Role of cAMP and calcium influx in endothelin-1-induced ANP release in rat cardiomyocytes

M. C. REBSAMEN,1 D. J. CHURCH,2 D. MORABITO,1 M. B. VALLOTTON,1 and U. LANG1
1Division of Endocrinology and Diabetology, University Hospital, and
2Geneva Biomedical Research Institute, CH-1211 Geneva 14, Switzerland

Rebsamen, M. C., D. J. Church, D. Morabito, M. B. Vallotton, and U. Lang. Role of cAMP and calcium influx in endothelin-1-induced ANP release in rat cardiomyocytes. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E922–E931, 1997—The mechanism of endothelin-1 (ET-1)-induced atrial natriuretic peptide (ANP) release was studied in neonatal rat ventricular cardiomyocytes. These cells expressed a single high-affinity class of ET_A receptor (dissociation constant = 54 ± 18 pM, n = 3), but no ET_B receptors. Incubation of cardiomyocytes with ET-1 led to concentration-dependent ANP release and prostacyclin production. ET-1-induced ANP release was affected by neither protein kinase C (PKC) inhibition or downregulation nor by cyclooxygenase inhibition, indicating that ET-1-stimulated ANP secretion is not a PKC-mediated, prostaglandin-dependent process. Further- more, ET-1 significantly stimulated adenosine 3',5'-cyclic monophosphate (cAMP) production and increased cytosolic calcium concentration in these preparations. Both ET-1-induced calcium influx and ANP release were decreased by the cAMP antagonist Rp-cAMPS, the Rp diastereoisomer of cAMP. Moreover, ET-1-induced ANP secretion was strongly inhibited in the presence of nifedipine as well as in the absence of extracellular calcium. Thus our results suggest that ET-1 stimulates ANP release in ventricular cardiomyocytes via an ET_A receptor-mediated pathway involving cAMP formation and activation of a nifedipine-sensitive calcium channel.

endothelin-1; receptor; prostacyclin formation; protein kinase C downregulation; adenosine 3',5'-cyclic monophosphate antagonist; nifedipine-sensitive calcium channel; signal transduction; atrial natriuretic peptide

ENDOTHELIN-1 (ET-1), the principal peptide of the endothelin family, has been shown to have a variety of biological activities in both vascular and nonvascular tissues, including the heart, the kidney, and the central nervous system (21). In cardiomyocytes, ET-1 has been found to increase cell contraction frequency (12) and to stimulate the release of atrial natriuretic peptide (ANP; see Ref. 11), a hormone that regulates salt and water balance and is synthesized and secreted by both atrial and ventricular myocytes.

At the cellular level, the effects of ET-1 appear to be mediated by two specific receptor subtypes termed ET_A and ET_B (21), both of which are functionally coupled to phospholipase C (PLC). Interestingly, the intracellular signaling pathways underlying ET-1-induced ANP release have been proposed to result at least in part from the activation of this effector (15), an event leading to increased phosphatidyl inositol 4,5-bisphosphate hydrolysis, which in turn promotes both inositol 1,4,5-trisphosphate-mediated Ca^{2+} mobilization and the diacylglycerol-dependent activation of protein kinase C (PKC). Although a number of studies have indicated a role for PKC in ET-1-induced ANP secretion in various cardiomyocyte models (11, 15, 26), the role of Ca^{2+} mobilization in ET-1-induced ANP release remains to be demonstrated. It is generally believed that Ca^{2+} plays an important role in ET-1-dependent ANP secretion (11, 13, 15, 26), and it is likely that this occurs through an influx of extracellular Ca^{2+} in most experimental models, since ET-1-induced ANP release is inhibited in the presence of the dihydropyridine calcium channel blocker nifedipine (11).

Despite the above, the mechanism of ET-1-induced ANP secretion remains incompletely understood, particularly with regard to the effect(s) of ET-1 on cytosolic free calcium concentration ([Ca^{2+}_c]), PKC activation, and/or increase in contraction frequency, as well as to the role of these responses in ET-1-induced ANP secretion. In ventricular cardiomyocytes, Damron et al. (7) found that inhibition of PKC reduces ET-1-induced increase in ATP-triggered calcium transients, whereas Xu et al. (30) report that PKC inhibition increases ET-1-stimulated calcium responses. In atrial cardiomyocytes, PKC inhibition was found to partially inhibit ET-1-induced ANP release (26).

Discrepancies have also been reported regarding ET-1-induced calcium responses. Uusimaa et al. (26) observed that ET-1 causes concentration-dependent increases in cytosolic calcium concentration in atrial myocytes, whereas McDonough et al. (15) found that ET-1 did not significantly increase basal cytoplasmic calcium in the same cells. Ono et al. (19) showed that ET-1 inhibits the L-type calcium channels in adult guinea pig atrial myocytes. In contrast, ET-1 has been found to increase calcium conductance through L-type calcium channels in ventricular cardiomyocytes (26).

Previous studies from our laboratory have shown that, in spontaneously beating neonatal rat ventricular cardiomyocytes, angiotensin II (ANG II), arginine vasoressin (AVP), and phorbol diester-induced ANP release are phenomena mediated by PKC-dependent prostaglandin formation, a response that appears to lead to cAMP production and nifedipine-sensitive calcium influx (2, 27). In the present study, we identified the ET-1 receptor subtype present in cultured, spontaneously beating neonatal rat ventricular cardiomyocytes and further investigated the role of PKC, intracellular calcium mobilization, calcium influx, prostaglandin formation, and cAMP production in ET-1-induced ANP secretion.

MATERIALS AND METHODS

Materials. Rat [3-125I-Tyr^{125}]ANP-(99–126) was purchased from Novabiochem (Basel, Switzerland). 3H-labeled 6-keto-
prostaglandin (PG) F_{1\alpha}, (6-keto-PGF_{1\alpha}) was obtained from Amersham International (Bucks, UK). ET-1, ET-3, succinyl-[Glu\beta,Ala\beta,\alpha]-endothelin-1-[8—21] (IRL-1620), and [\alpha-Trp^{95}(Ac)]endothelin-1-[16—21] were from Bachem (Bubendorf, Switzerland). 45-Phorbol 12-myristate 13-acetate (PMA), 3-isobutyl-1-methylxanthine (IBMX), Indomethacin, nifedipine, ethylene glycol-bis-(\alpha-aminoethyl) ether-N,N,N',N' -tetraacetic acid (EGTA), insulin-transferrin-selenium medium supplement (ITS; 5 \mu g/ml, 5 \mu g/ml, and 5 ng/ml, respectively), bovine serum albumin (BSA) fraction V, aprotinin, bacitracin, and probenecid were from Sigma (St. Louis, MO). Fura 2-acetoxyethyl ester (fura 2-AM), fluo 3-acetoxyethylmethyl (fluo 3-AM) and puronic acid were obtained from Molecular Probes (Eugene, OR). Calphostin C, chelerythrine, and the Rp diastereoisomer of adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS) were from LC Laboratories (Woburn, MA). The PKC inhibitor CGP-41251 was a gift from Ciba-Geigy (Basel, Switzerland). Thapsigargin was from Anawa Trading (Wangen, Switzerland). Ionomycin and cyclosporin A were from Calbiochem (San Diego, CA). McCoy's modified 5A medium, Hanks' Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM)-F12, fetal calf serum (FCS), trypsin, and deoxyribonuclease (type I) were from GIBCO (Basel, Switzerland). Anti-ANP antiserum was obtained from Peninsula Labs (Belmont, CA). Anti-6-ketoprostaglandin (PGF\textsubscript{1\alpha}) antiserum was from Oxford Biomedical Research (Oxford, MI). 125\textsuperscript{I}-labeled cAMP, [Tyr\textsuperscript{13}]-endothelin-1, and 125\textsuperscript{I}-IRL-1620 were from Du Pont-NEN (Regensdorf, Switzerland). Antibiotics (penicillin and streptomycin) were from Hoechst (Frankfurt, Germany) and Grunenthal (Glarus, Switzerland), respectively. Anti-adenosine 3',5'-cyclic monophosphate (cAMP) antisera was a generous gift from Dr. A. Baukal (National Institutes of Health, Bethesda, MD). Bicinchoninic acid (BCA) protein assay reagent was from Pierce Chemical (Rockford, IL).

Cell culture. Neonatal rat cardiomyocytes were obtained from 1- to 2-day-old Wistar rats, as previously described (27). Briefly, the lower two-thirds of 30–50 neonatal rat hearts were excised and placed in 40 ml of sterile Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free HBSS containing 100 IU/ml of penicillin and 10 \mu g/ml of streptomycin at 4°C. The tissue was washed with 20 ml of HBSS, cut into small pieces, further washed with 40 ml of HBSS and enzymatically digested for 8 min with 10 ml of trypsin/DNase HBSS solution (2.5 mg/ml and 0.03 mg/ml, respectively) at 37°C in a 50-ml sterile conical tube subjected to constant stirring. The supernatant from the first incubation procedure was repeated. Subsequent supernatant volumes were collected and centrifuged at 200 g for 5 min, and the resulting cell pellets were resuspended in McCoy's modified 5A medium containing 10% FCS and 1% ITS at 37°C. Once the sequential digestions were terminated, the cells were pooled, washed with 40 ml of McCoy's modified 5A medium containing 10% FCS, 1% ITS, 100 IU/ml penicillin, 10 \mu g/ml streptomycin, and 0.5 \mu g/ml fungizone and seeded in 90-mm plastic petri dishes or six-well culture plates (Costar, Cambridge, MA). The majority of cultured cells (i.e., >90%) began to contract spontaneously within 24–48 h of plating (30–50 beats/min) and exhibited positive staining for pro-ANP, as we have previously shown (27). Confluent, spontaneously beating cells were used on the 3rd day of culture for all experiments described herein.

Receptor-binding assays. Cell membranes from cultured neonatal ventricular cardiomyocytes were obtained as described by Thibault et al. (24). Protein concentration was determined with BCA protein assay reagent using BSA as a standard. For saturation analysis, membrane proteins (20 \mu g) were resuspended in a binding buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 50 \mu M aprotinin, 0.1% bacitracin, 5 mM MgCl\textsubscript{2}, 0.5% BSA, pH 7.2) and incubated for 1 h at 37°C in the presence of increasing concentrations of 125\textsuperscript{I}-labeled [Tyr\textsuperscript{13}]-endothelin-1 or 125\textsuperscript{I}-IRL-1620 (2,200 Ci/mmol), a specific ET\textsubscript{B} receptor agonist. After the incubation, the tubes were filtered on Whatman GF/C glass fiber filters with a cell harvester (Brandel). The filters were washed six times with 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, and radioactivity was measured in a gamma counter. For nonspecific binding determination, a large excess of unlabeled ET-1 or ET-3 (1 \mu M) was added. For competition analysis 20 \mu g of membrane protein were incubated with 10 \mu M 125\textsuperscript{I}-labeled [Tyr\textsuperscript{13}]-endothelin-1 in the presence of increasing concentrations of different competitors. All other parameters were identical to those used in the saturation experiments.

Determination of ANP release. For assessment of ANP release, six-well tissue culture plates containing confluent, spontaneously contracting cardiomyocyte monolayers were washed with 2 ml of Krebs-Ringer buffer containing 0.2% BSA and 0.2% glucose, as previously described (2). After the supernatant was replaced with 1 ml of fresh buffer, the cells were incubated at 37°C for the indicated times in the presence of the various pharmacological agents, and 500-\mu l aliquots of the supernatant were collected and assayed for ANP content, according to radioimmunological methods already described (2). Agents used in these studies were dissolved in either water, dimethyl sulfoxide (DMSO), or mixtures thereof so that the final concentration of DMSO did not exceed 0.5%. When tested for ANP release-inhibiting or -stimulating properties, a 1% concentration of DMSO induced a maximal response for ANP tracer displacement occurred at 4 and 5%, respectively (data not shown). Relative affinity of the anti-ANP antiserum for rat atriotepitide was given as follows: 100% for \alpha-human ANP and rat ANP (Ile\textsuperscript{124}-\alpha-human ANP), 100% for rat atriotepitide III, 60% for rat ANP fragment 18—28, 5% for rat atriotepitide I, <0.001% for rat ANP fragment 13—28, 0% for rat brain natriuretic peptide, rat C-type natriuretic peptide, ANP fragment 1—11, rat atriotepitide I and substance P. The detection limit for ANP determinations was 3 pg/ml of incubation medium. The intra- and interassay coefficients of variation were 4% for rat atriotepitide I and 5% for ANP, respectively (n = 8). Nonspecific binding was typically 4% of maximal rat [3-125\textsuperscript{I}]-Tyr\textsuperscript{125}]-ANP (99—126) binding. The dose eliciting 50% of the maximal response for ANP tracer displacement occurred at 48 ± 5.2 pg (n = 5). Serial dilutions of the incubation media yielded results that paralleled those of synthetic ANP standard.

Determination of prostacyclin production. For assessment of PG\textsubscript{1\alpha} formation, six-well tissue culture plates containing spontaneously contracting cardiomyocyte monolayers were washed with 2 ml of Krebs-Ringer buffer containing 0.2% BSA and 0.2% glucose, as previously described (2). After the supernatant was replaced with 1 ml of fresh buffer, the cells were incubated at 37°C for 60 min in the presence of various pharmacological agents. PG\textsubscript{1\alpha} production was determined by radioimmunoassay of 50-\mu l aliquots of the incubation medium for 6-keto-PGF\textsubscript{1\alpha}, the stable metabolite of PG\textsubscript{1\alpha}. Relative affinity of the anti-6-keto-PGF\textsubscript{1\alpha} for eicosanoids was given as follows: 100% for 6-keto-PGF\textsubscript{1\alpha}, <1% for PGE\textsubscript{2}, 20\textsuperscript{\mu g} of membrane protein were incubated in a binding buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 50 \mu M aprotinin, 0.1% bacitracin, 5 mM MgCl\textsubscript{2}, 0.5% BSA, pH 7.2) and incubated for 1 h at 37°C in the presence of increasing concentrations of 125\textsuperscript{I}-labeled [Tyr\textsuperscript{13}]-endothelin-1 or 125\textsuperscript{I}-IRL-1620 (2,200 Ci/mmol), a specific ET\textsubscript{B} receptor agonist. After the incubation, the tubes were filtered on Whatman GF/C glass fiber filters with a cell harvester (Brandel). The filters were washed six times with 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, and radioactivity was measured in a gamma counter. For nonspecific binding determination, a large excess of unlabeled ET-1 or ET-3 (1 \mu M) was added. For competition analysis 20 \mu g of membrane protein were incubated with 10 \mu M 125\textsuperscript{I}-labeled [Tyr\textsuperscript{13}]-endothelin-1 in the presence of increasing concentrations of different competitors. All other parameters were identical to those used in the saturation experiments.
Determination of cAMP formation. For cAMP determination, contracting cells were grown to confluency in six-well tissue culture plates, washed twice with 2 ml Krebs-Ringer buffer, and incubated with various pharmacological agents at 37°C for 30 min in the presence of 0.2 mM IBMX. cAMP was measured according to Harper and Brooker (9), with acetylation of the samples. Dioxan (70% vol/vol) was used for separation, and bound radioactivity was counted with a multigamma counter. The limit of detection was 30 fmol/tube. Specific binding ranged from 15.4 to 28.9%, and nonspecific binding ranged from 2.1 to 3.3%. Fifty-percent tracer displacement occurred at 477.7 ± 53.3 fmol (means ± SE, n = 18). Intra- and interassay coefficients of variation were 10 and 12%, respectively.

Calcium fluorometry. Determinations of [Ca^{2+}]_{i} were carried out in confluent monolayers of spontaneously contracting cardiomyocytes grown on glass slides with the use of the fluorescent Ca^{2+} probe fura 2, adapting the method described elsewhere (3). Briefly, the monolayers were washed twice with 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 MgEDTA, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5.5 mM glucose, and 0.5% BSA at pH 7.4], covered with 400 µl of modified Krebs-Ringer buffer containing 3 µM fura 2-AM and 0.5% BSA, and incubated for 15 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 thermostated holder of an LS-3 Perkin-Elmer fluorescence spectrophotometer. Continuous stirring was achieved by means of a magnetic stirrer. Fluorescence of fura 2-loaded monolayers was measured 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 dissociation constant (K_{d}) of fura 2 for Ca^{2+} was assumed to be 224 nM. Mean diastolic [Ca^{2+}]_{i} was estimated by averaging basal (resting) [Ca^{2+}]_{i} values for each tracing over a 30-s interval preceding stimulation with the various pharmacological agents.

Alternatively, cytosolic free calcium determinations were conducted by plating cells in black-walled 96-well microtiter plates and by washing confluent monolayers of cardiomyocytes with 150 µl/well serum-free DMEM-F12 medium containing 2 mM glutamine and 100 U/ml penicillin and/or streptomycin. The washes were removed, and the monolayers were incubated at 37°C for 60 min with 100 µl/well of serum-free DMEM-F12 medium containing 2 mM glutamine, 1 µM cyclosporin, 1 µM probenecid, and 4 µM fluo-3-AM previously dissolved in 20% puronic acid (1 mg/ml stock). After the loading procedure, the monolayers were washed four times at room temperature with 150 µl of HEPES buffer (10 mM, pH 7.4) containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl_{2}, 1 mM MgCl_{2}, 10 mM glucose, 1 µM cyclosporin A, and 1 µM probenecid (assay buffer), after which 180 µl of the same buffer were added to each well, and the plates were used immediately.

ET-1-induced calcium mobilization and influx were monitored at room temperature by means of a FLIPR fluorescent imaging plate reader (Molecular Devices, UK). The excitation wavelength of 488 nm was supplied by a 3-W argon laser (Coherent, Lasarne, Switzerland) set at 0.8–1.2 W, according to the level of cell confluency. Fluorescent emission was measured at 512 nm. All assays were conducted at a mean fluorescent baseline signal of 3.0–3.5 × 10^{4} fluorescent counts per minute per well and per plate. Lens aperture was maintained constant at f/1.4, and shutter speed was set at 0.3–0.4 s. A total of 120 sampling points were collected for each well over 6 to 8-min periods. Sample injection speed was set at 175 µl/min. Under these conditions, maximal signal amplitude was an additional 2–5 × 10^{4} fluorescent counts on addition of 0.1 µM ET-1 to wells containing only vehicle.

Statistical analysis. Student-Fisher unpaired bilateral t-tests and/or analysis of variance using the Scheffé F-test criterion for unbalanced groups was used were applicable. A value of P < 0.05 was accepted as statistically significant. Results represent the means ± SE of at least three experiments performed in duplicate or triplicate determinations.

RESULTS

Determination of the endothelin receptor subtype present in neonatal ventricular cardiomyocytes. As is shown in Fig. 1A, binding experiments showed that 125I-ET-1 bound specifically to cultured neonatal rat ventricular cardiomyocyte membranes. Scatchard analysis of the data obtained from these preparations indicated the presence of a single class of high affinity binding site for ET-1 displaying a K_{d} value of 54 ± 18 pM and a maximum binding capacity of 108 ± 51 fmol/mg protein (n = 3, Fig. 1B). In contrast, no specific binding was observed with the ET_{B} receptor-selective agonist 125I-IRL-1620 (Fig. 2).

In competition experiments (Fig. 3), ET-1 potently displaced 125I-ET-1 binding with a half-maximal inhibi-
tion ($K_i$) of $81 \pm 8 \text{ pM (n = 4)}$. With the use of \([D-Trp16(Ac)]ET-1-(16—21)\), a compound known to have a high affinity for the ETA receptor (6), a $K_i$ of $65 \pm 10 \text{ nM}$ was observed (n = 4). Finally, the competition binding assay results further indicated that IRL-1620 displaced labeled ET-1 binding with a $K_i$ of $1.56^{\pm}0.3 \mu\text{M}$, a value consistent with that determined by Takai et al. (23) for IRL-1620 binding to the ETA receptor subtype. Thus these data present strong evidence that ETA is the endothelin receptor subtype present in rat ventricular cardiomyocyte membranes.

Role of PKC and PGI$_2$ in ET-1-induced ANP secretion. As illustrated in Fig. 4, ET-1 induced a concentration-dependent increase in ANP secretion in neonatal rat ventricular cardiomyocytes, exhibiting a 50% effective concentration (EC$_{50}$) value of 0.11 nM. Incubation of cells with 1 nM of ET-1 significantly increased ANP release to 135$\pm$7% of basal values (n = 5).

It has previously been shown that PKC-dependent prostaglandin formation is at the basis of ANG II- and AVP-induced ANP secretion in cardiomyocytes (2, 27). In this light, we further investigated whether the ETA receptor couples to the same transduction pathway to induce ANP release by testing the effects of the PKC inhibitors calphostin C (500 nM), chelerythrine (1 µM), and CGP-41251 (1 µM), a derivative of staurosporine (2) (Fig. 5). The influence of PKC was also studied by using cardiomyocytes in which PKC had been downregulated by prolonged, 24-h incubation with 200 nM PMA. This experimental procedure has been shown to induce downregulation of PKC$\alpha$, $\delta$, and $\epsilon$ in neonatal rat ventricular cardiomyocytes (20). In this context it is
inhibitor indomethacin. As shown in Fig. 6C, incubation of cardiomyocytes with 5 µM indomethacin, a concentration that almost abolishes ET-1-induced PGI2 production (Fig. 6B; n = 10), had no effect on ET-1-induced ANP release (n = 7). These results strongly suggest that, in contrast to what has been previously observed for other PLC-stimulating agonists, ET-1-stimulated ANP secretion is neither PKC nor prostacyclin dependent.

Role of cAMP and [Ca2+]i in ET-1-induced ANP secretion. To identify the signaling pathway by which ET-1 induces an increase in cardiomyocyte ANP release, we investigated whether ET-1 promotes cAMP formation in these cells. As shown in Fig. 7A, ET-1 induced a 50 ± 5% increase of cAMP formation in the presence of 0.2 mM IBMX in these preparations (n = 10). The hypothesis that cAMP formation is responsible for ET-1-induced ANP secretion was tested by incubating cardiomyocytes with 0.1 µM ET-1 in the presence of Rp-cAMPS (0.5 µM), a membrane-permeant phosphodiesterase-resistant cAMP analog displaying cAMP-antagonizing properties (3). As shown in Fig. 7B, Rp-cAMPS was found to decrease ET-1-induced ANP release by 51 ± 3% (n = 9). In addition to this, we have previously shown that both the adenylyl cyclase activator forskolin (0.1 µM) and the cell-permeant cAMP analog dibutyryl cAMP (DBcAMP; 10 µM) promote significant increases in cardiomyocyte ANP secretion (3). Taken together, these findings suggest that an increase in cAMP production is involved in ET-1-induced ANP release in this system.

Because it has been suggested that calcium influx is an important signaling component in agonist-induced ANP release (14), we further tested the effect of ET-1 on [Ca2+]i in cultured cardiomyocytes. Calcium fluorometry using the calcium-sensitive fluorescent probe fura...
revealed that cultured cardiomyocytes behave much like a syncytium, such that each contraction of the whole cell population was accompanied by a single Ca\textsuperscript{2+} transient. As shown in Fig. 8, stimulation of cardiomyocyte monolayers with 0.1 µM ET-1 induced a biphasic calcium response: a rapid and transient increase in [Ca\textsuperscript{2+}]\textsubscript{c} followed by a sustained phase of elevated [Ca\textsuperscript{2+}]\textsubscript{c} (\(n = 13\)). In addition to this, ET-1 increased the frequency of calcium spikes as well as the contraction frequency of the beating monolayers by \(-40\% (n = 4, \text{ data not shown}).

To obtain more information concerning the effect of ET-1 on the different components of the calcium response, we studied the influence of ET-1 on [Ca\textsuperscript{2+}]\textsubscript{c} in the presence of 0.1 µM nifedipine and in the absence of extracellular calcium (0.2 mM EGTA). Under these conditions calcium influx can be excluded from partaking in ET-1-induced calcium responses, whereas release of Ca\textsuperscript{2+} from intracellular stores remains possible. As is shown in Fig. 9A, nifedipine abolished the sustained phase of the ET-1-induced [Ca\textsuperscript{2+}]\textsubscript{c} response in these cells (plateau), and this without affecting the rapid transient increase in [Ca\textsuperscript{2+}] due to calcium release from intracellular stores (initial peak, \(n = 7\)). The same response was obtained in the absence of extracellular calcium (\(n = 6\); Fig. 9B). Indeed, the amplitude of the ET-1-induced Ca\textsuperscript{2+} peak was the same in the presence and in the absence of extracellular Ca\textsuperscript{2+}, whereas ET-1-induced Ca\textsuperscript{2+} influx (plateau phase) was observed only in the presence of extracellular Ca\textsuperscript{2+} (Fig. 8 vs. Fig. 9). In addition to this, nifedipine abolished Ca\textsuperscript{2+} spikes and cell contractions in these preparations (data not shown).

In the same context, we studied the effect of calcium influx on ANP secretion in cultured cardiomyocytes. Fig. 10 illustrates that the calcium ionophore A-23187 (5 µM) induced an ANP response of a similar magnitude as that elicited by 0.1 µM ET-1. Moreover, in the absence of extracellular calcium (0.2 mM EGTA), as well as in the presence of nifedipine (0.1 µM), ET-1-induced ANP release was markedly inhibited. To confirm the hypothesis that calcium influx but not Ca\textsuperscript{2+} release from intracellular stores is implicated in ET-1-induced ANP release, we further tested the effect of 100 nM thapsigargin on cardiomyocyte ANP secretion. Thapsigargin, which is known to increase [Ca\textsuperscript{2+}] in cardiomyocytes by discharging intracellular Ca\textsuperscript{2+} stores (29), strongly inhibited ET-1-induced release of calcium from intracellular stores (\(n = 4\), Fig. 11A) yet was ineffective at inducing ANP secretion in ventricular cardiomyocytes on its own (from 387 ± 25 to 354 ± 43 pg/well, \(n = 6\), Fig. 11B). Along the same lines, depleting intracellular Ca\textsuperscript{2+} stores by adding thapsigargin before stimulation with 100 nM ET-1 did not affect ET-1-induced ANP secretion.
release (547 ± 64 pg/well compared with 499 ± 58 pg/well in the presence of 100 nM thapsigargin, n = 6, Fig. 11B). Taken together, these data indicate that Ca\(^{2+}\) release from intracellular stores is not involved in ET-1-induced ANP secretion.

Experiments using different concentrations of nifedipine and Rp-cAMPS (see Table 1) revealed that nifedipine inhibited ET-1-stimulated ANP release by 62 ± 5 and 82 ± 1% at 0.1 and 1 µM, respectively, whereas Rp-cAMPS reduced ET-1-induced ANP secretion by 62 ± 12 and 73 ± 5% at 0.5 and 5 µM, respectively (n = 4). Table 1 also illustrates that the inhibitory effects of nifedipine and Rp-cAMPS on ET-1-induced ANP release were not additive, suggesting that cAMP production and calcium influx are involved in the same signaling cascade leading to increased ANP secretion in ET-1-stimulated cardiomyocytes.

To better understand the interaction between calcium influx and cAMP formation in ET-1-induced ANP secretion, we studied the effect of nifedipine on cAMP production. We observed that nifedipine (0.1 µM) had no effect on basal cardiomyocyte cAMP production, whereas, in the presence of IBMX (0.2 mM), it induced a 24–93% augmentation of cAMP formation in ET-1 and forskolin-stimulated cells (data not shown). Interestingly, this result is in agreement with the observation that cardiac tissue contains an adenylyl cyclase that is inhibited by increases in cytosolic free Ca\(^{2+}\) (31).

Because this suggests that calcium influx is not responsible for increased cAMP formation in ET-1-stimulated cells, we ultimately studied the influence of cAMP on ET-1-induced calcium influx by exposing cardiomyocytes to Rp-cAMPS (Fig. 12). Although Rp-cAMPS (5 µM) had no effect on basal [Ca\(^{2+}\)]\(_c\) in unstimulated cardiomyocytes (data not shown), it reduced ET-1-induced calcium influx by 34 ± 2% (n = 5, Fig. 12). Conversely, Rp-cAMPS (5 µM) did not affect the calcium influx caused by application of 50 mM of K\(^+\) to the monolayers, a concentration that depolarizes the cell membrane (data not shown). This observation indicates that Rp-cAMPS has no effect on the opening of voltage-operated calcium channels due to depolarization, but decreases ET-1-induced calcium influx through its cAMP-antagonizing properties.

In agreement with these findings as well as with the presumption that ANP secretion is related to cell contraction, Rp-cAMPS was found to have a similar

![Fig. 11. Effect of thapsigargin on ET-1-induced calcium mobilization and ANP release.](image1)

**Table 1. Effect of nifedipine and Rp-cAMPS on ET-1-induced ANP release**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nifedipine</th>
<th>Rp-cAMPS</th>
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<tr>
<td></td>
<td>0.1 µM</td>
<td>1.0 µM</td>
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<td>Inhibition of ET-1-induced ANP release, %</td>
<td>62 ± 5</td>
<td>82 ± 1</td>
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Values are means ± SE determined from 4 experiments performed in triplicate determinations. ET-1, endothelin-1; ANP, atrial natriuretic peptide.
inhibitory effect on ET-1-induced increases in cell contraction frequency (38 ± 3% inhibition, n = 4). Thus these results suggest that cAMP is involved in ET-1-induced increases in cell contraction frequency and calcium influx leading to ANP release.

**DISCUSSION**

Taken together, the present results indicate that ET-1 promotes ANP release in cultured spontaneously beating neonatal rat ventricular cardiomyocytes via an ETA receptor-mediated pathway involving cAMP formation and the activation of a nifedipine-sensitive calcium channel.

We showed that neonatal rat ventricular cardiomyocytes express a single high affinity class of ET{sub A} receptors but no ET{sub B} receptors, whereas Touyz et al. (25) summarized in a short report that these cells possess both ET{sub A} and ET{sub B} receptors, predominantly the ET{sub A} subtype. Substantiating our conclusion, the ET{sub A} receptor subtype agonist [d-Trp{sup 16}(Ac)ET-1-(16-21)] inhibited 125I-ET-1 binding with high affinity, whereas the ET{sub B}-selective agonist IRL-1620 inhibited binding with low affinity. In addition, no specific binding was observed with the labeled ET{sub B} agonist 125I-IRL-1620. In this context, it is also noteworthy that the EC{sub 50} for the stimulation of ANP secretion by ET-1 (110 pM) is in the same range of concentration as the concentration of 125I-ET-1, resulting in half-maximal binding (K{sub d} = 54 pM).

Experiments conducted in PKC-downregulated cardiomyocytes or in the presence of PKC and cyclooxygenase inhibitors indicated that ET-1-induced ANP secretion is not a PKC-mediated, prostaglandin-dependent event as we have previously shown is the case for ANG II- and AVP-stimulated ANP release (2, 27). Our observation that cyclooxygenase inhibitor has no effect on ET-1-induced ANP release is in agreement with the findings of Uusimaa et al. (26) in ventricular and atrial cardiomyocytes.

In contrast to the results obtained with PKC and cyclooxygenase inhibitors, the membrane-permanent, phosphodiesterase-resistant cAMP antagonist Rp-cAMPs substantially reduced ET-1-induced ANP secretion, pointing to a role for cAMP in ET-1-stimulated ANP release in neonatal ventricular cardiomyocytes. This is further substantiated by the fact that both forskolin and dBcAMP induce ANP secretion in neonatal spontaneously beating ventricular cardiomyocytes, as we have shown previously (3).

We further showed that ET-1-induced ANP release was strongly inhibited both in the absence of extracellular Ca{sup 2+} and in the presence of the calcium channel blocker nifedipine, whereas the ionophore A-23187 stimulated ANP secretion in spontaneously beating cardiomyocytes. These results are in agreement with a study reporting that calcium influx plays an important role in myocardial ANP secretion (14). The fact that thapsigargin, while depletion intracellular Ca{sup 2+} stores, had no effect on basal and ET-1-induced cardiomyocyte ANP secretion further confirmed that calcium influx, but not Ca{sup 2+} release from intracellular stores, is involved in ET-1-stimulated ANP secretion.

In this context, it is important to note that our observations with neonatal rat ventricular cardiomyocytes concern acute ANP granule release, and not ANP message for long-term secretion, since Nakagawa et al. (16), using the same cells, have shown that ANP mRNA begins to increase only 3 h after stimulation with ET-1. Similarly, it has been shown that both cAMP and extracellular Ca{sup 2+} also play an important role in increasing ANP message for long-term ANP release in cultured cardiomyocytes (4, 13).

The observation that Rp-cAMPS and nifedipine both inhibited ET-1-induced ANP release without having an additive effect suggests that both cAMP and calcium influx are involved in the same signaling cascade leading to ANP release in ET-1-stimulated cardiomyocytes. However, the mechanism by which ET-1-induced activation of myocardial, cAMP-activated, voltage-operated calcium channels leads to ANP secretion remains to be fully elucidated. For example, the cAMP analog Rp-cAMPS inhibited ET-1-stimulated ANP release by ~73%, whereas it reduced ET-1-induced calcium influx and increase in cell contraction frequency by only 35 and 38%, respectively. Conversely, we have previously shown (3) that forskolin induces a small increase in cardiomyocyte calcium influx leading to a significant ANP secretion, a response that is completely abolished in the presence of nifedipine. All these findings could be explained by the hypothesis that calcium influx-mediated ANP release is a very sensitive process, insofar as small changes in calcium influx, possibly interacting with cell contraction, very strongly affect ANP secretion.

In this context, it is also very interesting to note that inhibition of calcium influx by nifedipine, while suppressing ANP release, augmented cAMP production in ET-1 and forskolin-stimulated cardiomyocytes. This observation is in agreement with previous studies reporting that cardiomyocytes express the types V and VI of adenylyl cyclase, which are known to be inhibited by Ca{sup 2+} influx (31). It has therefore been suggested that in cardiomyocytes calcium influx acts as a negative feedback control on the activation of adenylyl cyclases. Thus it appears that ET-1, while stimulating adenylyl cyclase through activation of G{sub s} (8, 18, 1), also has an inhibitory effect on cAMP production via an increase in Ca{sup 2+} influx.

This complex mechanism of action could at least partially explain the controversial observations concerning the effects of ET-1 receptor activation on cAMP formation discussed in the next paragraph.

Our data are in agreement with the findings that, in several cell systems such as vascular smooth muscle cells (8), embryonic bovine tracheal cells (18), and transfected Chinese hamster ovary cells (1), the ET{sub A} receptor is functionally coupled to the adenylyl cyclase system via G{sub s}. In contrast, the ET{sub B} receptor appears to be linked to adenylyl cyclase by G{sub i} (8). In rat atrial slices, Sokolovsky et al. (22) observed both ET-1-induced stimulation and inhibition of cAMP formation.
depending on ET-1 concentration, whereas Ono et al. (19) showed that ET-1 decreased basal and isoproterenol-stimulated cAMP production in adult guinea pig atria. In contrast, Iorns et al. (11) observed no effect of ET-1 on basal or forskolin-stimulated cAMP production in rat atrial myocytes, whereas Hilal-Dandan et al. (10) found that, in adult ventricular cardiomyocytes, ET-1 reduced isoproterenol- and forskolin-stimulated cAMP production.

Overall, our results indicate that, in neonatal rat ventricular cardiomyocytes, the ET-1-ETA receptor complex activates $G_s$, leading to stimulation of the cAMP cascade. They further suggest that in these cells calcium mobilization from intracellular stores and stimulation of cAMP production represent two cellular events linked to two different G proteins that are coupled to one receptor, the ETA receptor. Oda et al. (18) have already shown that in embryonic bovine tracheal cells the ETA receptor is linked to two effectors systems, phospholipase C and adenylyl cyclase. The coupling of a single receptor to multiple signal transduction pathways has been shown with other receptors.

Direct linkage to both phosphoinositide hydrolysis and cAMP cascade has been reported for the thyrotropin receptor (28) and tachykinin receptors (17).

Our results further indicate that Ca$^{2+}$ influx is one of the main mechanisms by which ET-1 induces acute ANP secretion in spontaneously beating neonatal cardiomyocytes and suggest that a substantial part of the ET-1-stimulated ANP release occurs via increased cAMP production leading to the activation of nifedipine-sensitive calcium channels.

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Address for reprint requests: M. Rebsamen, Division of Endocrinology and Diabetology, Univ. Hospital, CH-1211 Geneva 14, Switzerland.

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REFERENCES


Downloaded from http://ajpendo.physiology.org/ by 10.220.33.19 on September 30, 2017
endothelin-1 (8–21), IRL 1620, for the ET$_{B}$ receptor. Biochem.
24. Thibault, G., A. F. Doubell, R. Garcia, R. Larivi`ere, and 
   E. L. Schiffrin. Endothelin-stimulated secretion of natriuretic 
   peptides by rat atrial myocytes is mediated by endothelin A 
25. Thibault, G., A. F. Doubell, R. Garcia, R. Larivi`ere, and 
   E. L. Schiffrin. Endothelin-stimulated secretion of natriuretic 
   peptides by rat atrial myocytes is mediated by endothelin A 
   Schiffrin. Modulation of Ca$^{2+}$ transients in neonatal and adult 
27. Uusimaa, P. A., I. E. Hassinen, O. Vuolteenaho, and H. 
   Ruskoaho. Endothelin-induced atrial natriuretic peptide re- 
   lease from cultured neonatal cardiac myocytes: the role of 
   extracellular calcium and protein kinase-C. Endocrinology 130: 
28. Van der Bent, V., D. J. Church, M. B. Vallotton, P. Meda, 
   D. C. Kem, A. M. Capponi, and U. Lang. [Ca$^{2+}$], and protein 
   kinase C in vasopressin-induced prostacyclin and ANP release in 
   rat cardiomyocytes. Am J. Physiol. 266 (Heart Circ. Physiol. 35): 
29. Vigne, P., J. P. Breittmayer, and C. Frelin. Thapsigargin, a 
   new inotropic agent, antagonizes action of endothelin-1 in rat 
   atrial cells. Am J. Physiol. 263 (Heart Circ. Physiol. 32): H1689–
30. Xu, Y., L. Sandirasegarane, and V. Gopalakrishnan. Protein 
   kinase C inhibitors enhance endothelin-1 and attenuate vasopres- 
   sin and angiotensin II evoked [Ca$^{2+}$] elevation in the rat cardio-
   calcium channels acts as a negative regulator of adenylyl cyclase 
   activity and cyclic AMP levels in cardiac myocytes. Mol. Pharma-