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Elevation of Intracellular Calcium in Clonal Neuronal and Embryonic Kidney Cells Involves Endogenous Endothelin-A Receptors Linked to Phospholipase C Through $G_{q/11}$

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Abstract

Endothelin-1 (ET-1) is a pain mediator, elevated in skin after injury, which potentiates noxious thermal and mechanical stimuli (hyperalgesia) through the activation of ET_A (and, perhaps, ET_B) receptors on pain fibers. Part of the mechanism underlying this effect has recently been shown to involve potentiation of neuronal TRPV1 by PKC ϵ . However, the early steps of this pathway, which is recapitulated in HEK 293 cells co-expressing TRPV1 and ET_A receptors, remain unexplored. To clarify these steps we investigated the pharmacological profile and signaling properties of native endothelin receptors in immortalized cell lines including HEK 293 and ND-7 model sensory neurons. Previously we showed that in ND7/104, a dorsal root ganglia-derived cell line, ET-1 elicits a rise in intracellular calcium ($[Ca^{2+}]_{in}$) which is blocked by BQ-123, an ET_A receptor antagonist, but not by BQ-788, an ET_B receptor antagonist, suggesting that ET_A receptors mediate this effect. Here we extend these findings to HEK 293T cells. Examination of the expression of ET_A and ET_B receptors by RT-PCR and [¹²⁵I]-ET-1 binding experiments confirms the slight predominance of ET_A receptor binding sites and messenger RNA in both ND7/104 and HEK 293T cells. In addition, selective agonists of the ET_B receptor (sarafotoxin 6c, BQ-3020 or IRL-1620) do not induce a transient increase in $[Ca^{2+}]_{in}$. Furthermore, reduction of ET_B mRNA levels by siRNA do not abrogate calcium mobilization by ET-1 in HEK 293T cells, corroborating the lack of an ET_B receptor role in this response. However, in cells with low endogenous ET_A mRNA levels, ET-1 does not induce a transient increase in $[Ca^{2+}]_{in}$. Observation of the $[Ca^{2+}]_{in}$ elevation in ND7/104 and HEK 293T cells in the absence of extracellular calcium suggests that ET-1 elicits a release of calcium from intracellular stores, and pretreatment of the cells with pertussis toxin or a selective inhibitor of phospholipase C (PLC) point to a mechanism involving $G_{q/11}$ coupling.

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These results are consistent with the hypothesis that a certain threshold of ET_A receptor expression is necessary to drive a transient $[Ca^{2+}]_{in}$ increase in these cells and that this process involves release of calcium from intracellular stores following Gαq/11 activation.

Keywords

Endothelin-1; ET_A receptor; ET_B receptor; HEK 293T; ND7/104; calcium; Gαq/11

1. Introduction

Endothelin-1 (ET-1), a 21 amino acid peptide [1], is produced by a wide range of cell types where it mediates myriad cellular effects [2]. Hence, ET-1 has been linked to many physiological processes with identified roles in health and disease [3,4]. ET-1 binds, with sub-nanomolar affinities, to two class A type G protein-coupled receptors known as ET_A and ET_B [5,6]. In humans these receptors share ~51% amino acid sequence identity and are encoded by two distinct genes (EDNRA and EDNRB) located on chromosome 4 (4q31.22) and 13 (13q22), respectively. ET_A and ET_B receptors display different selectivity for endothelins [7] as well as for many competitive peptide or non-peptide antagonists which are important tools to study the function of each receptor type [8].

Over the years the relevance of endogenous ET-1 in pain processing has become apparent, including its role in inflammatory, cancer and neuropathic pain (see [9] for a recent review). In the skin, where ET-1 initiates local nociception following injury [10] and modulates the duration of inflammatory pain [11], two different mechanisms appear to be at play. Subcutaneous injection of high doses of ET-1 (100 to 500 μM) into the rat's plantar region elicits pain-like flinching behavior and induces selective firing of nociceptive fibers (C- and A delta-) but not of the non-nociceptive A beta-fibers [12]. This effect, which is blocked by BQ-123, a selective ET_A receptor antagonist, is likely directly mediated by the activation of ET_A receptors present in a subset of small peptidergic (CGRP+) and non-peptidergic neurons [13–15]. Activation of ET_A leads to increased neuronal excitability by lowering the threshold of activation of tetrodotoxin-insensitive sodium channels [16] and by reducing the current from delayed rectifier potassium channels [17]. In addition, a mechanism involving TRPV1, a non-specific cation channel activated by a variety of noxious stimuli and predominantly found on unmyelinated C-nociceptors [18], has been demonstrated. TRPV1-deficient mice show attenuated ET-1-induced nociceptive behaviors such as licking, flinching and biting [19].

Subcutaneous injection of relatively low concentrations (2–10 μM) of ET-1 induces thermal and mechanical hyperalgesia. These elevated responses to applied stimuli occur with little of the overt flinching or licking seen with higher concentrations [20–23]. Tissue specific ablation of ET_A receptors in Na_v1.8-expressing nociceptors completely suppresses tactile allodynia as well as late phase thermal hyperalgesia caused by ET-1's local injection [11]. Acute thermal hyperalgesia, which is likely mediated by TRPV1 receptors, since it is capsazepine-sensitive, is attenuated by antagonists of both ET_A and ET_B receptors [23]. About half of the dorsal root ganglia (DRG) sensory neuron population expressing TRPV1 is immuno-positive for ET_A receptors, and activation of ET_A receptors with 10 nM ET-1 enhances a capsaicin-induced increase in intracellular calcium in dissociated mouse DRG neurons [24]. This effect is mediated by ET_A receptor activating PKCε, which in turn phosphorylates TRPV1 at residue S800, thus potentiating TRPV1 activity [24,25]. This mechanism was initially recognized in HEK 293 cells which, like DRG neurons, express the diacylglycerol (DAG)-activated PKCε [26]. Interestingly, heterologous co-expression of

ET_B receptors and TRPV1 in HEK293 cells demonstrates that ET_B can also sensitize TRPV1 through this pathway, although to a lesser extent than ET_A receptors [25].

Despite these significant breakthroughs, the cascade of events leading to the production of DAG and subsequent translocation of PKC ϵ from the cytosol to the cell membrane following ET-1 application remains relatively unexplored. Our laboratory reported previously that in the DRG-derived cell line ND7/104, ET-1 induces a transient rise of intracellular calcium, [Ca²⁺]_{in}, emanating from intracellular stores, which is blocked by BQ-123 but not by the ET_B selective antagonist BQ-788 [27]. More recent work has demonstrated that in freshly dissociated mouse DRG neurons 10 nM ET-1 also induces a very small intracellular calcium increase from intracellular stores which is exclusively dependent on ET_A receptors. Conversely, satellite cells, which are thought to express exclusively ET_B receptors [13–15], show a large intracellular calcium increase stimulated by 10 nM ET-1 that is ET_B receptor-dependent [24]. Taken together, these data emphasize the importance of intracellular calcium modulation in ET receptor-mediated pain signaling (see [28] for a recent review).

In the present paper we use quantitative PCR (qPCR) and radio-ligand binding to show that both ET_A and ET_B receptors are expressed by ND7/104 and HEK 293T cells. In both cell types, however, ET-1-induced calcium responses are only mediated by ET_A receptors, possibly due to sub-threshold ET_B receptor expression. We also show that this ET-1-stimulated calcium response depends on PLC and is likely mediated through the G α /11 signaling pathway.

2. Material and Methods

2.1. Pharmacological reagents

Endothelin-1 was purchased from Alexis Biochemicals (Lausen, Switzerland). BQ-123, BQ-788, IRL-1620 and BQ-3020 were from American Peptide Co. (Sunnyvale, CA). IRL-2500 was from Tocris (Ellsville, MO). Sarafotoxin 6c was from RBI (Natick, MA). Forskolin was from MP Biomedicals (Santa Ana, CA). ATP, Bradykinin, Ionomycin, Cholera toxin, Pertussis toxin, MDL-12,330A and U73122 were from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Cell cultures

ND7/104 (a hybrid cell line derived from rat dorsal root ganglia neurons hybridized with N18TG2 mouse neuroblastoma cells) was a generous gift from Dr. P. Hogan (Harvard Medical School, Boston, MA). The HEK 293T cell line was generously donated by Dr. Ging-Kuo Wang (Brigham and Women's Hospital, Boston, MA), and the HEK 293 cell line was provided by Dr. K. Sugimoto (Department of Anesthesiology, Nagoya University Hospital, Nagoya, Japan). All cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100U), and streptomycin (100 μ g) (Invitrogen, Carlsbad, CA), at 37°C in 5% CO₂.

2.3. Quantitative RT-PCR

Cells were grown to sub-confluence in 6-well plates before harvest and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quality and concentration were determined using a nanodrop and quality assessed by visual inspection after separation on an agarose gel. cDNA synthesis on 500 ng of total RNA was carried out with random hexamers using a First strand cDNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was performed in triplicate on a fraction of the cDNA in a Miniopticon thermocycler

(Bio-Rad, Hercules, CA) using Evagreen™ PCR mix (Bio-Rad, Hercules, CA) under the following conditions: 94 °C for 3 min, 45 cycles (94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min) followed by a melting curve to verify the specificity of the primers. All the primer pairs employed gave rise to a single peak confirming their specificity (data not shown). All primer pairs were designed to amplify rodent and human templates. The ETA primers used were ETAFRW: 5'-GGCTTCGTCATGGTACCC-3' and ETAREV2: 5'-CAAAGAGCCACCAGTCCT-3 which amplify a 116 bp DNA fragment (NM_010332; NM_001957). The ETB primers were ETBFRW4: 5'-GACTGGCCATTTGGAGCTG-3' and ETBREV4: 5'-GGAACCCCAATTCCTTTAATTC-3 which amplify a 145 bp DNA fragment (NM_007904; BC014472). Cyclophilin A primers were QCYCLOAFRW: 5'-ATCTGCACTGCCAAGACTGA-3' and QCYCLOAREV: 5'-ATGGTGATCTTCTTGCTGGTCT-3' which amplify a 134 bp DNA fragment (BC137057; BC099478). Internal controls included omitting reverse-transcriptase or templates to check for contamination of the starting material. Efficiency of amplification was determined for each primer pair in triplicate, using 10-fold serial dilution of starting cDNA, using the REST-MCS software [29]. The relative expression of the target gene was calculated based on real-time PCR efficiencies and the threshold value of the unknown sample versus the standard sample [30]. Data are expressed in fold-changes $\pm$ SEM corresponding to the ratio between experimental groups or cell lines. Student t-test with unequal variance was used for analyses (Welch t-test, Microsoft Excel).

2.4. Calcium imaging experiments

Twenty four to forty eight hours prior to calcium imaging the cells were seeded at a density of 10^6 cells/plate onto a Poly-L-Lysine-coated coverslip (VWR, Radnor, PA) and were ~80% confluent at the time of assay. Cells were rinsed twice with Ringer's solution (NaCl 155 mM, KCl 4.5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, D-glucose 10 mM, Hepes 5 mM) and loaded for 30 min at 37 °C with 4 μ M Fura 2-AM (Invitrogen, Carlsbad, CA) dissolved in Ringer with 0.025 % (w/v) Pluronic F-127 (Invitrogen, Carlsbad, CA). Then cells were incubated in the dark at 25 °C for an additional 15 min prior to Ca²⁺ imaging.

Solutions diluted in normal Ringer or calcium-free Ringer solution (NaCl 155 mM, KCl 4.5 mM, MgCl₂ 3 mM, D-glucose 10 mM, Hepes 5 mM) were applied using a micropipette to the coverslip fitted in a custom-made recording chamber. The excess solution was aspirated by an attached vacuum line. Cells were monitored on an inverted Diaphot 300 microscope (Nikon, Melville, NY) equipped with a PTI-IC100 digital camera (PTI, Birmingham, NJ). Acquisition of the fluorescence intensity following 340/380-nm excitation and 535-nm emission wavelengths was recorded using the PTI software for 240 seconds. At the end of the experiment ATP (40 μ M) was applied as a control to stimulate endogenous purinergic receptors.

The Ca²⁺ changes are expressed as the fractional change in fluorescence light intensity over the baseline intensity: $\Delta F/F = (F - F_0)/F_0$, where F is the fluorescence light intensity at each time point and F₀ is the fluorescence intensity averaged over 30 seconds before the stimulus application. The peak intensity following stimulus administration was averaged with the measurements immediately before and after it to correct for intrinsic fluctuations of the fluorescent signal. The data were normalized to the response elicited by ATP by dividing the peak value (averaged over these 3 data points) of the stimulus response by the peak value of the ATP response for each cell. In cells treated with U73122 the data were normalized to the response elicited by 10 μ M ionomycin instead of ATP. Data are presented as the mean maximum normalized fluorescence increase averaged for at least 15 representative cells from 3 independent experiments, unless noted otherwise. The response to ET-1 (30 nM) was from at least 40 cells from 8–9 independent experiments. The ATP response, presumably mediated by endogenous P2X and P2Y receptors, was comparable across cell types.

Statistical significance between the control group (typically ET-1 30 nM) and the experimental groups was established following two-way ANOVA with Bonferroni post-tests, calculated using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA). Responses were highly reproducible, reflecting the low variability observed when using clonal cell lines.

2.5. ^{125}I -ET-1 binding assays

Equilibrium binding studies were conducted on 90 % confluent cells in 12-well plates, 24 h after seeding. All binding studies were performed at 4 °C to prevent receptor internalization. Cells were incubated for 3 h in 1 mL of 0.1 % BSA in culture medium, with concentrations of [^{125}I]-ET-1 (Perkin Elmer, Waltham, MA) ranging from 10 to 600 pM in saturation binding studies or constant at 30 pM in unlabelled ligand competition studies. After incubation, supernatants were pooled from quadruplicates of each condition and measured for radioactivity to determine free [^{125}I]-ET-1. Cells were washed thoroughly two times with 0.1 % BSA in culture medium and solubilized overnight with 0.5 mL 1.0 N NaOH. The culture dishes were scraped the following day to collect all the cellular materials and the collected solution was then neutralized with 0.5 mL 1.0 N HCl. These samples were transferred to 7 mL scintillation vials and mixed thoroughly with Ecolite(+)™ Liquid Scintillation Cocktail (MP Biomedicals, Solon, OH). Radioactivity was measured for 5 min/sample in a Beckman Model LS8500 (Beckman, Inc; Palo Alto, CA) scintillation counter. Specific binding was defined as the difference between total binding and nonspecific binding, the latter determined by co-incubation with 500 nM unlabelled ET-1. Protein concentration was measured using a BCA protein assay (Pierce Biotechnology, Rockford, IL).

Specific binding *versus* free [^{125}I -ET-1] data were fit by the following logistics equation (Eq.1), analogous to the Hill equation:

$$U_{\text{sat}} = B_{\text{max}} \frac{[*\text{ET} - 1]^{n_H}}{(K_D^{n_H} + [*\text{ET} - 1]^{n_H})} \quad (\text{Eq.1})$$

where [$*\text{ET}-1$] is the free ^{125}I -ET-1 concentration in the supernatant, U_{sat} is the amount of ^{125}I -ET-1 bound to receptors, B_{max} is the total number of binding sites, n_H is an empirical parameter that reflects cooperativity in binding and K_D is the equilibrium dissociation constant.

For the competition binding studies, which used increasing concentrations of the ET_A -selective antagonist BQ-123 and a nominally constant concentration of ^{125}I -ET-1, data were fit by the following equation:

$$Y = \left[1 - \frac{[\text{Ligand}]}{[\text{Ligand}] + K_i \left(1 + \frac{[\text{ET1}]}{K_{\text{DET1}}} \right)} \right] \times 100 \quad (\text{Eq.2})$$

Optimal fits of these equations to the experimental data generated by Graphpad Prism (Graphpad Software Inc., La Jolla, CA) provided estimates of K_D , B_{max} , n_H , and K_i from individual experiments. Three such experiments were separately analyzed for the parameter values which were then averaged and reported in Table 1.

2.6. Intracellular cAMP measurements

Ten thousand cells/well were plated onto 96-well plates 24–36 h prior to experimentation. Activation of the receptors was conducted by adding the appropriate agonist in solution in PBS to the wells and incubating the plate for 10 min at 37 °C. cAMP levels were subsequently measured by using a cAMP enzyme immunoassay (GE healthcare, Rochester, NY) following the manufacturer's instructions. cAMP production was normalized to the protein concentration in each well, determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL). Briefly, 50 µl of protein samples were added to 200 µl of 50:1 Pierce's reagents A, and B, incubated for half an hour at 37 °C, and protein concentration was determined at 562-nm using a Bio-Rad microplate reader. Three independent experiments were performed in triplicate. Statistical analysis of the data was performed with Graphpad Prism (Graphpad Software Inc., La Jolla, CA).

2.7. Small interfering RNA (siRNA) transfections

HEK 293T cells were reverse transfected at the time that they were seeded onto coverslips prior to imaging. Following the recommendations of the manufacturer ON-TARGETplus SMARTpool ETRB siRNA or ON-TARGETplus siCONTROL non-targeting siRNA (Dharmacon, Lafayette, CO) was first diluted in Opti-MEM I Medium without serum and then combined with diluted RNAiMAX lipofectamine (Invitrogen, Carlsbad, CA) and incubated at room temperature for 15 minutes. The complex was then diluted in culture media (DMEM +10% FBS without antibiotics) containing suspended cells which were then plated onto poly-L-lysine-coated coverslips. Six hours after plating, the media containing the transfection material was removed and the cells treated as described under 2.3. Calcium imaging experiments were conducted 48 h later as described under 2.4 and the cells collected for qPCR analysis (see procedure under 2.3).

3. Results and Discussion

3. 1. ET_A and ET_B receptors are expressed at different levels in ND7/104, HEK 293T and HEK 293 cells

To determine the repertoire of ET receptors in ND7/104, HEK 293T and HEK 293 cells we first conducted quantitative RT-PCR on total RNA extracted from these cells. We used primers specific for ET_A or ET_B receptors as well as for cyclophilin A, a housekeeping gene used for the normalization of the samples. As shown in Figure 1, messenger RNA for the endothelin A and B receptors was detected in all three cell lines. ET_A mRNA levels were comparable between ND7/104 and HEK 293T cells (ANOVA, $p > 0.05$), but HEK 293 cells had significantly less (ANOVA, $p < 0.01$). In the same manner, ND7/104 and HEK 293T have significantly more ET_B mRNA than HEK 293 cells (ANOVA, $p < 0.001$) while ND7/104 and HEK 293T have comparable ET_B mRNA levels (ANOVA, $p > 0.05$). Comparison of ET_A and ET_B messenger RNA levels within cell lines indicated that levels are comparable in ND7/104 and HEK 293 cells but that ET_A is significantly more abundant than ET_B in HEK 293T cells (Welsh t-test, $p < 0.05$).

Unfortunately, we were unable to validate the presence of the receptor proteins in these cells by Western blot analysis due to the lack of specificity of the commercial antibodies we tested. All anti-ET_B receptor antibodies tested failed to recognize an HA-tagged ET_B receptor protein otherwise detected by the anti-HA antibody (data not shown). This problem corroborates recent concerns about commercial ET receptor antibodies raised by another group using immunoprecipitation to study receptor heterodimerization [31].

Next we sought to determine whether mRNA expression levels are correlated with ET-1 binding sites. To do so, intact ND7/104, HEK 293T and HEK 293 cells were incubated with

[¹²⁵I]-ET-1 for 3 h, at 4 °C to prevent receptor internalization while reaching equilibrium binding. All parameters from the binding experiments are collected in Table 1.

Saturation binding data were analyzed by fits of the logistics equation (Eq 1, see Methods) to determine a binding capacity, $B_{\max}$. This measure of total ET receptor number on the plasma membrane equalled 38.4 ± 4.1 fmol/mg ($n=3$) in ND7/104 cells (Figure 2) and 33.7 ± 10.0 fmol/mg ($n=3$) in HEK 293T cells (Figure 2), whereas much less (3.6 ± 0.5 fmol/mg; $n=3$) was detected in HEK 293 cells (Figure 2, Table 1). Equilibrium dissociation constants, K_D , were similar for ND7/104 and HEK 293 cells, at 70 ± 10 pM and 58 ± 18 pM, respectively. In HEK 293T cells the affinity was slightly lower, with $K_D = 170 \pm 100$ pM (Table 1). Our results are in line with [¹²⁵I]-ET-1 binding experiments conducted on pituitary tissue sections, reporting a K_D of 71 pM and a $B_{\max}$ of 120 fmol/mg [32]. In contrast, membranes of HEK 293 cells over-expressing cloned tagged-ET_A or -ET_B receptors displayed much higher $B_{\max}$ values, as expected, (~ 7 pmol/mg) and slightly lower K_{Ds} (~ 30 pM for ET_A receptors and ~ 10 pM for ET_B receptors; ref. [33]).

Fits of the binding data showed a Hill coefficient, n_H , around 2 (with a large error in this parameter for HEK-293 cells where the binding was very low) (Table 1). It thus appears that there is some positive cooperativity in the binding of [¹²⁵I]-ET-1 to the receptors in these cells.

Competition studies were subsequently performed using a range of concentrations of BQ-123, an ET_A receptor-selective antagonist, and a constant [¹²⁵I]-ET-1]. These experiments determined both the inhibitory equilibrium dissociation constants, K_i , for this antagonist, and the proportions of ET_A and ET_B receptors in the different cell lines (Figure 3). In ND-7/104 cells, K_i for BQ-123 = 2.26 ± 0.33 nM; in HEK 293T cells, $K_i = 5.47 \pm 1.52$ nM, and in HEK 293 cells, $K_i = 0.43 \pm 0.07$ nM (Table 1). Fitting of the competition binding data by a one-site or a two-site model indicated a better fit for the latter in ND7/104 and HEK 293 cells, where BQ-123 bound to a high ($K_i < 10$ nM) and a low ($K_i > 100$ nM) affinity site (Figure 3). The K_i for low affinity binding in HEK 293T cells was too high to allow its estimation (see Figure 3).

Based on these results, and the assumption that the high-affinity site corresponds to ET_A receptors, we used 100 nM BQ-123 in subsequent experiments to determine ET_A and ET_B receptor densities in these cells (Figure 4). ET_B receptor-specific binding was inferred by subtraction of the ET_A receptor density from the total. (Competition studies were attempted with BQ-788, the ET_B receptor antagonists, and several ET_B receptor agonists, but the results were anomalous and will not be described here.) In ND7/104 cells ET_A binding accounted for 75.2 ± 22.2 %, with the remainder, 24.8 ± 7.3 %, assigned as ET_B. In HEK 293T cells the percentage of ET_A receptor sites was of 75 ± 9 % while that of ET_B sites was of 25 ± 3 %. In HEK 293 cells ET_A and ET_B receptor-selective binding accounted for 85.3 ± 19 % and 14.7 ± 3.3 %, respectively (Figure 4). Thus, ET_A and ET_B receptor binding sites are represented at a proportion close to 3:1 in all three cell lines.

This ratio is in the same direction as the proportions of mRNA obtained by qPCR for the different receptor sub-types where the observed ratio is close to 2:1 in HEK 293T and HEK 293 cells. However, in ND7/104 the ratio of mRNA for the receptor sub-types is closer to 1:1, although with a large error for ET_B. To our knowledge this is the first quantitative report of endogenous ET receptor messenger RNA levels and ET-1 binding sites in these cell lines, some of which have been used extensively for ET receptor heterologous expression studies [24,25,33–37]. The presence of both ET_A and ET_B receptors in ND7/104 cells indicates that this cell line might be more representative of a subset of trigeminal ganglionic neurons which, unlike lumbar non-myelinated primary sensory neurons such as

those found in DRG, express both ET_A and ET_B receptors [38]. Note that high and low affinity sites for BQ-123, as we observe here, have been reported in the past for cells co-expressing both receptor subtypes, such as canine spleen cells [39]. However, in that study the affinity of the weaker binding site was quite low, similar to that obtained from Chinese hamster ovary cells over-expressing the cloned human ET_B receptor ($K_i \sim 60 \mu\text{M}$) [40]; this is a K_i much higher than what we calculate in ND7/104 and HEK 293 cells ($< 2 \mu\text{M}$). Further experiments will determine whether this low affinity binding is attributable to ET_B or ET_A/ET_B heteromers.

Published ligand binding studies have reported atypical pharmacological profiles for ET-1 receptors in cells expressing both receptor subtypes [32,41]. Splice variants and heterodimerization of ET_A and ET_B have been proposed to explain these phenomena [33], but the precise molecular mechanisms underlying these effects remain elusive. ND7/104 and HEK 293T cells endogenously co-express both receptor subtypes and represent a valuable and dependable tool to study at high throughput receptor/ligand interactions and putative cooperativity between the two subtypes.

3.2. ET-1 elicits an ET_A mediated rise of intracellular calcium in ND7/104 and HEK 293T, but not in HEK 293 cells

Previous studies using ND7/104 and HEK 293T cells reported that ET-1 evokes an increase in intracellular calcium levels in these cells, which is blocked by BQ-123 in ND7/104 cells [27,42]. Given the different receptor expression levels among the three cell lines used here, we sought to compare their $[\text{Ca}^{2+}]_{\text{in}}$ mobilization responses to ET-1. Cells were submitted to ratiometric calcium imaging analysis during exposure to 30 or 100 nM ET-1. Note that because ET-1 induces a well-documented, long-lasting down regulation of the endothelin receptors, different coverslips were used for the different concentrations of ligands tested. In addition, to account for potential variability in the loading of the cells which might confound comparison between experiments, individual cellular responses to endothelin receptor agonists or antagonists were normalized to their response to ATP (40 μM). ATP induced a strong and comparable calcium response in all three cell lines through endogenous purinergic receptors [43] and was therefore also used as an indicator of the cell viability.

Endothelin-1 at 30 nM and 100 nM elicited $[\text{Ca}^{2+}]_{\text{in}}$ mobilization in $\sim 35\%$ of both the ND7/104 and HEK 293T cells. The amplitudes of these responses were similar to that elicited by ATP (40 μM ; Figures 5, 6A). (Importantly, neither the amplitude or duration of the calcium transient induced by ATP (40 μM) were altered following exposure to the agonist ET-1 or the antagonist BQ-123 (Figure 5)). Of note, neither of these concentrations of ET-1 produced a detectable increase of $[\text{Ca}^{2+}]_{\text{in}}$ in HEK 293 cells (Figures 5E, 6A). The density of surface ET receptors in HEK 293, indicated by the lower mRNA and ET-1 binding sites, may be inadequate to trigger a detectable rise in $[\text{Ca}^{2+}]_{\text{in}}$.

To investigate which receptor subtype is responsible for the ET-1 stimulated rise in $[\text{Ca}^{2+}]_{\text{in}}$, selective antagonists were applied first alone and subsequently in combination with 30 nM ET-1 on the same coverslip. Recorded responses (Figure 5) show that none of the antagonists applied alone triggered a rise in $[\text{Ca}^{2+}]_{\text{in}}$ (data not shown). Furthermore, the ET-1 stimulated calcium increase was blocked by 50 nM BQ-123 but not affected by BQ-788 (100 nM) nor by IRL-2500 (50 nM), two potent ET_B-selective antagonists (Figure 6B). Therefore, in these cells endogenous ET_A receptors appear to be responsible for the rise in intracellular calcium. Note that at higher concentration IRL-2500 (100 nM) partially blocked the response to ET-1 (30 nM), probably as a result of its lack of selectivity for ET_B receptors at that concentration.

These findings confirm the ET_A receptor dependence of calcium responses in ND7/104 and HEK 293T cells. Although both ND7/104 and HEK 293T cells also have ET_B receptors, these receptors were present at a lower level and may therefore not be sufficiently abundant to support a calcium response. The concept of a threshold expression for ET receptor in calcium mobilization is supported by the lack of an ET-1 calcium response in HEK 293 cells, which have 10-fold lower levels of both ET_A and ET_B receptors (Table 1) and which lack any ET-1 – induced intracellular calcium increases.

In a context where accumulating evidence points to GPCR expression levels as a determining factor in the selectivity of coupling to G proteins, and signaling pathway engagement [44,45], these cells represent a useful tool. This important point is further emphasized by the fact that these cells are often used for heterologous expression studies [43] as well as by a recent report demonstrating that, when over-expressed in CHO cells, ET_A receptors have the ability to recruit additional G proteins [46].

3.3. Activation of the endogenous ET_B receptor with selective agonists does not trigger a rise in intracellular calcium concentration

To confirm the lack of an ET_B receptor contribution to the ET-1 mediated calcium signal in these cells, we tested whether activation of ET_B receptors with selective agonists could trigger a rise in intracellular calcium. First, cells were stimulated with 50 nM BQ-3020, a potent and highly selective ET_B receptor agonist. Subsequently, we used IRL-1620, also a highly selective ET_B receptor agonist. Finally, we used the highly ET_B-selective sarafotoxin 6c, a 21 amino acid peptide agonist isolated from snake venom with a structure similar to ET-1. As shown in Figure 7, none of these agonists elicited a rise of $[Ca^{2+}]_{in}$, thus corroborating the results obtained with ET_B selective antagonists.

3.4. Down-regulation of ET_B mRNA levels in HEK 293T cells does not alter the intracellular calcium release triggered by ET-1

Since ET_B receptors have been proposed to interact with ET_A receptors through the formation of heterodimers [34,36], we sought to analyze any potential contribution of ET_B receptors to the coupling of ET_A receptors to calcium's increase. To do so, HEK 293T cells were transiently transfected with either siRNA targeting the human ET_B receptor or a “control” pool of non-targeting siRNA. Forty eight hours following transfection the cells underwent calcium imaging. Stimulation of the cells with ET-1 (30 nM) elicited a calcium transient in both siRNA-treated and control cells (Figure 8A) suggesting that ET_B is not necessary for the coupling of ET_A to the calcium pathway. This is consistent with previous findings in HEK 293 cells showing that ET-1 can trigger a calcium response in HEK cells transfected with only ET_A receptors [33]. ET_A and ET_B mRNA expression levels in the cells used for calcium imaging were determined by quantitative RT-PCR (qPCR). While mRNA levels for ET_B were substantially reduced in these cells ($p < 0.01$), those for ET_A were unchanged as compared to the cells transfected with the non-targeting pool siRNAs (Figure 8B). These results further confirm that ET_B receptors do not influence coupling of ET_A receptors to intracellular calcium release in these cells.

3.5. ET-1 elicits a release of calcium from intracellular stores in ND7/104 and HEK 293T cell via Phospholipase C and Gα_{q/11}

To clarify the intracellular mechanisms responsible for the rise of $[Ca^{2+}]_{in}$ upon ET-1 stimulation in ND7/104 and HEK 293T cells, we used selective inhibitors of potential signaling components downstream of the receptor. First, we sought to determine whether the rise of intracellular free calcium concentration resulted from Ca^{2+} release from the endoplasmic reticulum (ER) or from a Ca^{2+} influx through voltage-independent Ca^{2+} -permeable cation channels. When calcium imaging experiments were performed in the

absence of extracellular calcium the response to ET-1 was not diminished in ND7/104 or in HEK 293T cells (Figure 9), implying that the elevated intracellular Ca^{2+} is released from the ER, most probably following activation of IP₃ receptors. This independence from extracellular calcium was also the case for the ATP response, indicating that in these cells endogenous P₂Y receptors are involved. RT-PCR with primers specific for P₂Y₁, a receptor previously reported to be endogenously expressed in HEK 293 cells [47], confirmed the presence of this receptor in our HEK 293T, HEK 293 and ND7/104 cultures (data not shown). Note that the elevated response ratio of ET-1:ATP recorded in ND7/104 cells is due to a lower ATP response in the absence of extracellular calcium, as opposed to a larger response to ET-1. One likely explanation for this effect could come from the fact that P₂X receptors contribute to the calcium response in these cells.

To further confirm that endothelin receptors are coupled to phospholipase C (PLC) activation, we examined the effects of the selective PLC inhibitor U73122. Cells were pretreated for 30 minutes with 10 μM U73122 prior to stimulation with ET-1 (30 nM). Under these conditions we observed a complete inhibition of the calcium transient induced by ET-1 in both cell lines (Figure 9), indicating that endothelin receptors are coupled to phospholipase C activation in these cells. The calcium response to ATP was also abrogated by U73122 in ND7/104 and HEK 293T cells suggesting that P₂Y receptors are operating via PLC as well. (Due to the absence of a response to ATP in ND7/104 and HEK 293T cells in the presence of U73122, the ET-1 response was standardized to that of ionomycin instead of ATP). Note that in ND7/104 cells, U73122 does not inhibit the response elicited by bradykinin, suggesting that its inhibitory action is not through some non-selective effect (data not shown).

G protein-coupled receptors such as the ET_A and ET_B receptors can stimulate phospholipase C's activity through two mechanisms [28]. One mechanism involves the G protein α -subunit $\text{G}\alpha_{q/11}$ while the other mechanism implicates the $\beta\gamma$ subunit of the heterotrimeric G protein, usually those interacting with $\text{G}\alpha_i$ subunits. To discriminate between these two possibilities, cells were pre-treated for 24–36 hrs with 10 ng/ml pertussis toxin (PTX) prior to stimulation with ET-1 (30 nM). PTX differentially inactivates $\text{G}\alpha_i$ while leaving $\text{G}\alpha_{q/11}$ signaling unaffected. As illustrated in Figure 9, the PTX treatment did not affect the response to ET-1, implying that in these cell lines ET-1 mediates its effect through $\text{G}\alpha_{q/11}$.

Altogether, these results demonstrate that ET_A receptor activation leads to the release of calcium from internal stores in ND7/104 and HEK 293T cells through the $\text{G}\alpha_{q/11}$ - PLC signaling pathway. It is likely that these steps also underlie the BQ-123-sensitive intracellular calcium increase emanating from intracellular stores recorded in freshly dissociated mouse DRG neurons exposed to 10 nM ET-1, a step that leads to TRPV1 potentiation [24]. Interestingly, Nishimura et al., reported that in HEK 293T cells Ric-8A, a GTP exchange factor for $\text{G}\alpha_q$, works as a positive regulator of ATP- and ET-1-induced intracellular calcium mobilization and of ERK activation [42]. Since Ric-8A is present in the nervous system in mice [48] it will be interesting to test whether this factor modulates ET-1 signaling in DRG neurons in an ET_A-selective manner. In addition, several other regulatory proteins in complex with $\text{G}\alpha_q$ [49] might also participate in the modulation of the ET_A mediated pain signaling pathway in DRG neurons. One promising candidate is the G protein-coupled receptor kinase 2 (GRK2). It is a well known modulator of GPCR activity [50] that is present in about 50 % of DRG neurons. GRK2 has been shown to have a functional role in increased sensitivity of nociceptors elicited by IL-1 β [51] and may similarly participate in nociceptor sensitization mediated by ET-1.

3.6. ET-1 does not engage the Epac pathway to trigger the release of calcium from intracellular stores in ND7/104 and HEK 293T cells

It was previously reported that in HEK 293 cells cAMP is able to elicit the release of calcium from intracellular stores through a pathway involving the cAMP-dependent RAP guanine-nucleotide-exchange factor known as Epac (exchange protein directly activated by cAMP). RAP activation ultimately leads to activation of phospholipase C ϵ which in turn produces IP3 and opens IP3 receptors on the ER [52]. Since U73122 is a broadly acting PLC blocker, the use of this compound does not allow discrimination between activation through G $\alpha_{q/11}$ and G α_s , both of which activate PLCs. To discriminate between these two possibilities, we first sought to determine whether ET-1 (0.3 nM to 300 nM) elicited a rise in cAMP levels in these cells using an Elisa-based cell assay to monitor intracellular levels of cAMP. Under these conditions we did not record a rise of intracellular cAMP in either ND7/104 or HEK 293T cells (Figure 10A), whilst a strong activator of adenylate cyclase, forskolin (10 μ M), produced a consistent and robust cAMP accumulation (data not shown). Next we tested whether blocking the cAMP pathway in HEK 293T and ND7/104 cells would affect negatively the calcium transient elicited by ET-1 (30 nM). First, cells were preincubated for 18hrs with 100 ng/ml cholera toxin (CTX), known to uncouple the receptor from G α_s . This treatment did not significantly alter the calcium transient elicited by ET-1 (30 nM) compared to untreated cells, in the presence or absence of extracellular calcium (Figure 10B). Additional experiments using the adenylate cyclase inhibitor MDL-12,330A (50 μ M), which led to the same outcome (Figure 10B) further argue that ET-1-induced calcium responses in HEK 293T and ND7/104 cells are not mediated through the Epac pathway.

Note that the statistically significant ($p < 0.05$) boost in the response ratio recorded in ND7/104 cells under zero extracellular calcium conditions is explained by a diminished response to ATP following this treatment rather than an increased response to ET-1. In these cells pretreatment with MDL 12,330A had a similar effect on the ATP response. Note that application of MDL 12,330A (50 μ M) alone did not elicit a significant rise of $[Ca^{2+}]_{in}$ above baseline levels (data not shown).

ET $_A$ and ET $_B$ heterologously expressed in CHO cells have been reported to couple to G α_s and G α_i , respectively [53]. If we make the assumption that endogenous ET receptors in HEK 293T and ND7/104 cells are coupled to the same G-proteins, then the net effect of their simultaneous activation on intracellular cAMP effect might be close to zero as these receptors would antagonize each other. Under these conditions activation of the Epac pathway by ET-1 would be unlikely.

4. Conclusion and perspectives

In conclusion, our data reveal the crucial role of endogenous ET $_A$ and G $\alpha_{q/11}$ in the ET-1-evoked calcium transient in ND7/104 and HEK 293T cells. If these findings are validated in pain models *in vivo* then these cell lines will become valuable tools for the screening and development of therapeutic compounds targeting this pain signaling pathway. In addition, ET $_A$ and ET $_B$ receptors have been recently reported to interact through their carboxy-terminal regions with a number of accessory proteins regulating dimerization, degradation, and sub-compartment localization [54–56]. It will be interesting to study if these accessory proteins modulate calcium signaling by endothelin receptors as well.

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Figure 1. ET_A and ET_B transcripts in HEK 293T, HEK 293, and ND7/104 cells

Quantitative RT-PCR analysis of ET_A and ET_B receptors transcript abundance in HEK 293T, ND7-104 and HEK 293 cells using specific primers. Results are normalized to cyclophilin A expression levels and expressed in arbitrary units as the mean $\pm$ SEM of 4 independent biological samples. White bars represent the ET_A messenger level and black bars are for ET_B. ET_A and ET_B receptor levels were significantly lower in HEK 293 cells compared to HEK 293T and ND7/104 cells. ET_B message levels were comparable to that of ET_A in ND7/104 cells, but were significantly less than ET_A levels in both HEK cell lines. (** $p < 0.01$, *** $p < 0.001$; ANOVA)

Figure 2. Specific binding of [¹²⁵I]ET-1 in intact ND7/104, HEK 293T, and HEK 293 cells
 Saturation binding of [¹²⁵I]ET-1 on intact ND7/104, HEK 293T, and HEK 293 cells respectively. Maximal binding capacity is 38.4 ± 4.1 fmol/mg ($n=3$), 33.7 ± 10.0 fmol/mg ($n=3$), and 3.6 ± 0.5 fmol/mg ($n=3$) respectively. Each data point represents the mean $\pm$ SEM of 3 independent experiments performed in triplicate.

Figure 3. Competition binding experiments on intact ND7/104, HEK 293T, and HEK 293 cells 30 pM $[^{125}\text{I}]\text{-ET-1}$ was used in the presence of increasing concentrations of BQ-123. In ND7/104 and HEK 293 cells BQ-123 appears to bind to a high ($K_i < 10$ nM) and a low ($K_i > 100$ nM) affinity site. Each data point represents the mean $\pm$ SEM of 3 independent experiments performed in triplicate.

Relative Binding of [125 I]-ET-1
to Receptor Subtypes (%)

Figure 4. Relative surface expression of endothelin receptor subtypes in ND7/104, HEK 293T, and HEK 293 cells

100 nM of the selective ET_A receptor antagonist BQ-123 was used to define ET_A receptor surface expression. Endothelin-A receptors account for 75 ± 22 % binding in ND7/104 cells, 75 ± 9 % binding in HEK 293T cells, and 85 ± 19 % binding in HEK 293 cells. Endothelin-B receptors account for 25 ± 7 % binding in ND7/104 cells, 25 ± 3 % binding in HEK 293T cells, and 15 ± 3 % binding in HEK 293 cells. Values are mean ± SEM of 3 independent experiments.

Figure 5. ET-1 elicits ET_A receptor mediated calcium transients in ND7/104 and HEK 293T but not HEK 293 cells

A. Ratiometric calcium imaging of cell lines loaded with Fura-2. Addition of ET-1 (30 nM) to ND7/104 (A) or HEK 293T (C) cells caused an increase in $[Ca]_{in}$. This effect is blocked by the ET_A selective antagonist BQ-123 (B, D). In HEK 293 cells (E) ET-1 does not promote intracellular calcium mobilization. Each panel represents traces from one single cell representative of the field. Cells were rinsed with Ringer prior to ATP addition.

Figure 6. ET-1 elicits calcium transients in ND7/104 and HEK 293T but not HEK 293 cells

A. Ratiometric calcium imaging on ND7/104 or HEK 293T cells loaded with Fura-2AM. Addition of ET-1 30 nM (small checkers bars) or 100 nM (large checkers bars) to ND7/104 or HEK 293T cells caused an increase in $[Ca]_{in}$. However this effect was not observed in HEK 293 cells which show lower message and binding for both ET_A and ET_B receptors. Values are mean $\pm$ SEM of 3 independent experiments analyzed by 2-way ANOVA with bonferroni correction.

B: Ratiometric calcium imaging on ND7/104 (small checkers bars) or HEK 293T (large checkers bars) cells loaded with Fura-2AM and preincubated with BQ-123 (50 nM), BQ-788 (100 and 200 nM) or IRL-2500 (50 and 100 nM) and then exposed to ET-1 (30 nM) alone or

in combination with the appropriate antagonist. The ET_A receptor selective antagonist BQ-123 completely prevented the ET-1 mediated calcium response, while the ET_B selective antagonists BQ-788 and IRL-2500 were without effect. Note that the application of the antagonist alone did not significantly alter the basal intracellular calcium levels (not shown). Values are mean $\pm$ SEM of 3 independent experiments analyzed by 2-way ANOVA with bonferroni correction (***p* < 0.001, * *p* < 0.05).

Figure 7. ET_B selective agonists do not elicit calcium transients in ND7/104 and HEK 293T cells
 Ratiometric calcium imaging on ND7/104 (white bars) and HEK 293T (black bars) cells loaded with Fura-2AM. Cells did not respond to the application of the ET_B selective agonists sarafotoxin 6 c (50 and 1000 nM), IRL-1620 (100 and 1000 nM) or BQ-3020 (100 and 1000 nM). Values are mean $\pm$ SEM of 2 (high concentration) or 3 (low concentration) independent experiments analyzed by 2-way ANOVA with bonferroni correction (***) $p < 0.001$).

Figure 8. The calcium response remains unchanged following down regulation of ET_B transcript levels

A. Ratiometric calcium imaging on HEK 293T cells transfected with control (NTP) or ET_B siRNA (large checkers and stripes). Cells were loaded with Fura-2AM 48 hrs following transfection. The response to ET-1 (30 nM) is not significantly different between the two treatments ($p>0.05$). Values are mean $\pm$ SEM of 2 independent experiments analyzed by student t-test versus control.

B. Quantitative RT-PCR analysis of ET_A and ET_B transcript abundance in HEK 293T cells transfected with ET_B specific SiRNA. Treatment of HEK 293T cells with ET_B specific siRNA reduced mRNA levels of ET_B, but not ET_A receptors. Values for ET_A and ET_B are

normalized to cyclophilin A expression levels and expressed in arbitrary units as the mean $\pm$ SEM of a biological sample performed in triplicate. 1 ET_B siRNA and 2 ET_B siRNA each represent an independent experiment in which cells were transfected with ET_B siRNA. NTP represent the control experiment which used a non-targeting pool of siRNA. (** p<0.01, *** p<0.001, student t-test versus NTP).

Figure 9. Effects of selective second messenger pathway modulators on the Ca²⁺ response to ET-1 in ND7/104 and HEK 293T cells

Ratiometric calcium imaging on ND7/104 (white bars) and HEK 293T (black bars) cells loaded with Fura-2AM. The absence of extracellular calcium or pretreatment with PTX did not affect the response while U73122, an inhibitor of PLC activity, prevented a rise of intracellular calcium concentration upon ET-1 stimulation. Values are mean $\pm$ SEM of 3 independent experiments analyzed by 2-way ANOVA with bonferroni correction (*** $p < 0.001$, * $p < 0.05$).

Figure 10. Effects of increasing concentrations of ET-1 and cAMP pathway modulators on intracellular cAMP accumulation and calcium release respectively in ND7/104 and HEK 293T cells

A. cAMP accumulation in response to increasing concentrations of ET-1 in ND7/104 (circles) and HEK 293T (squares) cells. Cells were seeded in 96 well plates and 24–36 hrs later challenged with increasing concentrations of ET-1 for 10 minutes at 37 °C. As a control some cells were stimulated with forskolin (10 μM), a direct activator of adenylate cyclase activity, under the same conditions. cAMP was measured using an enzyme immunoassay kit. None of the ET-1 concentrations tested produced a significant effect on intracellular cAMP accumulation whereas forskolin was a potent stimulator with 71.3 ± 6.2

fmol/ μ g and 15.5 ± 2.5 fmol/ μ g in ND7/104 and HEK 293T cells respectively. Each data point represents the mean $\pm$ SEM of 4 independent experiments performed in triplicate.

B. Ratiometric calcium imaging on ND7/104 (small checkers) and HEK 293T (large checkers) cells loaded with Fura-2AM. Pretreatment of the cells with CTX (100 ng/ml) for 24–36 hrs or MDL 12,330A (50 μ M) five minutes before ET-1 (30 nM) stimulation did not affect negatively the rise of intracellular calcium concentration elicited by ET-1 in a significant way. The boost recorded following MDL 12,330A preincubation in ND7/104 cells is caused by a markedly diminished response to ATP in these cells. This phenomenon is also observed in absence of extracellular calcium. Note that MDL 12,330A alone did not produce a significant effect on basal intracellular calcium levels (not shown). Values are mean $\pm$ SEM of 3 independent experiments analyzed by 2-way ANOVA with bonferroni correction (** $p < 0.001$, * $p < 0.05$).

Table 1Parameters for [125 I]-ET-1 Binding to Intact Cells (mean $\pm$ SEM; n=3)

Cell Type	ND-7/104	HEK 293T	HEK 293
$B_{\max}$	38.4 $\pm$ 4.1 fmol/mg	33.7 $\pm$ 10.0 fmol/mg	3.6 $\pm$ 0.5 fmol/mg
K_D (I-ET-1)	0.07 $\pm$ 0.01 nM	0.17 $\pm$ 0.10 nM	0.06 $\pm$ 0.02 nM
n_H	1.75 $\pm$ 0.39	1.24 $\pm$ 0.48	3.48 $\pm$ 3.78
K_I (BQ-123)	0.35 $\pm$ 0.05 nM	0.21 $\pm$ 0.01 nM	0.39 $\pm$ 0.14 nM
n_H	-0.82 $\pm$ 0.04	-0.73 $\pm$ 0.22	-0.56 $\pm$ 0.16
High affinity $K_{I\text{ BQ-123}}$	2.26 $\pm$ 0.33 nM	5.47 $\pm$ 1.52 nM	0.43 $\pm$ 0.07 nM
Low affinity $K_{I\text{ BQ-123}}$	1899.00 $\pm$ 3812.00 nM	N.D.	195.10 $\pm$ 135.60 nM

N.D.: not determined