ANP release. The slopes of the relationships were significantly different between the groups of forskolin and staurosporine plus forskolin ($-0.029 \pm 0.012$ for staurosporine plus forskolin, $n = 7$ vs. $-0.279 \pm 0.050$ for forskolin, $n = 14$; $P < 0.01$). Forskolin-induced decrease in the concentration of ANP was significantly attenuated in the presence of staurosporine (see Figs. 8, 9, and 10). The decrease in the concentration of ANP in the presence of staurosporine plus forskolin was not significantly different from that in the presence of staurosporine alone.

A minor effect of PKA inhibition on the cAMP-induced decrease in ANP release. To define involvement of the PKA activity in inhibition of ANP release by forskolin, the PKA-specific inhibitor KT-5720 or Rp-cAMP was administered. KT-5720 blocked forskolin-induced accentuation of atrial dynamics (in difference in percent changes over the control, $1.71 \pm 0.81\%$ for KT-5720 plus forskolin, $n = 6$ vs. $27.94 \pm 3.49\%$ for forskolin alone, $n = 14$; $P < 0.001$). KT-5720 slightly but not significantly attenuated forskolin-induced decrease in atrial ANP release (in difference in percent changes, $-62.84 \pm 4.08\%$ for KT-5720 plus forskolin,
n = 6 vs. -68.48 ± 2.56% for forskolin alone, n = 14; P > 0.05; Fig. 10B). Another PKA inhibitor, Rp-cAMPS, slightly but significantly attenuated the forskolin-induced decrease in ANP release (in percent changes of the control; P > 0.05, Fig. 10A; in difference in percent changes over the control, 4.05 ± 2.27% for Rp-cAMPS plus forskolin, n = 5 vs. 27.94 ± 3.49% for forskolin alone, n = 14; P < 0.01). In the presence of PKA inhibitors, forskolin significantly increased cAMP production (data not shown).

Absence of effect of inhibition of PKC-α or Ca²⁺/calmodulin kinase on cAMP-induced decrease in ANP release. To define the involvement of PKC in inhibition of ANP release by forskolin, Gö-6976, a specific PKC-α inhibitor, was administered. Gö-6976 failed to modulate the forskolin-induced decrease in ANP release (Fig. 10). KN-62, a specific Ca²⁺/calmodulin inhibitor, also failed to modulate the forskolin-induced decrease in ANP release (Fig. 10B).

An activation of PKC with PMA accentuates ANP release and its specific blockade blocks increase in ANP release. To test the effect of staurosporine and Gö-6976 in relation to an accentuation of ANP release by PKC activation, PMA, an activator of PKC, was administered. PMA accentuated atrial ANP release (Fig. 10B). In the presence of Gö-6976 or staurosporine (0.1 μM), PMA failed to accentuate atrial ANP release.
DISCUSSION

The present data indicate that cAMP inhibits atrial myocytic ANP release via protein kinase-dependent and L-type Ca\(^{2+}\)-channel-dependent and -independent signaling pathways in rabbit atria.

cAMP Inhibits Myocytic ANP Release

The present study clearly shows that cAMP-elevating agents inhibit release of ANP. Both AC activation with forskolin and PDE inhibition with IBMX inhibited release of ANP. Both agents decreased the concentration of ANP in perfusate in terms of the ECF translocation and shifted the relationship between release of ANP and translocation of the ECF downward and rightward. This means that cAMP-elevating agents inhibit myocytic release of ANP. An inhibition of myocytic ANP release by AC activation is a function of an increase in cAMP production. Because cAMP efflux reflects the intracellular cAMP content, amplitude of cAMP efflux appears to represent atrial cAMP production (6). The present data are in agreement with previous reports showing an inhibition in ANP secretion by forskolin (12, 20, 25, 30) or IBMX (12) in cultured atrial myocytes or perfused rat hearts. However, the present data contrast with the previous reports on the stimulation of ANP release by cAMP-elevating agents (1, 5, 24) in cultured cardiac myocytes, isolated atria, or perfused rat hearts. The reasons for the discrepancy are not clear at present. Differences in methodology and/or basal status of atrial or cellular dynamics may account for the discrepancy. Although intracellular cAMP has been claimed to be a negative or positive regulator for ANP release, the mechanism by which cAMP controls atrial myocytic ANP release must be defined.

cAMP Inhibits ANP Release by a Protein Kinase-Dependent and L-Type Ca\(^{2+}\)-Channel-Dependent and -Independent Mechanism

The present study clearly shows that cAMP inhibits myocytic ANP release via protein kinase-dependent signaling. The data also suggest that Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels may be involved in a minor portion of the cAMP-induced inhibition of ANP release. A major portion of cAMP-induced inhibition of ANP release is related to protein kinase-dependent signaling. Inhibition of PKA attenuated a minor portion of the cAMP-induced inhibition of ANP release, which may be relevant to the inhibition of L-type Ca\(^{2+}\)-channel activity. Gö-6976, a specific PKC-α inhibitor, and KN-62, a Ca\(^{2+}\)/calmodulin kinase inhibitor, failed to modulate cAMP-induced inhibition of ANP release. This means that the mechanism by which staurosporine blocks the cAMP-induced inhibition of ANP release may be related to protein kinase(s) other than PKA, PKC-α, and Ca\(^{2+}\)/calmodulin kinase.

Because the cAMP signaling pathway contains cAMP-related activation of L-type Ca\(^{2+}\) channels (11, 22) and also because an increase in the intracellular Ca\(^{2+}\) concentration inhibits ANP release in beating atria and perfused heart (8, 13, 17, 25, 33), it was expected that cAMP-induced inhibition of ANP release would be related to Ca\(^{2+}\) influx via the L-type channel. Treatment with L-type Ca\(^{2+}\) channel or PKA inhibitors only slightly attenuated the inhibition of ANP release by forskolin. L-type Ca\(^{2+}\)-channel inhibitors failed to change the relationship between changes in cAMP production and ANP release. On this occasion, treatment with Ca\(^{2+}\)-channel or PKA inhibitors inhibited forskolin-induced increases in atrial dynamics. This suggests that cAMP negatively regulates myocytic ANP release via largely Ca\(^{2+}\)-independent pathways. An increase in ANP secretion by L-type Ca\(^{2+}\)-channel inhibitors is consistent with the previous report (33).

cAMP-induced inhibition of myocytic ANP release as well as an increase in atrial dynamics were not observed in the presence of staurosporine, a nonspecific protein kinase inhibitor. The effect was dose dependent. Staurosporine significantly shifted the relationship between changes in cAMP production and ANP release. An accentuation of ANP release by PMA is consistent with the previous report (23, 24). The findings that both the specific PKC-α inhibitor Gö-6976 and the nonspecific protein kinase inhibitor staurosporine (0.1 μM) completely blocked the PMA-induced increase of ANP release and that Gö-6976 but not staurosporine failed to modulate the forskolin-induced decrease of ANP release suggest that at least PKC-α is not involved in cAMP-induced inhibition of ANP release. Although the specific protein kinase responsible for the effect has yet to be defined, the present data indicate that cAMP-induced inhibition of ANP release is related to the activation of protein kinase(s) other than PKA, PKC-α, and Ca\(^{2+}\)/calmodulin kinase. From these data it is hypothesized that AC-activated cAMP production has two signaling pathways in the cardiac atrium: one is to increase Ca\(^{2+}\) influx leading to an increase in atrial dynamics, and the other is to activate protein kinase(s) leading to inhibition of secretory function. Therefore, these findings suggest that the mechanisms by which cAMP or Ca\(^{2+}\) inhibits myocytic release of ANP may not be the same. These data for the first time indicate that intracellular cAMP inhibits ANP release independently from the activation of L-type Ca\(^{2+}\) channels. These data suggest that a cAMP signaling pathway other than the activation of the L-type Ca\(^{2+}\) channel is very potent and distinct in the regulation of ANP secretion. Alternatively, activated cAMP signaling may sensitize the Ca\(^{2+}\)-related inhibitory mechanism so that only a minor fraction of Ca\(^{2+}\) is enough for the inhibition. Or, it seems likely that cAMP signaling is distal to the role of Ca\(^{2+}\) in the regulation of ANP release.

Previously it was shown that forskolin-induced inhibition of ANP secretion was not observed in the cultured noncontracting rat atrial myocytes treated with ryanodine and Ca\(^{2+}\) depletion (12), which suggested that cAMP-induced inhibition of ANP secretion was related to the increases in Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release. The present data contrast with that
Protein kinase-dependent inhibition of ANP release

The reasons for the discrepancy are not clear at present. Both differences in species and methodology may account for the discrepancy.

The blockade of the forskolin-induced accentuation of atrial dynamics by L-type Ca\(^{2+}\) channels, PKA inhibitors, or staurosporine that was observed in the present study is consistent with the notion (11, 22) that cAMP activates L-type Ca\(^{2+}\) channels via PKA. In the presence of staurosporine, forskolin actually decreased the atrial dynamics. The mechanism responsible for this phenomenon is unclear at present.

Taken together with previous reports showing inhibition of ANP release by cGMP (12, 17, 21, 24), it is clear that cyclic nucleotides are inhibitory regulators for the ANP secretion.

In conclusion, the present study shows that cAMP inhibits atrial myocytic ANP release via protein kinase-dependent signaling. The present study also suggests that an accentuation of Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels by AC-activated cAMP production is related to the control of dynamics with only a minor contribution to the control of secretory function of the cardiac atrium.

Perspectives

It has been shown that ANP secretion is controlled by a “two-step sequential mechanism” (2, 3). The first step is atrial myocytic ANP release into the interstitial space, and the second is convective translocation of the ECF with the released ANP into the atrial lumen. Atrial myocytic ANP release is under the control of variable second messengers (23). The second step, convective ECF translocation, is controlled by changes in atrial dynamics and the size of the extracellular space; therefore it is expected that factors affecting atrial dynamics such as cyclic nucleotide and Ca\(^{2+}\) modulating agents may influence atrial myocytic ANP release in a complex way. However, the exact nature of the roles for second messengers in the regulation of myocytic ANP release is largely unknown. Ca\(^{2+}\) is involved in both negative and positive regulation of atrial ANP release (33). As shown in the present study, cAMP is involved in the negative regulation of ANP release. This pathway is distinct from L-type Ca\(^{2+}\) channels. In the cardiac atrium, cAMP as an inhibitor for the regulation of ANP release may work through protein kinase signaling. However, it looks as though the signaling pathway is unusual. This suggests that within the cardiac atrium, roles for cAMP in the regulation of secretory function are complex. The signaling pathway for the AC-cAMP system in the endocrine atrium has to be defined.

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