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**Endothelin-1-Induced Inhibition of ATP Release from PC-12 Cells
is Mediated by the ET_B Receptor: Differential Response to
Endothelin-1 on ATP, NPY and Dopamine Levels**

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Running title: ET-1 mediates selective inhibition of ATP release via ET_BR

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Number of text pages: 40

Number of figures: 9

Number of references: 40

Number of words Abstract: 219

Number of words Introduction: 730

Number of words Discussion: 1,500

Nonstandard abbreviations: NE, norepinephrine; NPY, neuropeptide Y; DA, dopamine;

NGF, nerve growth factor; ET, endothelin; ET_BR, endothelin ET_B-receptor

Section Assignment: Neuropharmacology

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ABSTRACT

During sympathetic neurotransmitter release, there is evidence for differential modulation of co-transmitter release by endothelin (ET-1). Using nerve growth factor (NGF) differentiated PC12 cells, the effects of ET-1 on K⁺-stimulated release of adenosine triphosphate (ATP), dopamine (DA), and neuropeptide Y (NPY) were quantified using HPLC or radioimmunoassay. ET-1, in a concentration-dependent manner, inhibited the release of ATP, but not DA and NPY. Preincubation with the ET_{A/B} antagonist, PD 142893, reversed the inhibitory effect of ET-1 on ATP release, which remained unaffected in the presence of the ET_A specific antagonist BQ123. The ET_B agonists, sarafotoxin 6c, BQ3020 and IRL 1620 decreased K⁺-stimulated release of ATP in a dose-dependent manner, and this effect was reversed by the ET_B antagonists RES 701-1 and BQ788. Preincubation of PC12 cells with pertussis toxin, reversed the ET-1-induced inhibition of the K⁺-evoked ATP release. Real-time intracellular calcium level recordings were performed on PC-12 cell suspensions, and ET-1 induced a dose-dependent decrease in the K⁺-evoked calcium levels. Nifedipine, the L-type voltage-dependent Ca²⁺ channel antagonist, caused inhibition of the K⁺-stimulated ATP release, but the N-type Ca²⁺ channel antagonist, ω-conotoxin GVIA, did not reverse the effect on ATP release. These data suggest that ET-1 modulates the release of ATP via the ET_B receptor and its associated G_{i/o} G-protein, through attenuation of the influx of extracellular Ca²⁺ through L-type channels.

Precise regulation of sympathetic neurotransmission is mediated through the concerted action of neurotransmitters and neuromodulators. At the neuroeffector junction during sympathetic transmitter release, it is established that the neurotransmitters, norepinephrine (NE), neuropeptide Y (NPY) and adenosine triphosphate (ATP) are co-released from sympathetic neurons. Evidence has accumulated indicating that in addition to the coexistence of NE, NPY, and ATP in sympathetic nerves there is differential co-transmission of each of these transmitters (Lundberg et al., 1996). Each neurotransmitter can be released independently under appropriate conditions, and the application of each transmitter mimics a phase of sympathetic nerve stimulation with each phase inhibited by appropriate antagonists. (Todorov et al., 1994).

Each of the transmitters, NE, NPY and ATP, negatively modulate their own release from sympathetic nerves, as well as the release of each other (Lundberg, 1996). At the vascular neuroeffector junction of the guinea pig vas-deferens and rabbit ileocolic artery, stimulation of α_2 -adrenergic receptors inhibited the release of NE and, to a lesser extent, ATP (Bulloch et al., 1990; Driessen et al., 1993). ATP has been observed to inhibit nerve-stimulated release of NPY from the guinea pig heart (Haass et al., 1989). In the rat mesenteric arterial bed and guinea pig vas deferens, NPY not only negatively regulated its own release but also produced a negative feed-back modulation of NE and ATP release (Ellis JL, 1990; Westfall et al., 1995).

Evidence indicates that release of neurotransmitters at the neuroeffector junction is subject to differential modulation by various mechanisms. During electrical field

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stimulation of the guinea pig vas deferens temporal differences in the release of ATP and NE from the sympathetic nerves have been demonstrated. At lower frequencies of stimulation ATP levels reached a peak at 20 s, after which levels diminished with continued stimulation; however, peak levels of NE were not observed until 40 s, and remained constant during the period of stimulation (Todorov et al., 1994). In contrast, NPY release from sympathetic nerves required higher frequencies of stimulation, which can be inhibited by the NPY Y₁ specific antagonist BIBP3226 (Smyth et al., 2000).

There is also evidence for modulation of sympathetic neurotransmission at the vascular neuroeffector junction by non-neuronal mediators. The vasoactive peptide endothelin-1 (ET-1) attenuates sympathetic neurotransmission pre-junctionally by decreasing nerve stimulation induced release of NE. This has been observed in the guinea pig pulmonary and femoral arteries (Wiklund et al., 1989), rat and guinea pig vas deferens (Wiklund et al., 1990), dog coronary artery (Aarnio et al., 1993), rat mesenteric artery (Tabuchi et al., 1989). In the rat mesenteric bed ET-1 negatively modulates the release of NPY, but not NE (Hoang et al., 2002). Post-junctionally ET's modulate sympathetic neurotransmission by enhancement of the contractile effect of both nerve stimulation and a variety of vasoactive agents (Henrion and Laher, 1993; Hoang et al., 2002). ET-1, through activation of the ET_A receptor, potentiates the post-junctional contractile effects of ATP (Hoang et al., 2002; Mutafova-Yambolieva and Radomirov, 1993).

The present investigation endeavored to delineate the molecular signaling mechanism by which ET-1 modulated sympathetic neurotransmitter release. NGF-differentiated rat pheochromocytoma (PC12) cells were chosen as a functional model of

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sympathetic neurotransmission, as they phenotypically resemble sympathetic neurons and contain both small vesicles and dense core granules (Greene and Tischler, 1976). They are a well established model to study intracellular mechanisms related to sympathetic neurotransmission signaling as they synthesize and release catecholamines, specifically dopamine (Chen et al., 1994), purines (Kasai et al., 2001), and NPY (Chen et al., 1997). PC12 cells express G-proteins (Li et al., 1995), voltage-dependent ion channels (McCullough et al., 1998) and various second messenger systems (Vaudrey et al, 2002).

NPY is co-stored with catecholamines, NE and dopamine, in large dense core vesicles and ATP is co-stored with catecholamines in small synaptic vesicles (Lundberg, 1996) in sympathetic neurons. Release of these transmitters is modulated by spatial differences in calcium concentrations (Lundberg, 1996). ATP and catecholamines are co-stored and co-released from secretory granules of adrenal chromaffin cells from which PC12 cells are derived. Much work has been carried out on vesicle exocytosis coupled with neurotransmitter release from both chromaffin and PC12 cells (Anhert-Hilger et al., 1985; Strasser et al., 1999).

We report that ET -1 differentially modulates the release of the sympathetic co-transmitters. Specifically, ET-1 functions as a negative modulator of sympathetic neurotransmission and inhibits the release of the sympathetic co-transmitter ATP from NGF-differentiated PC12 cells. The mechanism of inhibition involves a specific endothelin receptor subtype and G-protein family, which modulate intracellular calcium levels.

Materials and Methods

Growth and differentiation of PC12 cells. PC12 cells were grown directly on six-well plates (treatment C, Mattek Corp., Ashland, MA) in Dulbecco's Modified Eagle's Medium (DMEM) and differentiated with NGF (50 ng/ml) for 5 days as described previously (Chen and Westfall, 1994).

Neurotransmitter Release Measurements -The media surrounding the cells was removed and replaced with 1.5 ml warmed (37 °C) Krebs' buffer. The composition of the Krebs' buffer was as follows: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃ and 10 mM HEPES; pH 7.4. The plates were then placed in a shaking water bath at 37 °C for 15 min to allow for equilibration. ET-I or the ET_B agonists were added 5 min prior to exposure to buffer containing KCl (50 mM). If an antagonist was to be studied then it was added 5 min prior to the addition of ET -1. After a further 5 min (catecholamine and ATP studies) or 15 min (NPY studies) incubation with KCl (50 mM) the plates were removed from the water bath and placed on ice. The buffer from each well was removed and spun at 1000 RPM for 2 min to remove any cells. The supernatant was then assayed for catecholamine, NPY or ATP content. The cells remaining on the plates were extracted with 1 ml 0.4N perchloric acid and added to the pellets in the tubes for determination of protein by the Lowry method, or total catecholamine or NPY content.

The Krebs solution was initially altered so as to account for alterations in ionic content. However, in comparison experiments where KCl was simply added to give a final concentration of 50mM no differences in neurotransmitter release or cell function were observed. As a result all subsequent experiments were carried out in this manner.

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The K⁺-stimulated neurotransmitter release was stable over time. Depending on the neurotransmitter analyzed, KCl was added for variable times. The rationale for the different periods was based on previous studies where it has been observed that the peak release of NPY₁ir occurred after incubation with the high-K⁺ buffer for 15 minutes (Chen et al., 1997); and the peak release of ATP and catecholamine after incubation with the high-K⁺ buffer for 5 minutes (Chen et al., 1994). Cells were pretreated with PTX for 20 h as the peak ribosylation of G α -subunits was shown to occur between 18-20 hours (Chen et al., 1994; McCullough et al., 1998).

Analysis of Purines -Purines were analyzed according to the method described previously by Levitt et al. (1984). Chloroacetaldehyde was used to form fluorescent 1,N6-ethenopurine (E-purine) analogs, which can be simultaneously separated from the same sample by reverse-phase HPLC and quantified by fluorescence detection. Chloroacetaldehyde (50 μ l ; synthesized according to the modified method by Secrist et al., (1973) was added to each cell sample which was then incubated in a dry bath at 80°C for 40 min. The reaction was stopped by placing the samples on ice and the samples were then analyzed by HPLC-fluorometric detection. Separation of purine compounds was achieved on a reverse-phase C-18 column. A dual buffer gradient system was used to separate and elute the purines from the column by gradually increasing the concentration of buffer B while decreasing the concentration of buffer A (Varian 9010 solvent delivery system). Buffer A (pH=6.0) consisted of 0.1 M phosphate buffer and buffer B (pH=6.0) consisted of 75% 0.1 M phosphate buffer and 25% methanol. The fluorescent purine derivatives were detected at an excitation wavelength of 300 nm and an emission wavelength of 420

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nm (Varian 9070 Fluorescence Detector). Identification of the purine peaks was carried out by comparison of retention times of purine standards. Sample purine content was quantified by peak integration (Varian Star Workstation Software). ATP analysis was performed on perfusates and normalized against protein concentrations.

Catecholamine Measurements -Endogenous catecholamines in cell extracts were identified and quantified by high pressure liquid chromatography with electrochemical detection (HPLC-EC) as previously published (Chen and Westfall, 1994). Separation of catecholamines was achieved on a reverse-phase C-18 column. The system consists of a Varian model 2510 solvent delivery system and a model 9090 autosampler (Varian, Walnut Creek, CA) coupled to a C 18 column and an ESA Coulochem II detector. Separations were performed isocratically using a filtered and degassed mobile phase consisting of 10% methanol, 0.1 M sodium phosphate, 0.2 mM sodium octyl sulfate and 0.1 mM EDTA, adjusted to pH 2.8 with phosphoric acid. The HPLC system is coupled to a computer with which chromatograms were recorded and analyzed with Varian Star workstation software. Dopamine analysis was performed on both cells (total release) and perfusates (fractional release), and amounts of neurotransmitter released calculated on the basis of $(\text{Fractional release}/[\text{Fractional release} + \text{total release}])$.

The dominant catecholamine neurotransmitter synthesized and released from the NGF-differentiated PC12 cell is dopamine (Greene and Tischler, 1976). While the NGF-differentiated PC12 cell has all the major enzymes necessary to produce all catecholamines, it lacks the essential co-factor ascorbate that is necessary in the

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conversion of dopamine to norepinephrine. Consequently, in the NGF-differentiated PC12 cell model, dopamine is the major catecholamine synthesized and negligible amounts of NE and epinephrine are produced. Dopamine, therefore, is utilized as an index of catecholamine release from the PC12 cell.

NPY Measurements -NPY -immunoreactivity (ir) was determined using the protocol of Chen et al. (1997) directly in acid extracts of release medium by radioimmunoassay using a specific antiserum, which was raised in rabbit against porcine NPY. Radioimmunoassays were performed using a 5-day disequilibrium method. Duplicated samples were incubated with the NPY antiserum. 24 hours later ^{125}I -NPY was added to each tube. Following a 72 hr (at 4 °C) incubation period, antibody bound NPY and free NPY were separated by a second antibody method and the radioactivity measured in a gamma counter. Triplicates were used in standard curves with a sensitivity range of 1-100 fmol. NPYir analysis was performed on both cells (total release) and perfusates (fractional release), and amounts of neurotransmitter released calculated on the basis of (Fractional release/[Fractional release + total release]).

Intracellular Calcium ($[\text{Ca}^{2+}]_i$) Measurements – Real-time $[\text{Ca}^{2+}]_i$ recording was performed on PC-12 cell suspension loaded with fura acetoxymethylester (fura 2-AM) during a 60-min incubation. A cell suspension of 5×10^6 cells/ml in DMEM-HEPES [10 mM], was loaded with fura 2-AM during a 60 min incubation with 10 μM fura 2-AM in DMEM at room temperature. Following the incubation period, the cells were micro-centrifuged and resuspended in 1 ml of BSS [139 mM NaCl, 5.5 mM KCl, 1 mM MgCl_2 , 0.75 mM CaCl_2 , 8 mM HEPES], and the wash and BSS suspension were repeated

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twice. Cells were then aliquoted into a new micro centrifuge tube at 1.5×10^6 cells/ml, and the volume increased to 2 ml with BSS. The cells were then incubated at room temperature, 45 min, to allow for esterase cleavage of the AM-ester. The suspension was then micro centrifuged, and suspended in 1 ml of BSS, and repeated twice.

A dual-excitation spectrofluorometric system (model CM111), Spex Industries, Edison, NJ) was used. In these experiments, the system was programmed to alternate between 340- and 380-nm excitation every second to obtain 1-s data points for each wavelength. After subtraction of background noise (see below), the 340-to-380 nm ratio was subsequently calculated for each pair of data points to obtain the final tracing. A steady-state system was used in this study. After a stable base period, cells were exposed to KCl, which produced an increase in $[Ca^{2+}]_i$. Alternatively, the cells were exposed to endothelin-1 (porcine, Calbiochem), and then stimulated with KCl. The fluorescence signal was calibrated according to Grynkiewicz's method by using the relationship $[Ca^{2+}]_i = K_d[(R - R_{min}) / (R_{max} - R)]^\beta$, where R is measured 340-to-380 nm ratio, R_{min} and R_{max} are the values of R at very low and saturating concentration of Ca^{2+} , respectively, and β is the ratio of emission intensities at 380-nm excitation in these two sets of conditions. The K_d for fura 2 + Ca^{2+} , is assumed to be 224nM. Calcium saturation was achieved by addition of 1 μ M ionomycin and zero Ca^{2+} by further addition of 5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA). $MnCl_2$ (5mM) was subsequently added to obtain background fluorescence. $[Ca^{2+}]_i$ was calculated by subtracting basal $[Ca^{2+}]_i$ from stimulated $[Ca^{2+}]_i$ using Prism software analysis.

Materials. Endothelin-1 (ET -1) was purchased from Peninsula Laboratories,

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Belmont, CA, USA. ET-1 (Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp) was made fresh from the lyophilized pure peptide, first as a concentrated solution in distilled water and then diluted with 0.1% bovine serum albumin in saline. The peptide was reconstituted prior to an experiment. Low concentrations of endothelin have been used in specific *in vivo* studies using this method (Mutafova-Yambolieva and Radomirov, 1993). PD142893 (N-Acetyl-b-Phenyl-D-Phe-Leu-Asp-Ile-Ile-Trp), BQ123 (Cyclo(D-Asp-Pro-D-Val-Leu-D-Trp)), Sarafotoxin 6c (Cys-Thr-Cys-Asn-Asp-Met-Thr-Asp-Glu-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp), BQ 3020 (N-Acetyl-[Ala^{11,15}]-Endothelin 1 fragment 6-21Ac-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp) and IRL 1620 (N-Succinyl-[Glu⁹, Ala^{11,15}]-Endothelin 1 fragment 8-21Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp) were all purchased from RBI, Natick, MA, USA. RES-701-1 was kindly donated by Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan. BQ 788 (N-[N-[N-[(2,6-Dimethyl-1-piperidiny)carbonyl]-4-methyl-L-leucyl]-1-(methoxycarbonyl)-D-tryptophyl]-D-norleucine sodium salt) was purchased from Alexis Corporation, San Diego, CA, USA. Pertussis toxin (PTX), and all buffer salts were purchased from Sigma Chemical Company, St Louis, MO, USA. Perchloric acid (HClO₄) was purchased from Fisher Scientific (Fair Lawn, NJ). Nerve growth factor (NGF) was purchased from Collaborator Biomedical Products (Bedford, MA). Reagents for tissue culture including Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, horse serum, glutamine and pyruvate, were obtained from JRH Biosciences (Lenexa, KS).

Statistics. Statistical analysis between treatments was determined by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test. ANOVA was

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performed on raw data in experiments where controls were normalized to 100%.

Statistical differences were accepted when $P < 0.05$.

Results

Endothelin Differentially Modulates the Release of Neurotransmitters and Neuromodulators. To determine whether ET-1 had a differential effect on the release of the neurotransmitters ATP, NPY and dopamine, the effects of ET-1 on the K⁺-evoked release of ATP from NGF differentiated PC12 cells was investigated. Incubation of PC12 cells with 50 mM KCl caused an increase in the release of ATP over basal. This increase in release was inhibited in a dose dependent manner in the presence of ET-1 (Figure 1A). In contrast, in the presence of ET-1 the release of the sympathetic co-transmitters dopamine and NPY was not significantly inhibited at these concentrations of the peptide (Figure 1B and 1C, respectively). These initial set of experiments indicated that the ET-1-induced inhibition was specific, for ATP, and thus in this case sympathetic co-transmission was differentially modulated.

A Specific Endothelin Receptor Subtype Mediates ET-1 Inhibition of ATP Release. A pharmacological approach was utilized to elucidate the mechanism by which ET-1 modulated the release of the co-transmitter ATP. Initially, the effect of the ET_A/ET_B antagonist, PD 142893, on the K⁺-evoked release of ATP from NGF differentiated PC12 cells was examined to assess whether this agent could reverse the ET-1-induced inhibition on the K⁺-evoked release of ATP from these cells. ET -1 reduced the K⁺-evoked release of ATP (Figure 2). Although the degree of inhibition was greater than the previous experiments it should be noted that the response of PC12 cells to K⁺-evoked depolarization was also variable in this experiment, and subsequent

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experiments, and therefore their susceptibility to ET-1. Pre-incubation of the PC12 cells with the ET_A/ET_B antagonist PD 142893, (Figure 2), reversed the inhibitory effect of ET-1 on ATP release.

To further elucidate the nature of the receptors involved in this effect, the ET_A specific antagonist BQ123 was used. In these experiments ET-1 reduced the K⁺-evoked release of ATP from the PC12 cells compared to basal. This reduction was not inhibited by BQ123, at two different concentrations (Figure 3), suggesting that the ET_A receptor does not mediate the ET-1-induced inhibition of ATP. To confirm whether the inhibitory effect of ET-1 on co-transmitter release was via the ET_B receptor, the effects of the ET_B specific agonists, Sarafotoxin 6c (STX 6c), BQ3020 and IRL 1620) on the K⁺-evoked release of ATP from PC12 cells were studied (Figure 4). Each of the ET_B agonists mimicked the inhibitory effect of ET-1 on ATP release. STX 6c, BQ3020, and IRL1620 reduced the K⁺-evoked release of ATP in a concentration-dependent effect (Figure 4A, 4B, and 4C, respectively).

To confirm that the ET_B receptor subtype was indeed mediating the effects of ET-1 in these experiments, the effect of specific ET_B receptor antagonists, RES-701-1 and BQ788, was analyzed (Figure 5). In these experiments ET-1 inhibited the K⁺-evoked ATP release from the PC12 cells, and the ET_B antagonist RES 701-1(Figure 5A) reversed this inhibition. Similarly, BQ788 reversed the effect of ET-1(Figure 5B).

A Pertussis-Toxin Sensitive, Calcium-Dependent Pathway Mediates ET-1 Inhibition of ATP Release. ET_A and ET_B receptors are members of the G-protein coupled receptor superfamily, therefore the next set of experiments investigated

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whether pertussis toxin (PTX) could alter the ET-1 induced inhibition of ATP release. In the absence of PTX, ET-1 inhibited the K^+ -evoked ATP release from the PC12 cells (Figure 6). Following pre-incubation of PC12 cells with 50 nM PTX, the ET-1-induced inhibition of the K^+ -evoked ATP release was reversed, which paralleled the K^+ -evoked ATP release control.

Essential to the exocytotic release of most neurotransmitters is the increase in intracellular calcium. If the mechanism of ET-1-induced inhibition of ATP release involves a decrease in the availability of Ca^{2+} , then an effect on intracellular levels of calcium $[Ca^{2+}]_i$ should be evident. Therefore, to ascertain whether ET-1 does alter levels of $[Ca^{2+}]_i$ we examined the effect of ET-1 via quantitative analysis of $[Ca^{2+}]_i$. Pre-incubation of a suspension of PC12 cells with ET-1 alone had no antagonistic effect on the baseline reading (data not shown). In the absence of ET-1, cells were exposed to KCl-induced depolarization, which produced a substantial increase in $[Ca^{2+}]_i$ release, above baseline (Figure 7A). Pretreatment of the total cell population with varying concentrations of ET-1 induced a dose-dependent decrease in the K^+ -evoked $[Ca^{2+}]_i$ response (Figure 8). As shown in a representative tracing, 100 nM ET-1 resulted in ablation of the initial calcium spike, but had no significant effect on the sustained phase of $[Ca^{2+}]_i$ release (Figure 7B).

To further our understanding of the mechanism by which by which ET-1 attenuates the K^+ -evoked release of ATP, we examined the effect of the calcium channel antagonists, ω -conotoxin GVIA (CTX) and nifedipine, that are specific for N- and L-type voltage-dependent channels, respectively. As shown in Figure 9A, the L-type Ca^{2+} channel antagonist nifedipine caused a concentration-dependent inhibition of the

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K⁺-evoked release of ATP, which mimicked the effect of ET-1. In contrast, pre-incubation of PC12 cells with the N-type Ca²⁺ channel antagonist CTX at concentrations of 5x10⁻⁹ and 5x10⁻⁸ M (Figure 9B) showed no significant inhibitory effect on the K⁺-stimulated release of ATP. At a concentration of 10⁻⁸ M, the inhibitor appeared to facilitate the K⁺-stimulated ATP release; however, analysis revealed that the apparent increase in K⁺-stimulated ATP release was not statistically significant. These results suggest that ET-1 may attenuate the influx of extracellular Ca²⁺ by modulating L-type Ca²⁺ channels, which consequently results in inhibition of ATP release in PC12 cells.

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Discussion

The results indicate that release of ATP from the NGF-differentiated PC12 cell can be modulated by ET-1. Furthermore, this modulation appears to be via the ET_B subtype of ET receptor and its associated G_{i/o} G-protein by means of the attenuation of the influx of extracellular Ca²⁺ possibly through voltage-sensitive channels. Moreover, inhibition of ATP release appears to be mediated through L-type voltage-dependent Ca²⁺ channels.

Interestingly, in this model of sympathetic neurotransmission it was demonstrated that ET-1 functions as a neuromodulator of transmitter release by differentially modulating the release of ATP, dopamine, and NPY. Until recently, the general consensus has been that ATP is co-stored and co-released with catecholamines in vesicles in the sympathetic nerve ending. However, recent evidence would suggest a more complex picture of neurotransmission occurs, and that differential release of the co-transmitters takes place at the neuroeffector junction. Todorov et al. (1994, 1996) demonstrated in the guinea pig vas-deferens that there is a temporal difference in the release of ATP and NE, with the transient release of ATP at initiation of nerve stimulation, followed by a sustained release of NE. Moreover, the authors demonstrated the ratio of the released NE to released purine from the guinea-pig vas deferens after sympathetic nerve stimulation did not remain constant, as would be expected if the co-transmitters originated from the same vesicle, but instead changed indicating a difference in the source of the two neurotransmitters (Todorov et al., 1994). These data

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suggest that in sympathetic nerves differential co-transmission occurs, and that ATP and NE may in fact be stored in separate vesicles.

These observations may provide an explanation for the difference in modulation by ET-1 on the release of the neurotransmitters demonstrated in this study. The differential modulation of the release of NE and ATP has been demonstrated by other substances in a variety of preparations. The α_2 agonist clonidine inhibits the release of NE but not ATP from the mesenteric plexus of the guinea pig ileum (Hammond et al., 1988). In other preparations, including the mouse vas deferens (Von Kugelgen et al., 1989), rabbit ileocolic artery (Bulloch and Starke, 1990) and guinea pig vas deferens (Driessan et al., 1993), activation of pre-junctional α_2 -adrenoceptors appears to decrease the release of NE more than the release of ATP. Similarly, inhibition of α_2 -adrenoceptors produces a greater overflow of NE than ATP suggesting that endogenously released NE normally has a greater inhibitory effect on its own release than on the release of ATP (Todorov et al., 1996). Recently, in the mesenteric rat arterial bed, ET-1 was shown to differentially modulate the release of the co-transmitters NPY and NE, by inhibiting the release of NPY but not NE (Hoang 2002). The findings in this study, therefore, are consistent with the existing evidence for differential modulation of sympathetic neurotransmission.

As previously discussed, ET's have been shown to modulate sympathetic neurotransmission by enhancement of the post-junctional contractile effect of nerve stimulation, or through a variety of vasoactive agents possibly by way of the ET_A receptor (Henrion and Laher, 1993). The current findings in this paper, and by other investigators, show a prejunctional inhibition of sympathetic neurotransmission, possibly

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via the ET_B receptor (Matsuo et al., 1997). The study by Nakamura et al. (2003) on gastric sympathetic neurotransmission demonstrated that both ET_A and ET_B receptor subtypes appear to participate in the inhibition of NE release from the rat stomach, although to different degrees. The selective ET_A receptor antagonist, BQ123, attenuated the concentration-dependent ET-1-induced inhibition of NE release, but the selective ET_B antagonist, BQ788 had no effect. However, activation of the ET_B receptor by the highly selective ET_B receptor agonist, sarafotoxin S6c, inhibited NE release from the stomach to a small but significant degree (Nakamura et al., 2003). These observations raise the possibility that ET -1, and its receptor subtypes, play a dual role in modulating sympathetic neurotransmission.

ET -1 is released from the vascular endothelium by a variety of stimuli. ATP is released both pre-junctionally from the sympathetic nerves (Burnstock and Kennedy, 1986) as well as post junctionally from smooth muscle upon contraction (Muramatsu et al., 1981). It is also known that the ATP metabolite, adenosine, can feed back through A₁ receptors to inhibit the further release of ATP from the nerve endings (Dalziel and Westfall, 1994). This, coupled with the fact that ET-1 also appears to feed back negatively on ATP release through the ET_B receptor, provides more insight into the control of the release of ATP at the neuroeffector junction. Moreover, these observations suggest that the release of ATP is modulated by independent mechanisms.

The ET-1 receptor is a G-protein-coupled receptor, and their associated G-proteins are pivotal in intracellular signaling events following agonist-induced receptor activation. The G_i or G_o family of G-proteins can be differentiated from the G_q, G_{12/13}

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subtypes by PTX-mediated ADP-ribosylation of their $G\alpha$ -subunits. In this study pre-treatment of NGF-differentiated cells reversed the ET-1-induced inhibition of the K^+ -evoked ATP release suggesting that PTX-sensitive G-proteins, G_i or G_o , are involved in intracellular signaling events caused by ET-1 to inhibit ATP release. The observations in this study are consistent with other investigations that have shown PTX-sensitive G-proteins involvement in the mechanism of ET-1 mediated events. PTX-sensitive G-proteins have been observed to prevent the ET-1-induced inhibition of NE release from the rat stomach (Nakamura et al., 2003), pig coronary artery smooth muscle contraction (Kasuya et al., 1992), and L-type voltage-dependent calcium currents in myocytes (Boixel et al., 2001).

Central to the exocytotic release of most neurotransmitters is the rise in intracellular Ca^{2+} concentrations (Parnas et al., 2000). The release of ATP from the sympathetic nerve terminals has been determined to be a Ca^{2+} -dependent process, and has been demonstrated in a variety of preparations including the bladder, vas deferens and neuromuscular junction (Smith, 1991). In this study, using NGF-differentiated PC12 cells, KCl-stimulation evoked a dose-dependent increase in intracellular Ca^{2+} levels (data not shown), suggesting intracellular Ca^{2+} levels are coupled to extracellular Ca^{2+} influx. The levels of $[Ca^{2+}]_i$ were negatively altered by ET-1, which was observed to inhibit $[Ca^{2+}]_i$ in a concentration-dependent manner. Interestingly, during ET-1-induced modulation of $[Ca^{2+}]_i$ levels, it was the transient calcium spike that was inhibited and not the sustained phase of $[Ca^{2+}]_i$. ET-1-induced stimulation of myocardial ventricular cells increases $[Ca^{2+}]_i$ levels in a biphasic manner, and the initial calcium spike has been suggested to be mediated by the ET_B receptor, as the ET_B receptor antagonist BQ788,

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abrogated this transient spike (Hong et al., 2002). These observations support the concept that a specific receptor can modulate calcium dynamics and in the PC12 cell ET-1, through the ET_B receptor, may negatively modulate ATP release through inhibiting the rise of intracellular Ca²⁺ concentrations.

In many excitable cells, neurotransmitter release is coupled to Ca²⁺ influx through voltage-dependent channels. NGF-differentiated PC12 cells possess a complement of functional voltage-dependent Ca²⁺ channels including the L-, N-, and P-types (McCullough et al., 1998), and are therefore a useful model to further explore the mechanism by which ET-1 inhibits ATP release. In a preliminary set of experiments, we tested the effect of ET-1 on whole cell Ba²⁺ current. ET-1, in a dose-dependent manner (100 and 300nM; data not shown), inhibited voltage-dependent Ca²⁺ channels in NGF-differentiated PC12 cells, suggesting that these channels may have a key role in the mechanism by which ET-1 negatively modulates ATP release. This observation, while preliminary, is consistent with those who demonstrated that ET-1 inhibits voltage-dependent Ca²⁺ channels, and that negative modulation of neurotransmitter release is mediated by these channels. N-type voltage-dependent Ca²⁺ channels have been shown to be involved in the mechanism by which ET-1 negatively modulates the release of NE from sympathetic gastric nerve terminals (Nakamura et al., 2003). In this study, the L-type voltage-dependent Ca²⁺ channel may be mediating the ET-1-induced inhibition of ATP release as nifedipine, an L-type voltage-dependent antagonist, dose-dependently inhibited ATP release. At concentrations of 5x10⁻⁹ and 5x10⁻⁸ M, the N-type Ca²⁺ channel antagonist, ω-conotoxin GVIA, did not cause a statistically significant inhibition of the K⁺-stimulated ATP release.

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The data presented indicate that ET-1-mediated attenuation of ATP is mimicked in a concentration-dependent manner by nifedipine, suggesting a potential role for the L-type voltage-dependent calcium channel in the molecular signaling mechanism. A possible mechanism for the inhibitory actions of ET-1 through the ET_B receptor could be through the inhibition of L-type voltage dependent Ca²⁺ channels in the nerve terminal thereby preventing Ca²⁺ influx. Interestingly it has been shown that ET -1 inhibits L-type Ca²⁺ channels in the heart, and that the ET-1-induced transient spike in [Ca²⁺]_i was inhibited by nifedipine (Boixel et al., 2001), suggesting a potential role for this specific voltage-dependent channel in the ET-induced inhibition of ATP release. Presently, however, we do not know whether the linkage between the endothelin receptor and the calcium channel is direct or indirect. However there are other studies where, in NGF-differentiated PC12 cells, NPY has been shown to indirectly inhibit extracellular Ca²⁺ influx through the L-type channel via a PKC and Ca²⁺ calmodulin-dependent protein kinase (McCullough and Westfall, 1996). Additionally, NPY can directly inhibit extracellular Ca²⁺ influx through the N-type voltage-dependent calcium channel (McCullough et al., 1998). Elucidation of the detailed molecular signaling mechanisms by which ET-1 has its effects is currently under investigation.

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Acknowledgements

We thank Dr's Yee Kim and. T.E. Egan for their technical assistance and advice.

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Footnotes

This work was supported by USPHS Grants numbers NHLBI 61836 and 60260

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Legends

Fig 1. Endothelin-induced inhibition of sympathetic co-transmitters. ET-1 (10^{-15} - 10^{-8} M; solid bars) caused a dose-dependent inhibition (10^{-15} - 10^{-8} M; 353 ± 58 %, 190 ± 36 %, 132 ± 39 % and 40 ± 17 %, respectively) of the K^+ -evoked release (open bars; 50 mM) of ATP (A) from NGF-differentiated PC12 cells. The fractional release of the catecholamine dopamine (B) and NPY (C) from NGF-differentiated PC12 cells was unaffected by the presence of ET-1. Each bar represents the mean \pm SEM of 12 wells from 4 individual experiments. * $P < 0.05$ versus K^+ -evoked release.

Fig 2. The effect of an ET_A/ET_B antagonist on ATP release. The ET_A/ET_B antagonist PD142893 (hatched bar; 10^{-5} M) reversed the inhibition, caused by ET-1 (solid bar; 10^{-10} M), of the K^+ -evoked release (open bar; 50 mM) of ATP from NGF-differentiated PC12 cells from -6 ± 22 % to 154 ± 43 %. Each bar represents the mean \pm SEM of 12-15 wells from 5 individual experiments. * $P < 0.05$ versus K^+ -evoked release, $^\dagger P < 0.05$ compared to ET-1.

Fig 3. The Effect of an ET_A antagonist on ATP release. In NGF-differentiated PC12 cells the ET_A antagonist BQ123 (hatched bars; 10^{-6} - 10^{-5} M; -24 ± 13 % and -16 ± 23 % respectively) did not reverse the inhibition caused by ET-1 (solid bar; 10^{-10} M) of the K^+ -evoked release (open bar; 50mM) of ATP from these cells. Each bar represents the mean \pm SEM; n = 6. * $P < 0.05$ versus K^+ -evoked release.

Fig 4. Pharmacological characterization of the ET_B agonists on ATP release.

The ET_B agonists (solid bars) STX 6c (A), BQ3020 (B) and IRL1620 (C) caused a dose dependent inhibition of the K⁺-evoked release (open bars; 50 mM) of ATP from NGF-differentiated PC12 cells: STX6c (10⁻¹²-10⁻⁸ M) – from 213 ± 63% to 69 ± 36 %, 33 ± 26% and -5 ± 26 % respectively; BQ3020 (10⁻¹²- 10⁻⁸ M) - from 213 ± 63% to 39± 19 %, 58 ± 22% and -5 ± 21% respectively; IRL 1620 (10⁻¹²-10⁻⁸ M) – from 213 ± 63% to 6 ± 22%, 16 ± 33 % and -24 ± 17% respectively. Each bar represents the mean ± SEM of 12-15 wells from 5 individual experiments.

*P<0.05 versus K⁺-evoked release.

Fig 5. Pharmacological characterization of the ET_B antagonists on ATP release.

The ET_B antagonists (hatched bars) RES701-1 (A) and BQ788 (B) reversed the inhibition caused by ET-1 (solid bar; 10⁻¹⁰ M; 11 ± 14 %) of the K⁺ evoked release (open bars; 50 mM; 156 ± 26%) of ATP from NGF-differentiated PC12 cells: RES701-1 (10⁻⁷-10⁻⁵ M; to 114 ± 35, 92 ± 23 and 91 ± 20%, respectively); BQ788 (10⁻⁷-10⁻⁵ M; to 59 ± 30, 107 ± 41 and 91 ± 20%, respectively). Each bar represents the mean ± SEM of 10-14 wells from 5 individual experiments.

*P<0.05 versus K⁺-evoked release, †P<0.05 compared to ET-1.

Fig 6. The Effect of Pertussis toxin (PTX) on ATP release. PC12 cells were preincubated with PTX (50 mM, 20 hr) and then stimulated with ET-1. PTX reversed the inhibition caused by ET-1 (solid bars, 10⁻¹⁰ M), of the K⁺-evoked

release (open bars; 50 mM) of ATP from NGF-differentiated PC12 cells from $26 \pm 50\%$, to $249 \pm 54\%$. Each bar represents the mean \pm SEM of 10-14 wells from 5 individual experiments. * $P < 0.05$ versus K^+ -evoked release.

Fig 7. Effect of Endothelin on Intracellular Calcium Levels $[Ca^{2+}]_i$. The effect of Endothelin-1 (ET-1; 100 nM) was tested on a suspension of NGF-differentiated PC12 cells (5×10^6 cells/ml) peloaded with fura 2-AM (10 μ M). A) A representative tracing on the effect of control cells stimulated with 25 mM KCl demonstrated a substantial increase in $[Ca^{2+}]_i$ (275 nM) above baseline. Baseline was normalized to 100nM. B) Preincubation of the NGF-differentiated PC12 cell suspension with 100 nM ET-1, and subsequent stimulation with 25 mM KCl, resulted in ablation of the initial calcium spike.

Fig 8. Endothelin in a dose-dependent manner decreased intracellular calcium $[Ca^{2+}]_i$. The effect of Endothelin-1 (ET-1) was tested on a suspension of NGF-differentiated PC12 cells (5×10^6 cells/ml) peloaded with Fura 2-AM (10 μ M). Control cells were stimulated with 25 mM KCl. Preincubation of the total cell population with ET-1 (1-100 nM; $88 \pm 6\%$, $65 \pm 7\%$, and $50 \pm 7\%$, respectively) produced a concentration-dependent decrease in $[Ca^{2+}]_i$. Values are means \pm SEM (bars) percentages of the stimulation of $[Ca^{2+}]_i$ over basal values by 25 mM KCl and were obtained from 5 independent experiments. Control samples were normalized to 100%. ANOVA was performed on raw data in experiments when controls were normalized to 100%. * $P < 0.05$ versus K^+ -evoked release.

Fig 9. Effect of calcium channel antagonists on ATP release. A) Nifedipine (Nif; 10^{-7} - 10^{-5} M; hatched bars), caused a dose-dependent inhibition of the K^{+} -evoked release (open bar; 50 mM) of ATP from NGF-differentiated PC12 cells ($428 \pm 86\%$ to $51 \pm 24\%$, $67 \pm 53\%$ and $38 \pm 66\%$, respectively). B) ATP release did not demonstrate statistical significant inhibition in the presence of ω -conotoxin GVIA (CTX; 5×10^{-5} - 10^{-8} M). Each bar represents the mean \pm SEM of 10-14 wells from 5 individual experiments. * $P < 0.05$ versus K^{+} -evoked release.

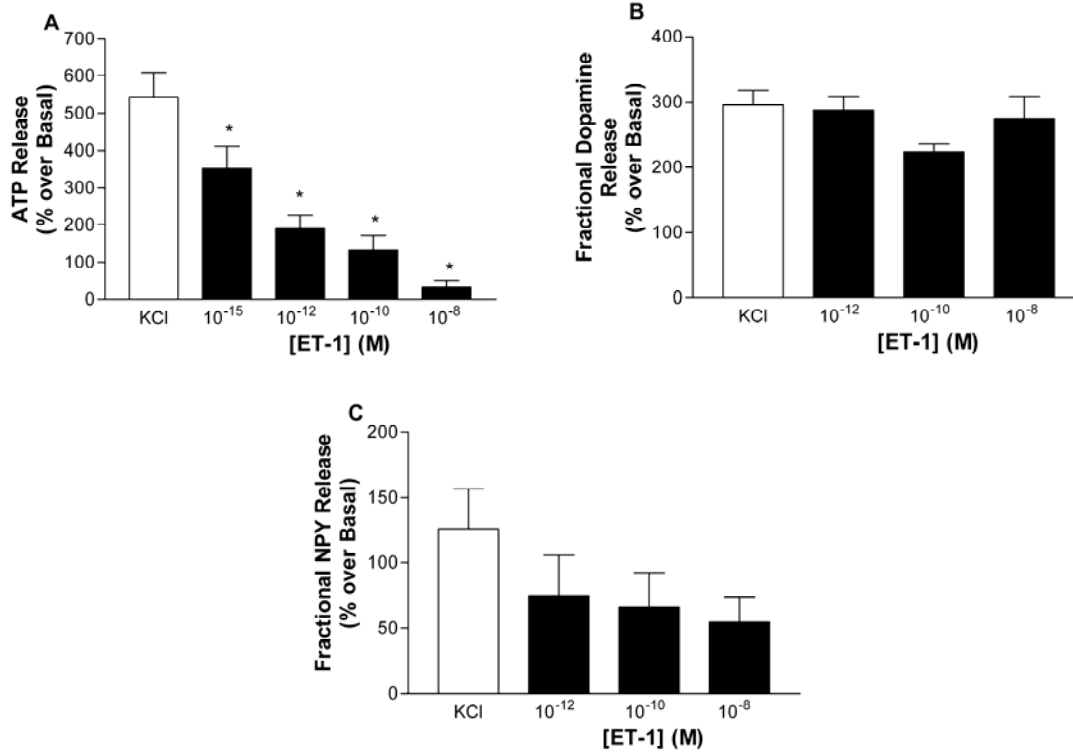


Figure 1

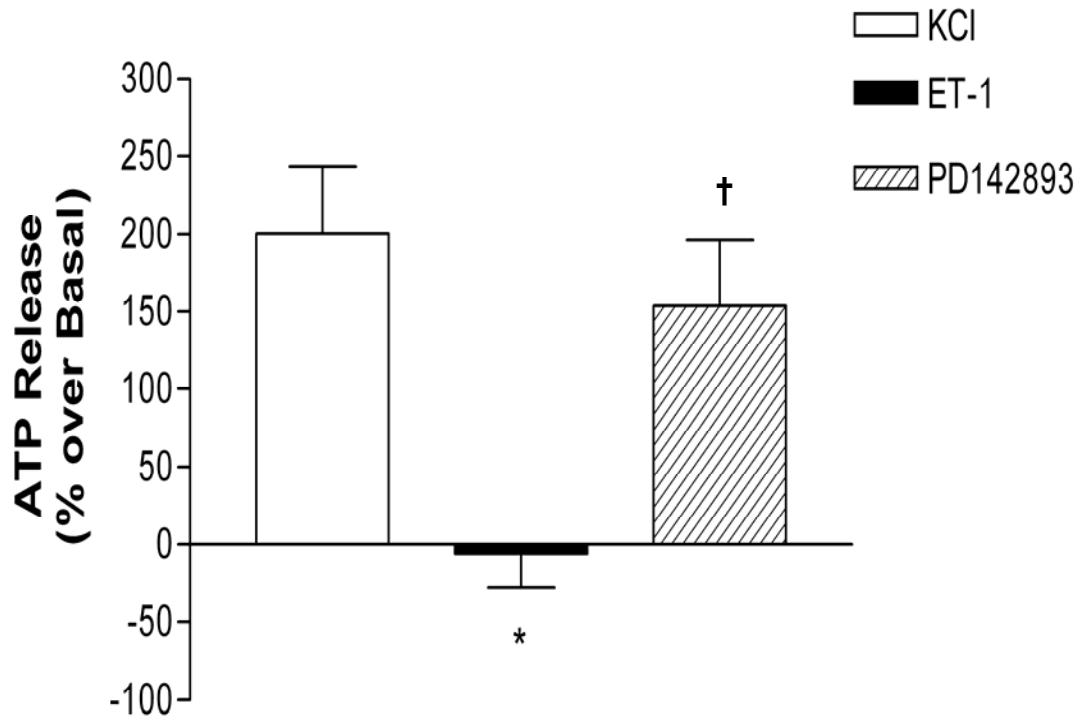


Figure 2

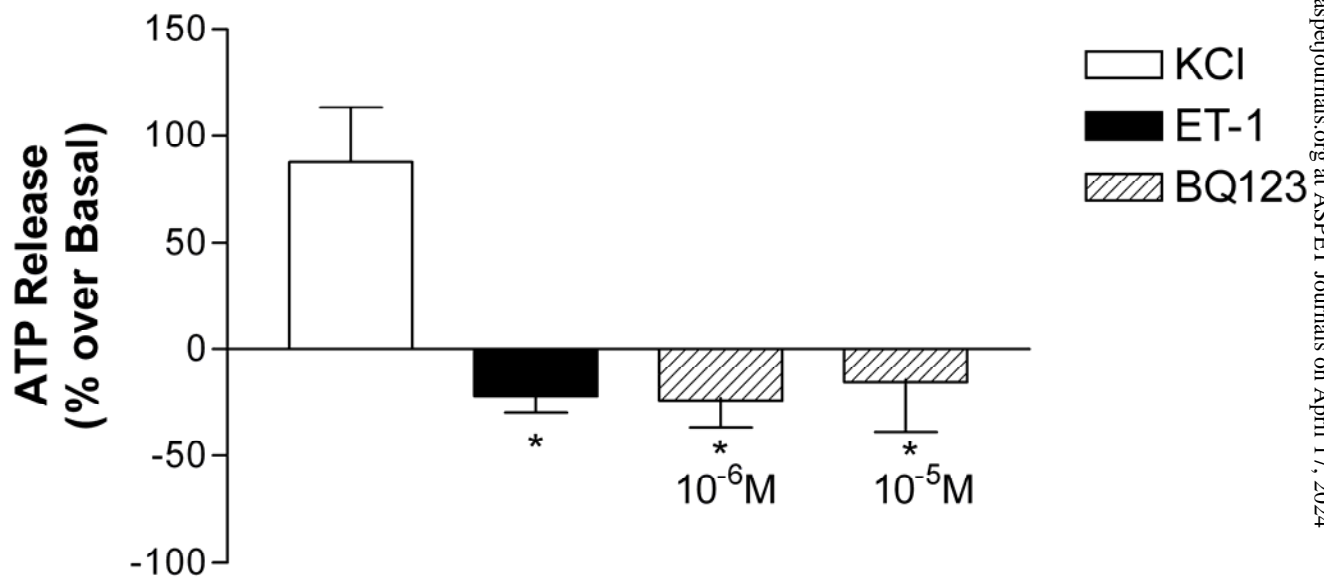


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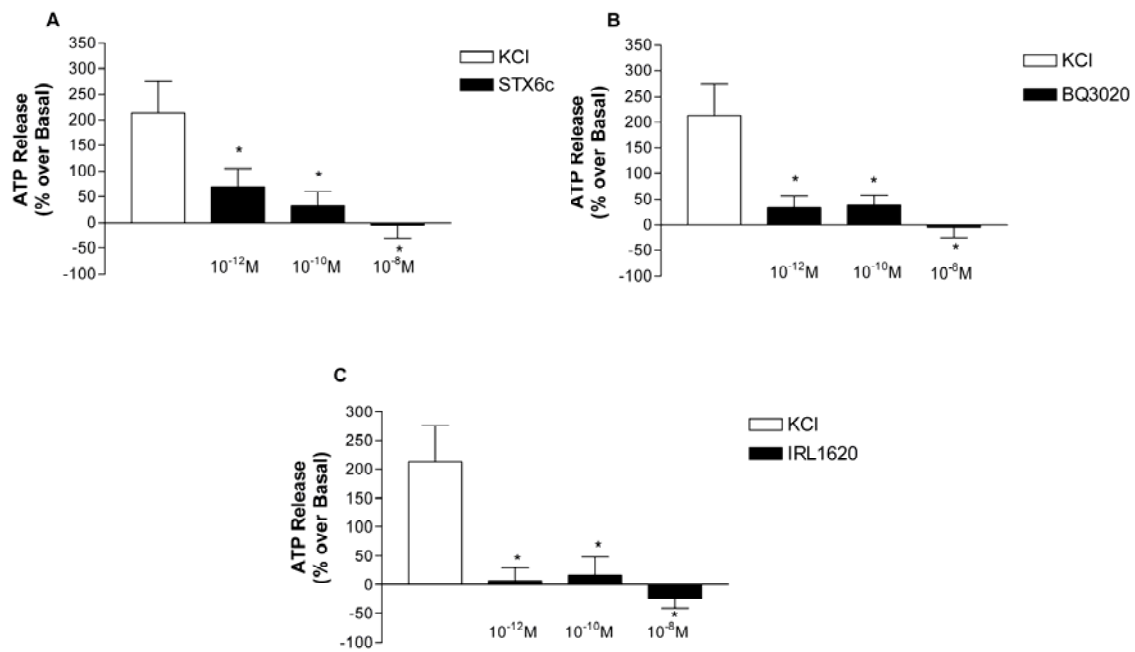


Figure 4

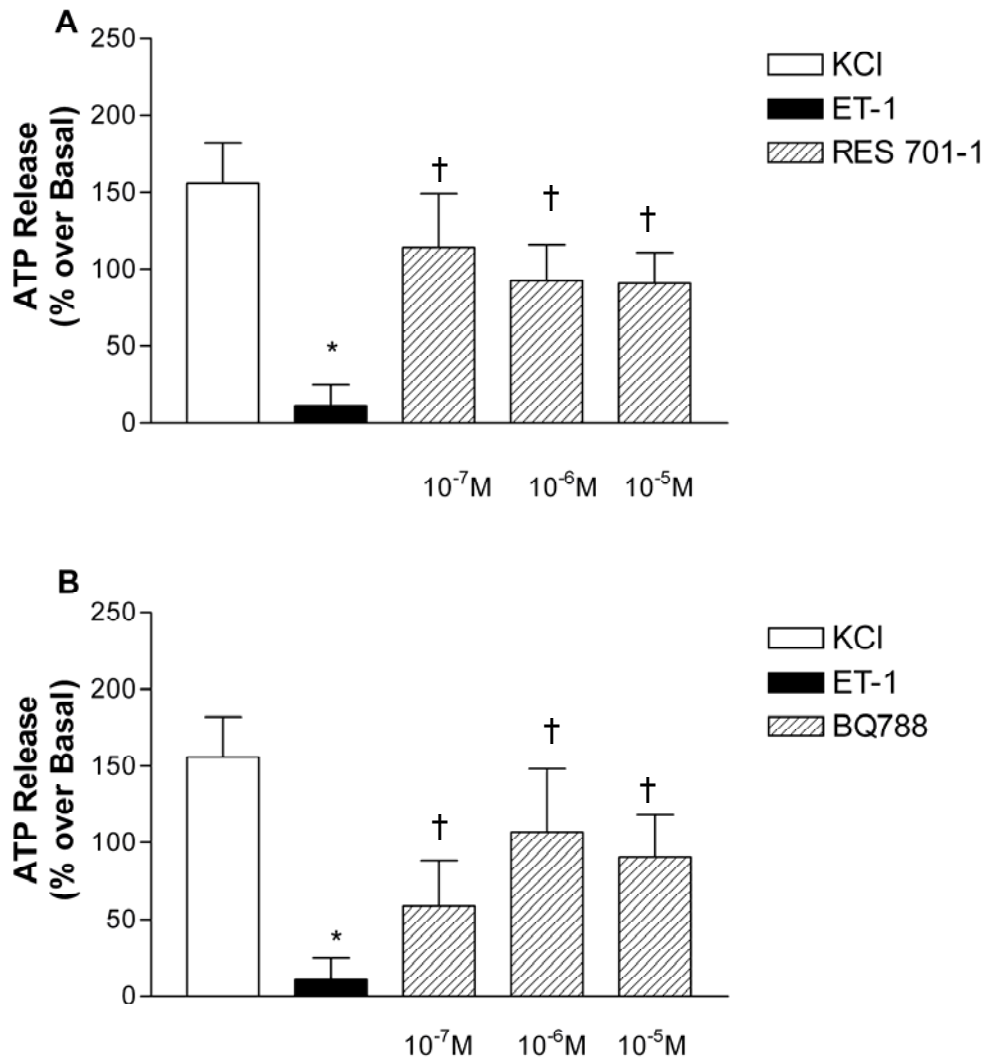


Figure 5

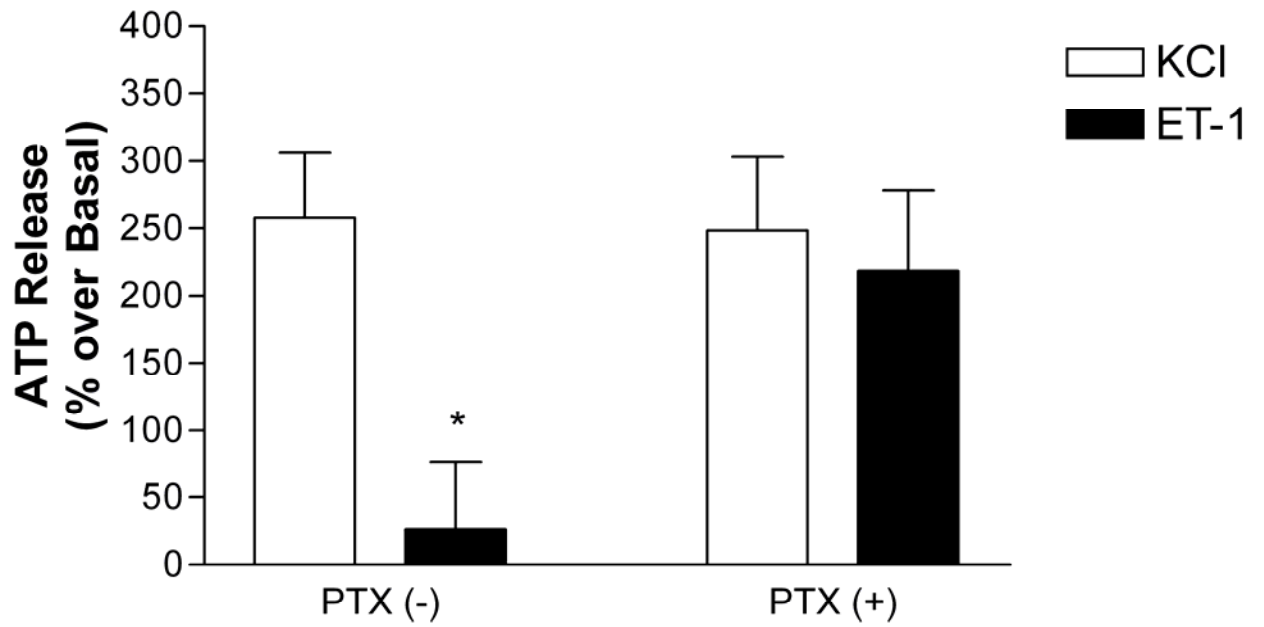


Figure 6

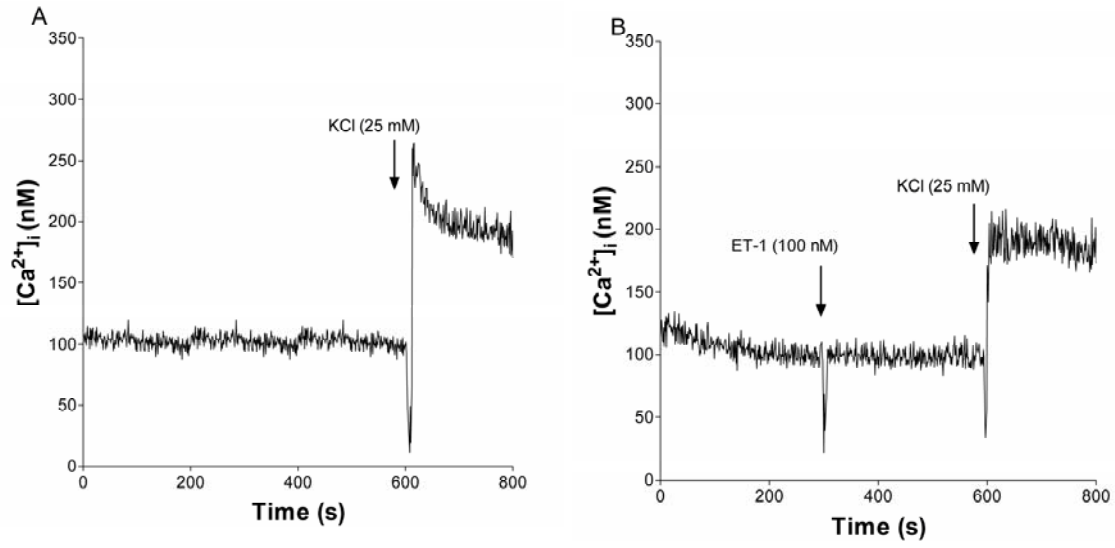


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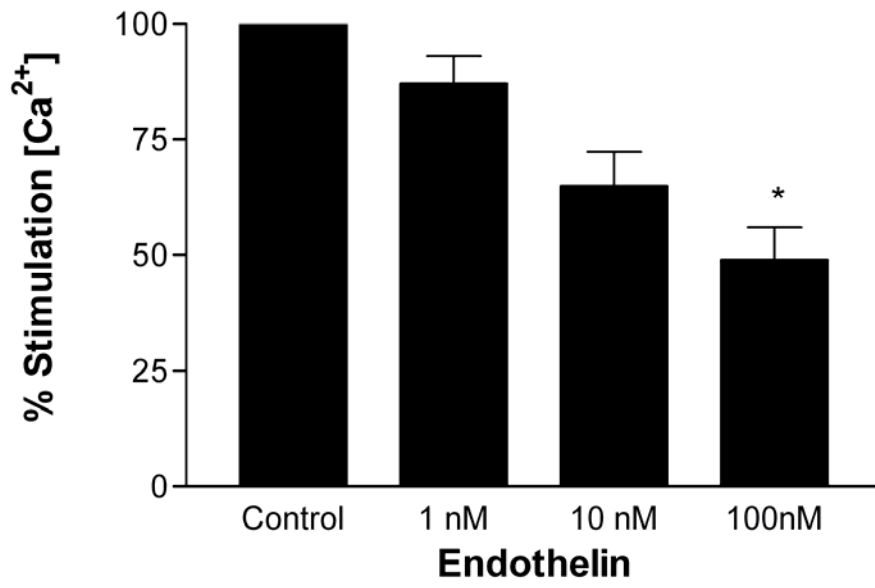


Figure 8

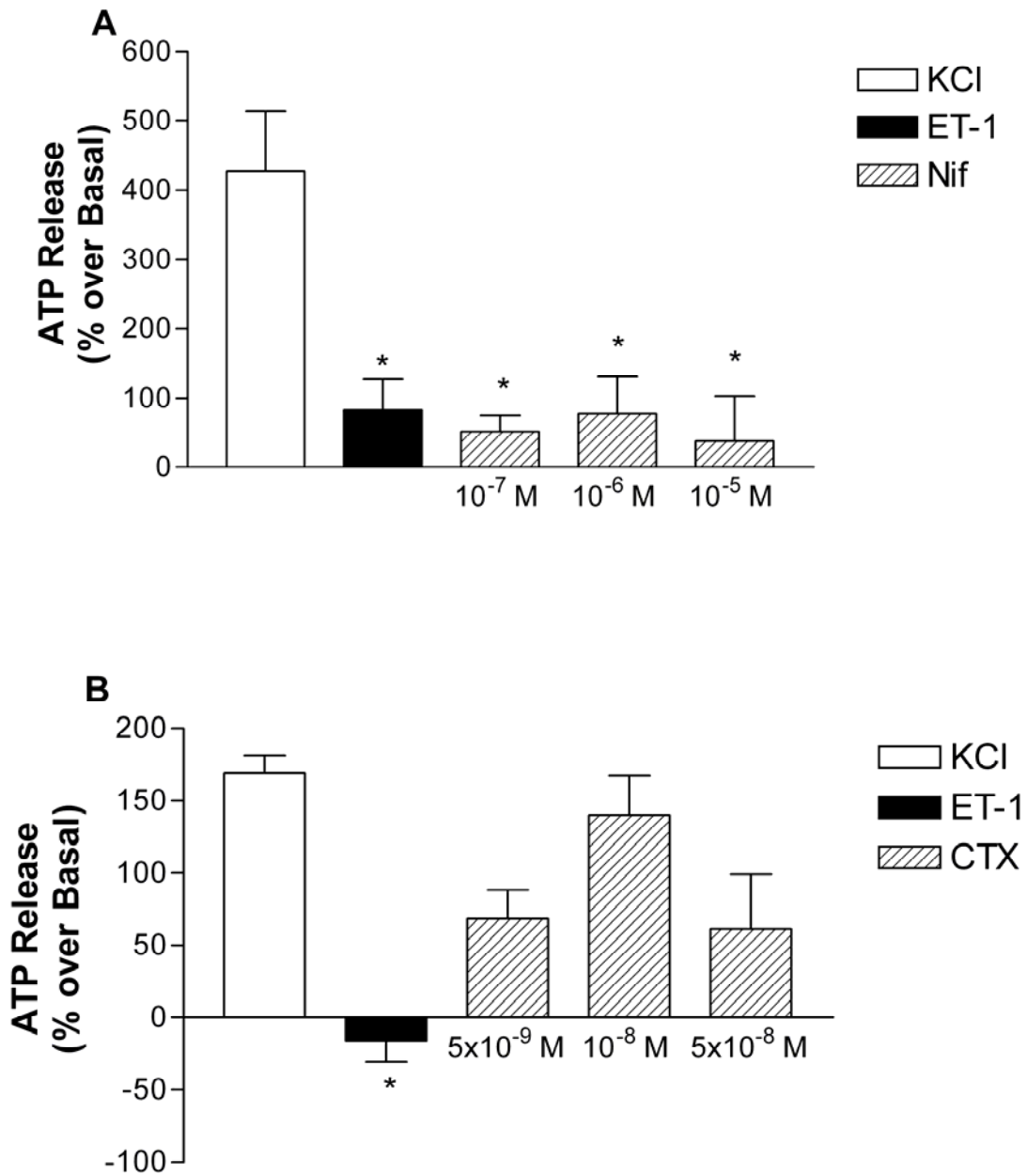


Figure 9