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# Stimulation of inositol phosphate production in clonal HSDM1C1 cells by endothelins and sarafotoxin

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Endothelin (ET*), a polypeptide of 21 amino acid residues, has been isolated recently from porcine vascular endothelial cells and has been shown to be identical in structure to human endothelin [1, 2]. Human and porcine ETs have been denoted as $ET_1$ [1, 2]. Rat ET ($ET_3$), on the other hand, has a slightly different amino acid sequence to $ET_1$ but still has a high degree of homology with the latter peptide [1, 2]. Subsequently ETs have been shown to be some of the most potent vasoconstrictor substances known to date and are capable of eliciting powerful responses *in vivo* and *in vitro* in a variety of animal/tissue preparations [1–5]. Although Yanagisawa *et al.* [1] originally proposed that perhaps $ET_1$ produced its biological actions by facilitating the entry of extracellular calcium into the cell by interacting with $Ca^{2+}$ channels, it has been shown that $ET_1$ does not bind directly to voltage-sensitive $Ca^{2+}$ channels, N-type $Ca^{2+}$-channels, or to sodium channels [5–7]. On the contrary, preliminary evidence, using cultured vascular smooth muscle cells [8–10], has indicated that $ET_1$ receptors may be coupled to the phosphoinositide (PI) turnover signalling mechanism resulting in mobilization of intracellular and/or extracellular $Ca^{2+}$ [9, 10].

Sarafotoxin S6b(SRFT) is also a 21 amino acid-containing peptide which has been purified from snake venom [11, 12]. SRFT has been shown to have a high degree of homology with $ET_1$ and, like the latter, is a potent vasoconstrictor [11, 12]. SRFT receptors in the rat brain and heart appear to mediate PI turnover [11, 12], and $ET_1$ and SRFT appear to have similar properties in terms of receptor binding activity and stimulation of PI hydrolysis in these tissues [13].

However, to date the physiological actions of $ET_1$ and SRFT, such as tissue contractions [1–5], *in vivo* hemodynamic and vascular effects [5, 11, 12], and the biochemical effects of these peptides, such as PI turnover and $Ca^{2+}$ mobilization [11, 12], have been conducted on isolated organs, tissue slices, cells in primary culture (after multiple passages in some cases) or using *in vivo* preparations. In the present study we have identified functional receptors for $ET_1$, $ET_3$ and SRFT in an immortal cell line, mouse fibrosarcoma cells (HSDM1C1), using PI turnover as an index of receptor activation. This is an important finding since clonal cell lines have several advantages over primary cultures of tissues and, therefore, HSDM1C1 cells may prove useful in further regulatory studies on the $ET_1$/SRFT receptor-coupled PI turnover system.

## *Materials and Methods*

Murine fibrosarcoma cells (from the American Type Culture Collection) were cultured in Ham's F10 medium supplemented with 15% donor horse serum and 2.5% fetal calf serum in 150 $cm^2$ Costar sterile culture flasks at 37° in a humidified atmosphere of 10% $CO_2$ and 90% air. The medium was changed every 3 days, and the cells were passaged by trypsinization every 5 days and subcultured at a density of 0.2–0.3 × $10^6$/well in 24-well plates. The cell inositol phospholipids were labeled at 37° with 0.45 to 0.8 μCi/mL of *myo*-[$^3$H]inositol (American Radiochemical Co.; 15 Ci/mmol) for 2 days.

For the phosphoinositide turnover experiments, the culture medium was aspirated, cells were washed gently with 2 mL of oxygenated Krebs-bicarbonate buffer (pH 7.4), and the cells were challenged with 0.25 to 0.5 mL of the drug solution made in Krebs buffer containing 10 mM LiCl. The cells were incubated for 30 min at 37° with the test compounds or buffer alone, and the assays were terminated by the addition of 1 mL of ice-cold chloroform/methanol (1 : 2, v/v). For antagonism studies, the putative antagonists were added to the cells 30 min prior to addition of agonists. After 5 min, the cell lysates were transferred to Biovials, and 0.3 mL of chloroform and 0.3 mL of water were added to the lysates. The contents of the vials were shaken for 5 sec and then left to stand at 23° for 1 hr in order to separate the aqueous and organic phases. After this time, 0.8 mL of the upper aqueous phase was transferred to Econo-columns containing 1 mL of AG-1-X8 anion exchange resin (200–400 mesh in formate form; Bio-Rad). The free *myo*-[$^3$H]inositol was eluted from the columns with 8–10 mL of unlabeled *myo*-inositol (20 mM) and discarded. Total [$^3$H]inositol phosphates retained on the resin were then eluted off using 4 mL of 1 M ammonium formate and 0.1 formic acid and counted and quantified as previously described [14–16]. The concentration–response curves were analyzed using an iterative curve-fitting computer program [17] and the concentrations of the agonists required to produce 50% of the maximal response ($EC_{50}$ values) obtained.

$ET_1$, $ET_3$, SRFT, bradykinin (BK), BK-antagonist and other peptides were purchased from Peninsula Laboratories and *cis*-dioxolane, histamine, glutamate, γ-aminobutyric acid (GABA), serotonin, norepinephrine, U69593 and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) were purchased from Research Biochemicals Inc. and the Sigma Co.

## *Results and Discussion*

Initial exploratory studies using a fixed concentration of test compounds (10 μM) showed that while $ET_1$, $ET_3$ and SRFT inducdd a 4.5- to 5.6-fold stimulation of PI turnover in HSDM1C1 cells (Fig. 1), several other neuroactive agents were inactive in this system (Table 1). The only other substances shown to be active in this system were BK and the muscarinic agonist, *cis*-dioxolane (CD) (Table 1). Detailed concentration–response studies revealed that $ET_1$, $ET_3$, SRFT, BK and CD possessed agonist activity, with

* Abbreviations: ET, endothelin; BK, bradykinin; PI, phosphoinositide; IP, inositol phosphate; and SRFT, sarafotoxin S6b.

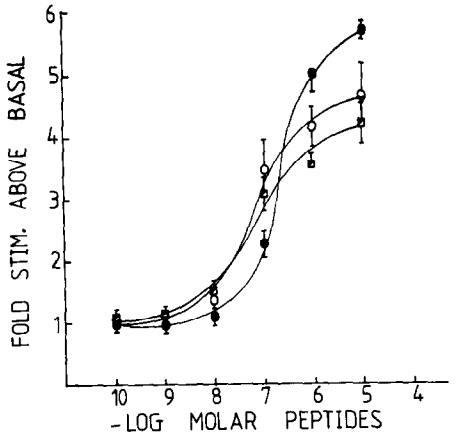


Fig. 1. Effects of $ET_1$, $ET_3$ and SRFT on PI turnover in HSDM1C1 cells. Data are means ± SE from 3–6 experiments. The x-ordinate depicts the −log peptide concentration and the y-ordinate depicts fold-stimulation of [$^3$H]IP accumulation above basal levels (see Table 1 for more details). Key: (◩) $ET_1$; (●) $ET_3$; and (○) SRFT.

the muscarinic agonist producing the highest maximum-fold stimulation of [$^3$H]IP generation (Table 1). Although, $ET_1$ and SRFT produced approximately the same maximum level of [$^3$H]IP accumulation, $ET_3$ induced a slightly higher level of PI turnover but was about 2.6 to 3.6 times less active than SRFT and $ET_1$ (Table 1, Fig. 1). Yanagisawa *et al.* [1] and other researchers [18] have also observed a similar relative lack of activity of $ET_3$ as compared to $ET_1$ in relation to their abilities to constrict rat aortic strips, to raise blood pressure, and to exert vasoconstriction in the rat mesentery. The equipotent effects of SRFT ($EC_{50}$ = 61.4 ± 5.3 nM) and $ET_1$ ($EC_{50}$ = 62.1 ± 7.4 nM) in the clonal HSDM1C1 cell PI turnover responses (Table 1) corroborate similar findings in rat brain and heart slices using the PI turnover technique (SRFT $EC_{50}$ = 100 ± 30 nM) [13], in porcine coronary artery ($ET_1$ $EC_{50}$ = 40 nM) [19], and in uterine slices ($ET_1$ $EC_{50}$ = 20–40 nM) [20]. $ET_1$ and SRFT also appear to have similar potencies in their abilities to contract several rat and guinea pig smooth muscle and vascular tissue preparations [5]. However, in view of the somewhat higher potency of $ET_1$ ($EC_{50}$ values of 1–5 nM) in certain cells and tissues of different species [1–5, 8–10, 18], these data may suggest the presence of multiple ET and/or SRFT receptors [5, 18]. This proposal has been supported recently by the detection of two [$^{125}$I]$ET_1$ binding sites, one with high affinity ($K_d$ = 0.8 nM) and the other with a lower affinity ($K_d$ = 45 nM) in rat kidney mesangial cells [21], and further by the finding that $ET_1$ and SRFT compete for [$^{125}$I]SRFT binding with inhibition constants of 42 and 40 nM, respectively, in rat cerebellar and aortic homogenates [22]. It may be also pertinent to note that activation of only the low-affinity $ET_1$ receptors in the rat mesangial cells results in inositol phosphate production [21]. Alternatively, species/tissue differences and/or differences in receptor reserves may account for the different biological potencies of these peptides [5, 18]. The apparent low potencies of $ET_1$ and SRFT in the HSDM1C1 cells may also be the result of cell transformation during the production of this clonal cell line in addition to some of the above factors.

The fact that the ETs and SRFT were acting through receptors which were distinct from that for BK and muscarinic drugs to cause PI hydrolysis in the HSDM1C1 cells was suggested by the observation of additive effects of different combinations of $EC_{50}$ concentrations of these agonists (Fig. 2). Furthermore, although the muscarinic receptor antagonist, 4-DAMP, and the $B_2$-selective BK antagonist, [D-Arg$^0$-Hyp$^3$-D-Phe$^7$]BK, competitively blocked [inhibition constants ($K_B$) [23] of 0.91 ± 0.15 (N = 4) nM and 370 ± 51 (N = 3) nM)] *cis*-dioxolane- and BK-induced PI turnover, respectively, no such influence on SRFT and $ET_1$ responses was observed with these compounds or with naloxone, U69593 and ω-conotoxin GV1A. The $K_B$ values of other muscarinic antagonists against *cis*-dioxolane-induced PI turnover (pirenzepine = 57 ± 9 nM; methoctramine = 560 ± 150 nM; *p*-fluorohexahydrosiladifenidol = 25 ± 5 nM) suggested the presence of putative $M_3$ muscarinic receptors in this cell line. However, although pre-

Table 1. Relative potencies of human endothelin, rat endothelin, sarafotoxin, bradykinin and *cis*-dioxolane on PI turnover in HSDM1C1 cells

| Test agonist | $EC_{50}$* (nM) | Max. fold-stim.* |
|---|---|---|
| Human endothelin ($ET_1$) | 62.1 ± 7.4 | 4.5 ± 0.4 |
| Rat endothelin ($ET_3$) | 224.1 ± 22.0† | 5.6 ± 0.1‡§ |
| Sarafotoxin S6b (SRFT) | 61.4 ± 5.3‖ | 4.9 ± 0.5 |
| Bradykinin (BK) | 61.9 ± 8.9 | 3.9 ± 0.3 |
| *cis*-Dioxolane (CD) | 1098 ± 279 | 17.1 ± 0.5¶ |

Data are means ± SE from 3–6 experiments, each conducted in triplicate. Typically, the basal dpm were 1000–1200 and in the presence of $ET_1$, $ET_3$ or SRFT (10 μM) the [$^3$H]IP dpm were increased to 5500–6800 dpm. Compounds that failed to induce PI turnover at ⩾10 μM included: histamine, glutamate, GABA, serotonin, norepinephrine, cholecystokinin, neurotensin, w-conotoxin GVIA, substance P, eledoisin, physaelemin, angiotensin II, urodilatin, thyrotropin-releasing hormone, proctolin, atrial naturetic peptide, naloxone, U69593, zacopride and vasopressin.

* $EC_{50}$ = concentration of agonist required to produce 50% of the maximal stimulation. Max. fold stim. = maximum fold-stimulation of PI turnover at the highest agonist concentration tested relative to the basal level.

†–‖ Statistical analysis: † $P < 0.01$ compared to $ET_1$; ‡ $P < 0.05$ compared to $ET_1$; § $P < 0.001$ compared to BK; ‖ $P < 0.01$ compared to $ET_3$; and ¶ $P < 0.001$–$0.05$ compared to $ET_1$, $ET_3$, SRFT and BK.

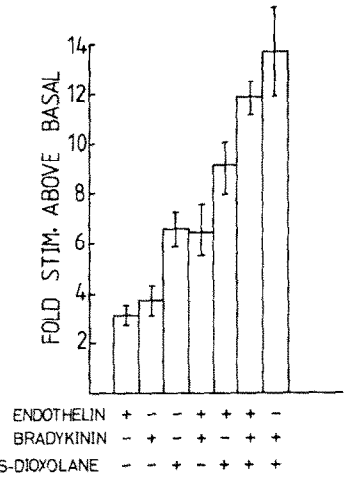


Fig. 2. Additive effects of $ET_1$, BK and *cis*-dioxolane on PI turnover in HSDM1C1 cells. Data are means ± SE from 3–4 experiments using $ET_1$ (90 nM), BK (50 nM) and *cis*-dioxolane (1 $\mu$M).

incubation of HSDM1C1 cells with the $Ca^{2+}$-channel blocker nifedipine (0.1 nM to 1 $\mu$M) failed to affect $ET_1$-induced PI breakdown (Fig. 3), the addition of 0.5 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) in the Kreb's buffer resulted in significant reductions in the potency of $ET_1$, BK and *cis*-dioxolane to promote [$^3$H]IP generation (Fig. 4). The responses to $ET_1$ and BK appeared to be more affected by this treatment than those of *cis*-dioxolane, perhaps providing further evidence for the distinct nature of the receptors and differential calcium requirements for inducing biological responses by these compounds.

The apparent lack of effect of nifedipine against the ET-induced PI turnover (Fig. 3) contrasts with the observations of the antagonism of ET-stimulated contractions of isolated rat and guinea pig tissues by nifedipine [5]. However, nifedipine and other calcium entry blockers have also been shown to be ineffective at inhibiting $ET_1$-induced PI turnover in rabbit aortic smooth muscle cells [24] and at blocking $ET_1$-induced vasoconstriction of isolated rat aorta [25]. Taken together, these studies suggest that although extracellular $Ca^{2+}$ is required for the full expression of many of the ET-induced biological responses (see above), extracellular $Ca^{2+}$ is not a prerequisite for ET-induced PI turnover [18], although tissue/species differences appear to prevail with respect to these parameters. Ambar *et al.* [15] have arrived at similar conclusions for $ET_1$ and SRFT using slices of rat heart and brain. In view of the existence of subtypes of the ET and SRFT receptors [18–22], it is feasible that while the activation of the high-affinity receptors may be sensitive to $Ca^{2+}$ entry blockers, the low-affinity receptor stimulation may be resistant to the dihydropyridines like nifedipine. However, further studies are necessary to resolve this issue.

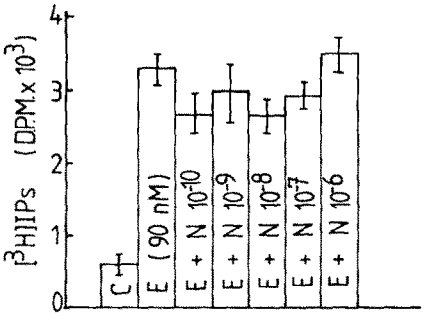


Fig. 3. Effect of nifedipine treatment on $ET_1$-induced PI turnover. [$^3$H]Inositol-labeled cells were exposed to nifedipine (0.1 nM to 1 $\mu$M) for 30 min at 37° before being challenged with $ET_1$ (90 nM). Note the lack of effect of the $Ca^{2+}$-channel blocker. Data are means ± SE of triplicate determinations from a single experiment. Two further experiments yielded similar results. E = $ET_1$; N = nifedipine.

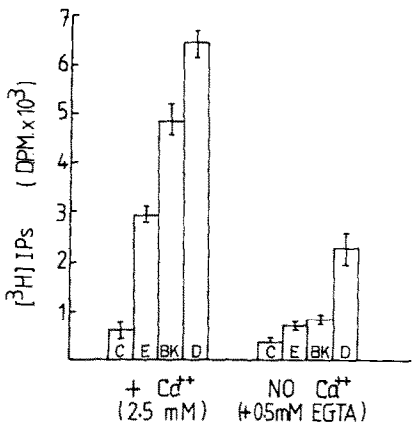


Fig. 4. Comparison of $ET_1$-, BK- and *cis*-dioxolane-induced PI turnover in the absence and presence of $Ca^{2+}$. Stimulation of PI turnover (in the presence of 10 mM LiCl) by $ET_1$ (E, 90 nM), BK (50 nM) and *cis*-dioxolane (D, 1 $\mu$M) in normal Kreb's buffer containing 2.5 mM $CaCl_2$ and Kreb's buffer lacking $CaCl_2$ but containing 0.5 mM EGTA was determined. Data are means ± SE of triplicate determinations from a single assay. Two additional assays yielded similar results.

In conclusion, the HSDM1C1 cell line contains specific $ET_1$, $ET_3$, SRFT, BK ($B_2$) and muscarinic receptors which are coupled to the PI turnover signal transduction mechanism. These cells should prove useful for further biochemical studies on the regulation of the receptor–effector coupling mechanisms.

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