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ENDOTHELIN-1 STIMULATION OF NORADRENALINE AND ADRENALINE RELEASE FROM ADRENAL CHROMAFFIN CELLS

MICHAEL R. BOARDER* and DEBORAH B. MARRIOTT

Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN, U.K.

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Abstract—Endothelin-1 (ET-1) stimulated release of both noradrenaline and adrenaline from cultured bovine adrenal chromaffin cells; stimulated release was small compared to that elicited by 50 mM potassium. Sarafotoxin-6b stimulated release to a similar extent as ET-1. The ET-1 stimulated release had an EC_{50} of about 1 nM. This calcium-dependent release was partially inhibited by nitrendipine (1 μM), but there was no synergistic interaction with the calcium channel agonist BAY K 8644 (1 μM). There was also no synergistic release seen when submaximal stimulation with potassium was combined with ET-1. Stimulation of fura-2 loaded cells with ET-1 produced an unusual timecourse of response which rose slowly to a maximum which was sustained. These results show that ET-1 may stimulate both noradrenaline and adrenaline containing chromaffin cells by a mechanism which, while partially dependent on dihydropyridine sensitive calcium channels, is distinct from the calcium channel agonist or membrane depolarization.

The adrenal medulla is a highly vascularized tissue in which the principle cell types are the microvascular endothelial cells and the neural crest-derived chromaffin cells. We have been interested in a possible interaction between the two cell types, in part as a model of interaction between endothelial cells and elements of the nervous system. For example, we have recently investigated the modulation of adrenal medulla endothelial cells by ATP released from the chromaffin cells [1]. A possible mechanism for regulation in the opposite direction (modulation of chromaffin cells by endothelial cells) is the secretion by the endothelium of a mediator such as endothelin, which interacts with the chromaffin cells.

Endothelin-1 (ET-1) is a 21 amino acid peptide isolated from vascular endothelial cells and shown to be a potent vasoconstrictor [2] and is now known to be one of a series of related peptides; three genes related to ET-1, coding in addition the closely eluted peptides endothelin-2 and endothelin-3 are found in the mammalian genome, and the sarafotoxins from snake venom show a remarkable structural and functional homology [3]. The demonstration that these peptides are active at receptors on vascular smooth muscle cells was followed by evidence that receptors for ET-1, and functional responses, may be found in many tissues. In the central nervous system binding sites for ET-1 and the related sequence sarafotoxin-6b have been described [4–9] and a functional response, (poly)phosphoinositide hydrolysis, has been reported [8–10]. Both ET-1, and to a lesser extent ET-3, have been localised in the spinal cord [11]. In the peripheral nervous system a further functional response to ET-1 has been described: depolarisation mediated by the release of substance P [12].

The cultured bovine adrenal chromaffin cell releases noradrenaline and adrenaline from separate cell subtypes in response to activation of a variety of cell surface receptors (e.g. Refs 13 and 14), which operate with different receptor-effector mechanisms. In a preliminary report [15] we showed that ET-1 could stimulate release of noradrenaline from chromaffin cells. Here we provide a partial characterisation of the stimulation of chromaffin cells by ET-1.

METHODS

Bovine adrenal chromaffin cells were digested with collagenase/protease by retrograde perfusion and purified by centrifugation followed by differential plating [16]. Cells were cultured on 24-well "Primaria" plates (0.5×10^6 cells/well) in Dulbecco's modified medium with 10% foetal calf serum, 27 mg/mL glutamine, 1 mL/100 mL of non-essential amino acids (GIBCO, at 100 × final concentration), 50 μg 100 mL of gentamycin, 2500 I.U./100 mL of penicillin, 2500 μg/100 mL of streptomycin, 2.5 μg/mL of fungizone, 5 μM fluorodeoxyiridine and 5 μM cytosine arabinoside. After three to seven days in culture, cells were washed twice in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffered balanced salt solution (BSS: NaCl, 125 mM: KCl, 5.4 mM; $NaHCO_3$, 16.2 mM; HEPES, 30 mM; NaH_2PO_4, 1 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 1.8 mM; glucose, 5.5 mM; buffered to pH 7.4). The stimulation period at 37° in the presence or absence of drugs in 0.5 mL BSS was for 5 min, after which the supernatant was removed to ice and taken to 0.1 M HCl. Where dihydropyridines were used they were present during a 10 min preincubation

* To whom correspondence should be addressed.

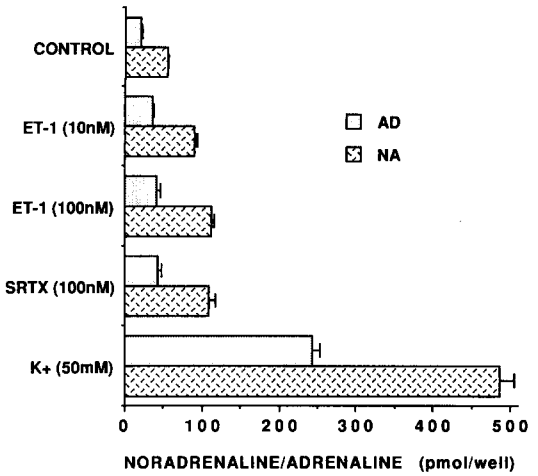

Fig. 1. Release of noradrenaline and adrenaline in response to ET-1, sarafotoxin-6b (SRTX) or depolarising concentrations of potassium $(K^+ 50\,mM)_m$ present during a 5 min incubation period (N = 4, mean ± SEM).

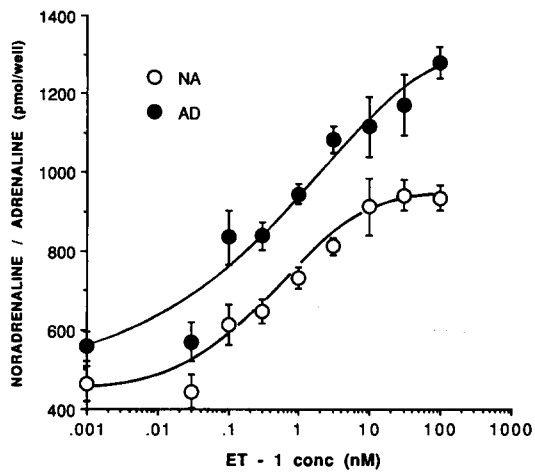

Fig. 2. Release of noradrenaline and adrenaline in response to different concentrations of ET-1 (N = 4, mean ± SE).

as well as during the 5 min stimulation period. Noradrenaline and adrenaline present in the acidified supernatants were estimated by high pressure liquid chromatography followed by electrochemical detection [17].

For the measurements of free intracellular calcium a method based on the fura-2 procedure of Grynkiewicz *et al.* (1985) [18] was used. Briefly, cells were cultured for 1–2 days on bacteriological dishes to which they did not adhere; suspended chromaffin cells were then loaded with 5 μM fura-2 acetoxymethyl ester in BSS for 45 min at 37°. After three washes the cells were resuspended at about 10^6/mL and placed in a cuvette maintained at 37° in a Perkin Elmer dual wavelength spectrometer, and fluorescence measured at emission wavelengths of 340 and 380 nM and excitation wavelengths of 500 nM. Maxima were recorded with 0.2% Triton X-100, followed by quenching with 7.5 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) for minima.

ET-1 and sarafotoxin 6b were from Peptide Research Institute (Osaka, Japan). All tissue culture reagents were from GIBCO (Paisley, U.K.) except Primaria plates (Falcon) which were from Becton–Dickinson (Oxford, U.K.). BAY K 8644 was a gift from Bayer (Leverkusen, F.R.G.). Other chemicals were from the Sigma Chemical Co. (Poole, Dorset, U.K.) or Fisons (Loughborough, U.K.).

RESULTS

Figure 1 shows that 5 min incubation with 10 nM or 100 nM ET-1 stimulates release of both noradrenaline and adrenaline. There was considerable variation in release. In a series of 17 consecutive experiments, each undertaken with quadruplicate determinations (utilising eight different cell preparations) four failed to show a significant increase in noradrenaline efflux over basal in response to ET-1. The remaining 13 showed a significant ($P < 0.05$) stimulated release to either 10 nM or 100 nM ET-1 (both maximally effective concentrations, see Fig. 2) of between 22 and 471 pmol noradrenaline/well (mean ± SEM = 183 ± 40), this being between 1.66 and 4.43 fold over control release (mean ± SEM = 2.4 ± 0.3). Expressed as a per cent of cell content the stimulated release varied from 1.66 to 8.5% (mean ± SEM = 2.4 ± 0.8). The variation in response in individual experiments with these small responses has precluded the pooling of data across experiments, and so data is shown from individual representative experiments (each with quadruplicate determinations).

Sarafotoxin-6b (100 nM) also causes a rise in efflux of both catecholamines to a similar degree to that elicited by ET-1. For comparison, Fig. 1 shows the consequences of stimulation with depolarizing concentrations of extracellular potassium. It can be seen that the ET-1 induced secretion is much smaller than that caused by depolarisation with 50 mM potassium; however, the ratio between released noradrenaline and adrenaline is similar, with noradrenaline being preferentially released by both types of stimulus. The ratio of noradrenaline to adrenaline in the cell extracts varied between different cultures. This resulted in a variation between cultures but not within the same culture, in the ratio of catecholamines released, as reflected in the data presented here.

Characterisation of the time course of release in response to ET-1 showed that by 5 min a maximal release was achieved with no further stimulation up to the maximum time measured of 20 min (data not shown). In Fig. 2 the dose–response relationship to ET-1 is shown, with a characteristic EC_{50} of about 1 nM, for both noradrenaline and adrenaline release.

The role of calcium entry in the ET-1 stimulation of release was investigated in a series of experiments. Chelation of extracellular calcium attenuates release of both noradrenaline and adrenaline, an effect which can also be seen by introducing the

Table 1. Stimulation of catecholamine efflux by ET-1: Effect of dihydropyridines

	Catecholamine release	
	Noradrenaline	Adrenaline
Control	170.4 ± 11.8	156.1 ± 15.3
ET-1 (10 nM)	253.8 ± 10.5	203.2 ± 11.7
ET-1 + Nitrendipine	217.4 ± 15.0*	175.3 ± 6.2
ET-1 + BAY K 8644	455.2 ± 28.0†	266.5 ± 18.5

The results are pmol/well (mean ± SEM, N = 4) of noradrenaline or adrenaline release during a 5 min incubation in the presence or absence (control) of 10 nM ET-1. Dihydropyridine at 1 μM were present as indicated during a 10 min preincubation as well as during the incubation period. Significantly different from stimulation in the absence of dihydropyridine: *$P < 0.05$; †$P < 0.001$; (Student's *t*-test).

inorganic calcium channel blocker cadmium. In other experiments it was shown that ET-1 stimulated catecholamine release could be attenuated by not adding calcium to the incubation medium (data not shown). Table 1 shows an experiment in which stimulation with ET-1 was in the presence of the dihydropyridine calcium channel blocker nitrendipine or the calcium channel enhancer BAY K 8644. The release of both catecholamines was blocked, in part, by nitrendipine. In three such experiments the reduction was to 37 ± 11% of control. Pre-release was greater in the presence of BAY K 8644. However, BAY K 8644 characteristically produces a stimulation of release on its own. Evaluation of an interaction between BAY K 8644 and ET-1 can more clearly be estimated in Fig. 3A, where it is apparent that the effects of the dihydropyridine agonist with ET-1 are additive, while combined with 25 mM potassium, BAY K 8644 is synergistic. In Fig. 3B the consequences of both ET-1 and a submaximal depolarizing concentration of potassium, are shown. Again, the two stimulants are additive in their effect on release, and not synergistic.

In order to further evaluate the effect of endothelium on chromaffin cells, Fig. 4 shows an example from a group of experiments measuring free intracellular calcium in cells loaded with the fluorescent dye, fura-2. For comparison the figure shows the effect of stimulation with nicotine, which produces a characteristically very rapid response,

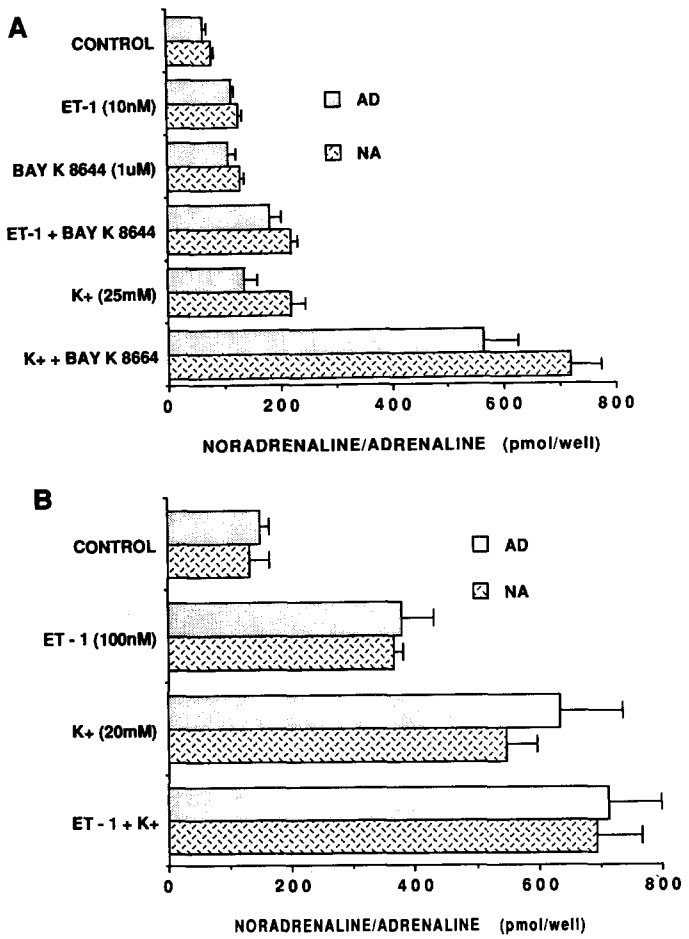

Fig. 3. Release of noradrenaline and adrenaline in response to different combinations of ET-1, high extracellular K^+ and BAY K 8644.

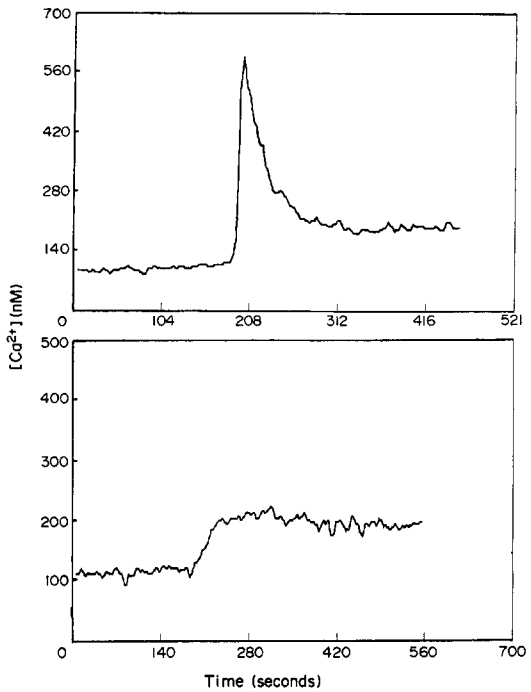

Fig. 4. Free intracellular calcium measurements in fura-2 loaded cells in response to 30 μM nicotine (upper panel) and 100 nM endothelin (lower panel). In each case the peptide was added at 180 sec.

with a maximum above 500 nM which rapidly declines with no discernible plateau at the maximum. By contrast, ET-1 caused a rise which, while essentially immediate in onset, was relatively slow in reaching a maximum of about 200 nM, which was sustained, with only a gradual decline. This unusual pattern of calcium elevation was very reproducible in all preparations which responded. However, for reasons which are not clear 5 out of 10 of the fura-2 loaded preparations tested failed to give a response to ET-1.

DISCUSSION

A large number of recent publications have investigated the role of calcium entry, mobilisation of intracellular calcium stores and phospholipid derived second messengers (such as inositol phosphates, diacylglycerol and arachidonic acid) in the endothelin stimulated contraction of vascular smooth muscle from a variety of sources. Some studies have suggested that contraction in response to ET-1 is not sensitive to dihydropyridine blockers [19, 20], while others report only partial sensitivity to drugs such as nitrendipine [21–25]. Many reports have shown that formation of inositol phosphates [19, 23, 26], mobilization of intracellular calcium [27] formation of diacylglycerol [25, 26, 28], and stimulation of phospholipase A_2 [29] may play a role in stimulus–contraction coupling in response to endothelins in vascular and cardiac muscle cells. However, studies on elements of the nervous system are few, and principally relate to stimulation of formation of inositol phosphates [8–10]. Studies on the interaction of endothelins with chromaffin cells has been limited to a preliminary communication from our laboratory showing that ET-1 can cause a small stimulation of release from chromaffin cells [15] and a report [30] showing that ET-1 can enhance free intracellular calcium.

The experiments reported here show that ET-1 [in common with sarafotoxin-6b] can enhance efflux from both noradrenaline and adrenaline secreting chromaffin cells in a manner dependent on extracellular calcium and sensitive to cadmium at a concentration effective at blocking both bradykinin and potassium stimulated release [16]. We have shown in previous studies that catecholamine release in response to agonists can be dependent on influx through dihydropyridine sensitive [31] or dihydropyridine insensitive [16] channels. Also, release in response to high extracellular potassium concentrations is mediated by calcium influx which is mainly, but not exclusively, through dihydropyridine sensitive channels [32]. In the experiments we have undertaken on ET-1 stimulated release the partial sensitivity to nitrendipine presumably reflects the involvement of both dihydropyridine sensitive and dihydropyridine insensitive channels. However, the stimulation of release by ET-1 is itself small compared to control, making a partial inhibition difficult to characterise.

The involvement of calcium channels, at least in part of a dihydropyridine sensitive type, has raised the possibility that ET-1 elicits release by a partial depolarization. While this has not been directly investigated, results presented here argue with this. We show that BAY K 8644 alone elicits a small release which is additive with the release in response to ET-1. If ET-1 were causing a submaximal depolarization then we would expect a large augmentation of release in the presence of BAY K 8644. Similarly, it has been suggested (2) that ET-1 might act directly at calcium channel sites to mimic the effect of dihydropyridine agonists such as BAY K 8644. Two observations presented here indicate that this is not the case. Firstly, the effect of ET-1 is still seen in the presence of maximally effective concentrations of BAY K 8644, indicating that they are acting by different mechanisms. Secondly the effect of BAY K 8644 with a submaximal depolarizing stimulus is synergistic, while the effect of ET-1 with submaximal depolarizing stimulation is seen here to be additive.

The relatively small amount of release (compared to other secretagogues studied) is reflected in the small calcium signal caused by ET-1. The calcium signal is also unusual in that it is slow to reach a maximum which is then sustained, in contrast to bradykinin where the signal rises rapidly to a maximum which is not sustained. It may be that the rapidly rising phase in response to bradykinin is due to mobilisation of intracellular calcium stores which is followed by the calcium influx necessary to elicit release, while in response to ET-1 only a small, slow onset and sustained calcium influx is seen in the absence of mobilization of intracellular pools. Consistent with this, our recent experiments suggest that endothelins act on chromaffin cells at a receptor

which differs from that on vascular smooth muscle cells not only with respect to radioreceptor assay binding characteristics but also in that it fails to stimulate hydrolysis of inositol phospholipids [33]. The pattern of calcium signal seen here is similar to the very small but sustained response reported by Rasmussen and Printz [30]. The release in response to ET-1 reported here is a small increase over control release compared to that elicited by other agents studied by us.

Using our procedure the control release is principally caused by the physical effects on the cells of changing medium, and is therefore much larger than the basal catecholamine efflux seen in undisturbed cells [16]. This means that the stimulation of release by ET-1 probably represents a several fold increase over true basal release from these cells. If therefore there is an endogenous stimulation of endothelin receptor on chromaffin cells *in vivo* then this is likely to be of significance. The small release compared to the experimental control does mean however, that the release is difficult to study and in cases where control release is high and noisy the stimulation may not be easily seen. This may explain why Rasmussen and Printz [30] see only a small release (1–2% over control release). Further comparison between their data and that reported here is complicated by differences in procedure: they used release of tritium after labelling with [^{3}H]noradrenaline, while we measured release of endogenous catecholamines; with their fura-2 calcium measurements they used room temperature, while we used 37°.

In conclusion, we have shown that ET-1 may stimulate the release of catecholamines from chromaffin cells by a mechanism distinct from depolarisation or calcium channel agonists. Consistent with this we have recently described a high affinity binding site for endothelins which has the characteristics of a receptor, which is distinct from the endothelin receptor found on vascular smooth muscle cells [33]. It is interesting to speculate that *in vivo* endothelial cells may modulate release of catecholamines from the adrenal medulla by endothelin production, and that perhaps other examples exist of regulation of neuronal elements by endothelial cells.

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