Accelerated Publications

Ligand-Specific Stimulation/Inhibition of cAMP Formation by a Novel Endothelin Receptor Subtype

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ABSTRACT: The possible involvement of a cAMP pathway in endothelin (ET) signal transduction was explored using rat atrial slices. We show that ET-1 induces both stimulation and inhibition of cAMP formation, depending on its concentration. Unexpectedly, the effects of ET-3 and of sarafotoxins *b* and *c* (SRTX-*b* and SRTX-*c*) on this pathway differ from that of ET-1. Moreover, we show that the ET-1-induced formation of cAMP results from catecholamine release in a process mediated by a Ca^{2+} channel coupled to a pertussis toxin sensitive G-protein. It is concluded that this pathway is mediated by a new ET_A receptor subtype (probably presynaptic), for which ET-1 is an agonist and ET-3, SRTX-*b*, and SRTX-*c* are antagonists.

Two endothelin receptor (ET-R) subtypes, ET_A-R and ET_B-R, mediate the stimulation of phosphoinositide hydrolysis in various tissues and cell types [as reviewed in Sokolovsky (1992a)]. Stimulation is achieved at nanomolar concentrations of ligands, e.g., endothelin-1 (ET-1). We recently described a novel subtype of ET-R (Sokolovsky et al., 1992). Because this novel subtype does not induce phosphoinositide hydrolysis, its signal transduction might involve cAMP formation. Effects on cAMP accumulation in response to relatively high concentrations of ET-1 were shown previously to occur in some tissues (Aramori & Nakanishi, 1992; Oda et al., 1992; Eguchi et al., 1993) but not in others and were even shown to inhibit this formation (Ladoux & Frelin, 1991; Hilal-Dandan et al., 1992; Irons et al., 1993). We therefore examined the possibility that the signal transduction pathway of ET_A-R in a rat heart preparation is coupled to cAMP formation. We show that ET-1 induces both stimulation and inhibition of cAMP formation, depending on its concentration. The effects of ET-3 and of sarafotoxins *b* and *c* (SRTX-*b* and SRTX-*c*) on this pathway differ from that of ET-1. Moreover, we show that the ET-1-induced formation of cAMP results from catecholamine release in a process mediated by a Ca^{2+} channel coupled to a pertussis toxin sensitive G-protein. It is suggested that this pathway is mediated by a new ET_A receptor subtype (probably presynaptic), for which ET-1 is an agonist and ET-3, SRTX-*b*, and SRTX-*c* are antagonists.

MATERIALS AND METHODS

ET-1, ET-3, SRTX-*b*, and SRTX-*c* were purchased from American Peptide Co. (Santa Clara, CA). cAMP was assayed by the RIA kit which was purchased from Du Pont-New England Nuclear (NEK-033). Pertussis toxin was purchased from List Biochemicals (Campbell, CA) and BQ-123 from Novabiochem (Switzerland).

cAMP Formation. Atrial slices were excised from adult male rats and transferred to Krebs buffer (NaCl, 123 mM; KCl, 5.0 mM; KH_2PO_4, 1.4 mM; $MgSO_4$, 1.3 mM; $CaCl_2$, 0.8 mM; glucose, 10 mM; HEPES, 20 mM; bubbled with 5% CO_2 and 95% O_2 to reach pH 7.4). Slices were washed once and incubated for 10 min in buffer containing the drugs at various concentrations. Incubation was terminated by the addition of 4 volumes of boiling Tris-HCl, 50 mM, and EDTA, 10 mM. Slices were homogenized by Brinkmann polytron PT-10 (setting no. 7, 0.5 min), and cAMP levels were determined by the radioimmunoassay kit. Protein concentrations were determined using BSA as a standard. Results are expressed as the amount of cAMP (picomoles) per milligram of protein.

[^{3}H]NE Release. Following decapitation of adult male rats, atrial slices were excised and transferred to Krebs buffer (NaCl, 124 mM; KCl, 5.0 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.3 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 26.0 mM; glucose, 10 mM; bubbled with 5% CO_2 and 95% O_2 to reach pH 7.4, 37 °C). The slices were washed in buffer with continuous bubbling for 20 min, with buffer changes every 10 min. Slices were incubated with 50 nM [^{3}H]NE in the same Krebs buffer, with the addition of 10 μM pargyline and 1 mg/mL ascorbic acid, for 1 h at 37 °C. After incubation the slices were washed with continuously bubbled buffer for 40 min with two changes of buffer. Aliquots of tissue were placed in baskets with mesh bottoms (100 μM pore size) and placed in buffer for 10 min to reach a steady basal release. Baskets were then transferred at 5-min intervals through a series of vials containing freshly bubbled buffer with or without ETs, SRTXs, or 50 mM KCl. Finally, the baskets were placed in 6 mL of 1 N NaOH overnight to solubilize the tissue. Radioactivity in the vials was determined by liquid scintillation spectrometry. The percentage of [^{3}H]NE was calculated as the amount released by the stimulant relative to the amount released during the basal interval. The rate of stimulated release was determined by subtracting the mean basal release from the release in the stimulated fraction. In each set of experiments, the release induced by 50 mM K^+ was used as a control to establish the level of releasable [^{3}H]NE.

RESULTS AND DISCUSSION

Intracellular cAMP levels were measured after application of agonist to rat atrial slices in the presence of the phosphodiesterase inhibitor IBMX (0.5 mM). As shown in Figure 1, ET-1 at picomolar concentrations induced a marked dose-

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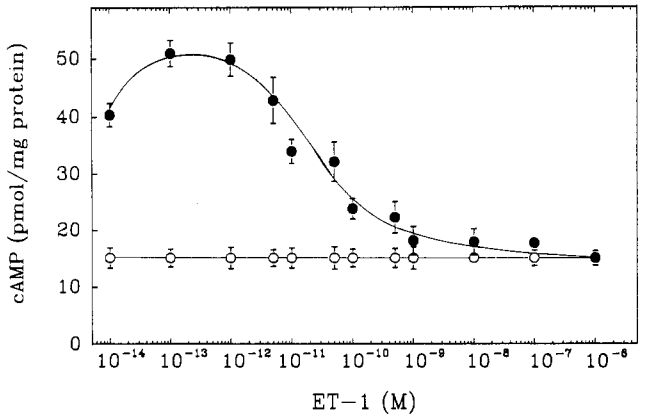

FIGURE 1: Dose–response curve of ET-1-induced stimulation of cAMP formation. cAMP was assayed by RIA (Du Pont-NEN). Each point is the mean ± SEM of four to seven experiments. Student's *t*-test was performed on each pair of stimulated vs basal values and in all concentrations up to 10^{-10} M (inclusive) yielded $p < 0.01$. Symbols: ●, ET-1 induced; ○, basal.

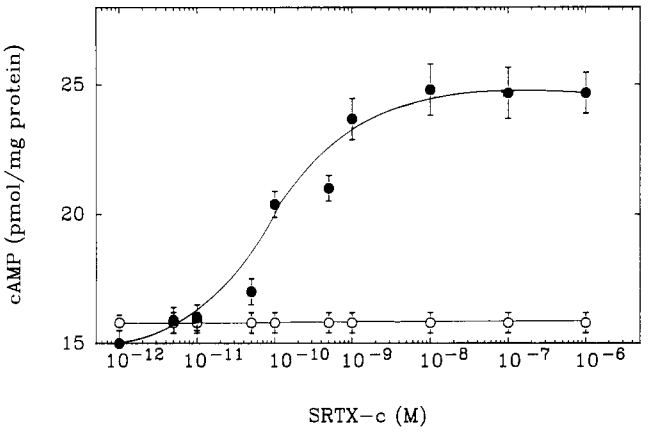

FIGURE 2: Dose–response curve of SRTX-*c*-stimulated cAMP formation. cAMP was assayed by RIA (Du Pont-NEN). Each point is the mean ± SEM of four to seven experiments (Student's *t*-test as in Figure 1 and in concentration range 10^{-10}–10^{-6} M yielded $p < 0.01$). Symbols: ●, SRTX-*c* induced; ○, basal.

dependent increase in cAMP. Maximal stimulation was 3–4-fold higher than basal cAMP levels. ET-1 concentrations higher than 10^{-11} M caused a dose-dependent inhibition of cAMP formation, which reached basal level at concentrations higher than 10^{-7} M (Figure 1). This unexpected behavior pattern is indicative of either the presence of more than one ET_A-R (one that stimulates and one that inhibits cAMP formation) or the existence of a rapid desensitization process. Both possibilities are under investigation in our laboratory.

Surprisingly, the peptide agonists SRTX-*b*, SRTX-*c*, and ET-3 showed a different pattern of behavior. With these agonists, no stimulation was observed at the picomolar range, followed by stimulation, though at lower efficiency, at the nanomolar range with maximal effect at 10^{-7}–10^{-6} M (Figure 2). The EC_{50} values (in nanomolar) for SRTX-*b*, SRTX-*c*, and ET-3 were 6, 1, and 5, respectively. Thus, signal transduction of ET_A-R appears to be ligand-specific. In view of the novel finding that a signal transduced by ET-1 differs from that transduced by its peptide analogs ET-3, SRTX-*b*, and SRTX-*c*, we examined the effects of SRTX-*b* or SRTX-*c* at concentrations of 10^{-10} M and higher on cAMP accumulation induced by 1 pM ET-1. The formation of cAMP was completely blocked, indicating that the difference in mode of signal transduction is not due to an inability of the peptides to bind to ET_A-R. Thus, ET-3, SRTX-*b*, and SRTX-*c* act as antagonists for the ET-1-stimulated cAMP accumulation.

Table 1: Effects of Various Agents on [^{3}H]NE Release and cAMP Accumulation Induced by ET-1[a]

	inhibition (%)	
	[^{3}H]NE released	cAMP accumulation
BQ-123 (5 nM)	100	96.8
BQ-123 (1 nM)	71.4	75.1
BQ-123 (0.1 nM)	2.3	28.6
BQ-123 (0.05 nM)	0	0
PT treatment	100	100
EGTA (1 mM)	100	100
nifedipine (50 μM)	98	75
verapamil (10 μM)	80	52.4
verapamil (1 μM)	10	33.6

[a] 100% is the response induced by 1 pM ET-1 minus the basal level. The values shown represent percent inhibition of the above.

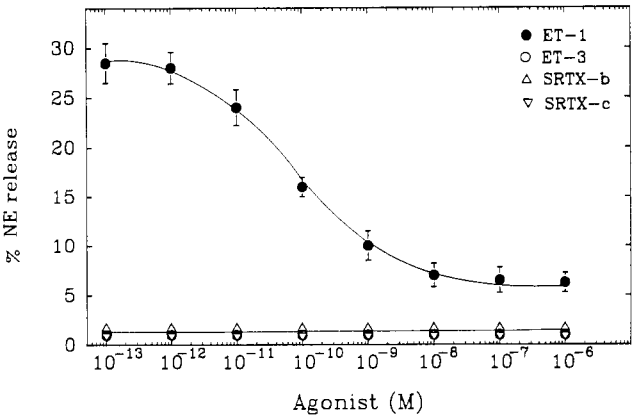

FIGURE 3: Dose–response curve of [^{3}H]NE release stimulated by four peptides. Each point is the mean ± SEM of three to five experiments (Student's *t*-test as in Figure 1, $p < 0.05$).

However, their ability to stimulate cAMP release at higher concentrations suggests that they may lead to the activation of different pathways.

The presence of the specific ET_A-R antagonist BQ-123 in the assay medium inhibited cAMP stimulation (Table 1), indicating that indeed this receptor subtype is involved. Analysis of the data yielded a Hill slope of 1.4, indicating cooperativity, in agreement with our recent binding and kinetic experiments in rat heart preparations (Sokolovsky, 1992b).

Because heart preparations are enriched with catecholamines, we examined the effect of the β-blocker propranolol on the cAMP formation stimulated by ET-1. cAMP accumulation was inhibited by 20 μM propranolol, and the EC_{50} value derived from dose-dependency curves was 7–8 μM. However, propranolol (10–50 μM) did not affect cAMP formation stimulated by ET-3, SRTX-*b*, or SRTX-*c*, suggesting that the stimulation of cAMP formation by ET-1 is a result of catecholamine release and again pointing to differences in signal transduction modes among endothelins and/or sarafotoxins. We then measured the ET-1-stimulated release of [^{3}H]norepinephrine (NE) into the assay medium. ET-1 released [^{3}H]NE from rat atrial slices in a dose-dependent manner, with maximal effect at the picomolar range and complete inhibition at 10^{-7}–10^{-6} M (Figure 3) and in a pattern very similar to that of cAMP accumulation (Figure 1). The amount of [^{3}H]NE released by ET-1 stimulation was high, about 80%–90% of that elicited by 50 mM K^+. Stimulation of NE release by ET was reported previously; for example, in cultured bovine adrenal chromaffin cells (Boarder & Marriot, 1991), ET-1 stimulated the release of catecholamines in a Ca^{2+}-dependent manner, but the stimulated release was small compared to that elicited by 50 mM K^+. As in the

case of cAMP formation, SRTX-*b* and SRTX-*c* at concentrations of 10^{-10} M and higher completely blocked [^{3}H]NE release induced by 1 pM ET-1.

Reserpine depletes the heart preparation of its catecholamine content, thus blocking further transmission by these agents. Rats were injected ip with reserpine (1 mg/kg) on two successive days, followed by no treatment on the third day. On the fourth day, animals were sacrificed, and atrial slices were prepared as described. In these preparations, stimulation of cAMP formation by ET-1 (10^{-13}–10^{-10} M) was completely inhibited. On the other hand, cAMP formation stimulated by SRTX-*b* or SRTX-*c* (10^{-9}–10^{-6} M) was similar to that obtained in control preparations from untreated rats.

Numerous studies on intact cells indicate that a rise in intracellular Ca^{2+} triggers the secretion of catecholamines. As shown in Table 1, nifedipine, verapamil, and EGTA abolished ET-1-stimulated catecholamine secretion in atrial preparations. This suggests that Ca^{2+} channels (including the L-type) may play an important role in amine release stimulated by ET-1 in rat atrial slices, possibly by coupling to G-protein(s). To determine whether the coupling of ET_A-R to cyclic AMP cascades is mediated by G-proteins, we examined the effect of pertussis toxin (PT)-catalyzed ADP-ribosylation of G-protein on ET-1-stimulated NE release and on cAMP accumulation, using our previously described experimental protocol (Sokolovsky, 1993). PT treatment of rat atrial slices (1 μg/mL, 1 h, 36 °C) completely inhibited ET-1-stimulated NE release and cAMP accumulation (Table 1) but did not affect the stimulation of cAMP formation by SRTX-*b*, ET-3, or SRTX-*c* (not shown). Thus, PT-sensitive G-protein is involved in mediating the catecholamine release. This is consistent with the suggested coupling of PT-sensitive G-protein(s) to the calcium channel (Hartzell & Fischmeister, 1992; Bernheim et al., 1992; Milligan, 1993).

We propose the following signal transduction pathway for this new ET_A-R subtype in the rat heart preparation: ET-1 binds to high-affinity binding sites (in the picomolar range) on the ET_A-R subtype, leading to activation of Ca^{2+} channels, which are coupled to G-protein. This activation results in the release of catecholamines, which in turn activate adenylyl cyclase, leading to the formation of cAMP and stimulation of the cAMP cascade.

This study demonstrates that the ET_A-R subtype with binding affinities in the picomolar range (designated ET_{A1}-R) plays a distinct role in signal transduction, indicating its physiological importance. From the dose–response curve shown in Figure 1 it is clear that the signal can be triggered by different stimulatory/inhibitory behavior, depending on the experimental concentration used. Thus, for example, ET-1 at 1 μM will neither stimulate nor inhibit cAMP formation in rat atrial slices. Moreover, the effects of ET-3, SRTX-*b*, and SRTX-*c* on the cAMP pathway differ from those of ET-1 (compare Figures 1 and 2). In this case, stimulation is at the nanomolar rather than the picomolar range. Moreover, each of the three peptide analogs blocked the stimulation of cAMP formation and [^{3}H]NE release induced by ET-1, indicating that they do bind at the low concentration range and that their binding prevents stimulation by ET-1. Thus, their binding appears to be mutually exclusive as expected for antagonists. The most plausible explanation is that they form different receptor–ligand complexes and that one type of complex might lead to stimulation of the cAMP cascade while another type might inhibit it. Because cAMP accumulation is induced by ET-3, SRTX-*b*, and SRTX-*c* only at nanomolar concentrations, a range at which phosphoinositide hydrolysis is evoked, we cannot exclude the possibility that triggering of the cAMP pathway is a secondary effect rather than a result of direct linkage to the pathway. Direct linkage of both phosphoinositide hydrolysis and cAMP signaling has been reported for the thyrotropin receptor (van Sande et al., 1990). Like ET_A-R, thyrotropin receptors require a higher concentration of ligand for activation of phosphoinositide hydrolysis activation than for stimulation of cAMP formation.

Endothelins are involved in numerous physiological responses (Sokolovsky, 1992a). Involvement of ET_A-R in cAMP formation as demonstrated in this study suggests the operation of a novel signaling pathway for such responses. Thus, in addition to the effects of ET-1 on functions such as contractility and ion handling in rat cardiac myocytes, it may be involved in pathways of cytosolic and nuclear signaling (Wang et al., 1982; Pribnow, 1992), such as gene expression and translation, which are typical of hypertrophic responses described, for example, in rat cardiac myocytes (Shubeita et al., 1990; Ito et al., 1991; Kramer et al., 1992; Sudgen et al., 1993).

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