Involvement of ET_A receptors in the facilitation by endothelin-1 of non-adrenergic non-cholinergic transmission in the rat urinary bladder

M.V. Donoso, C. Salas, G. Sepúlveda, J. Lewin, *A. Fournier & ¹J.P. Huidobro-Toro

Unidad de Regulación Neurohumoral, Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago-1, Chile and *INRS-Santé, Université du Québec, 245 Boulevard Hymus, Pointe-Claire, Québec H9R 1G6, Canada

1 Endothelin-1 (ET-1; 3–10 nM) raised the tone of rat bladders bathed in buffer containing atropine (1 µM) plus guanethidine (3.4 µM). In addition, ET-1 potentiated, in a concentration-dependent fashion (1–10 nM), the contractions evoked by both transmural nerve stimulation and applications of exogenous adenosine 5'-triphosphate (ATP).

2 The threshold concentration of ET-1 required to facilitate non-adrenergic non-cholinergic (NANC) transmission and potentiate ATP-induced contractions, was about 10 fold lower than that required to increase the bladder tone (3 nM).

3 The ET-1-induced increase in basal tension reached its maximal effect within 60–90 s. In contrast, the 7.8 µM ATP-induced contractions increased by 50% within the first minute following incubation with 10 nM ET-1 but required about 5 min to develop the maximal effect.

4 The ET-1-induced potentiation of NANC or ATP responses was long-lasting and persisted in spite of extensive washing. The recovery of the bladder excitability depended on the concentration of ET-1. Following the application of 3 nM ET-1, recovery required 30 min; applications of 10 nM ET-1 required at least 60 min for full recovery.

5 The ET-1-induced potentiation of responses was selective for ATP and related structural analogues. ET-1 did not modify the contractions induced by acetylcholine, 5-hydroxytryptamine, prostaglandin F_{2α} or bradykinin.

6 The potency of ET-2 was similar to that of ET-1. ET-3 and ET-C-terminal hexapeptide were inactive up to 100 M. Sarafotoxin S6b was 2 to 3 fold less potent than ET-1 whereas sarafotoxin S6c (100 nM) was inactive. AGETB-9 and AGETB-89, two ET_B receptor agonists, were also inactive (up to 100 nM).

7 Removal of one or both disulphide bonds in ET-1 and tryptophan-21 formylation of ET-1, resulted in inactive peptides (up to 100 nM).

8 The ET-1 receptor antagonists, BE-18257B and FR 139317, blocked both the ET-1-induced rise in tone and the potentiation of ATP responses in a concentration-dependent fashion. FR 139317 was at least 30 fold more potent than BE-18257B. Both antagonists blocked at lower concentrations the ET-1 increase in bladder tone as compared to the ATP potentiation. The antagonism was slowly reversible.

9 Results are consistent with the presence of ET_A receptors in the rat bladder, which mediate both actions of ET-1. The interaction of ET-1 with purinergic mechanisms is discussed.

Keywords: Endothelin-1; bladder neurotransmission; purinergic mechanisms; urinary bladder; ATP potentiation; NANC transmission; ET-A antagonists

Introduction

Adenosine 5'-triphosphate (ATP) has long been proposed as a non-adrenergic non-cholinergic (NANC) neurotransmitter (Burnstock *et al.*, 1978b). Recent evidence suggests that ATP may participate in synaptic physiology as a neurotransmitter and/or as a co-transmitter of central and peripheral neurones (Burnstock, 1990a,b; Evans *et al.*, 1992; Evans & Surprenant, 1992). ATP activates membrane bound receptors different from those of adenosine; the ATP receptors are classified into two main types, P_{2x} and P_{2y} (Burnstock & Kennedy, 1985). The P_{2x}-purinoceptor is thought to mediate rapid-onset excitatory responses and is characterized by a fast rate of desensitization following prolonged exposure to ATP or α,β-methyleneadenosine 5'-triphosphate. In addition, the P_{2x}-purinoceptor is selectively blocked in a competitive manner by suramin (Dunn & Blakeley, 1988). Recent solubilization of the ATP receptor suggests that it is an ionophore, sharing features with other channel-operating receptors (Bo *et al.*, 1992).

The role of ATP in neurotransmission has been investigated in the peripheral nervous system (Burnstock *et al.*, 1978a; Brown *et al.*, 1979; Hoyle & Burnstock, 1985). The most solid evidence supporting ATP as a neurotransmitter is derived from investigations in the vas deferens (Fedan *et al.*, 1981; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Astrand, 1984), the mesenteric artery (Ramme *et al.*, 1987), and the rat bladder (Burnstock *et al.*, 1978b; Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985; Acevedo & Contreras 1985).

Previous studies from our laboratory demonstrated that while bradykinin facilitated purinergic mechanisms operating in the bladder, ET-1 potentiated the vas deferens neurotransmission (Acevedo *et al.*, 1990; Donoso *et al.*, 1992). The recent discovery that the novel 21 amino acid peptide endothelin-1 (ET-1) is synthesized in the bladder epithelia, as well as in non-vascular bladder smooth muscle (Saenz de Tejada *et al.*, 1992), raised the possibility of studying the putative role of this peptide in the bladder NANC transmission. Although ET-1 was linked originally only to the control of blood flow (Yanagisawa & Masaki, 1989; Lerman *et al.*,

¹ Author for correspondence.

1990), it was soon recognized that ET-1 is synthesized in various organs. ET exerts effects on smooth muscles in the respiratory, intestinal and urogenital tracts, as well as neurones and epithelial cells (for recent reviews see Simonson & Dunn, 1990; Highsmith *et al.*, 1992; Filep, 1992). In addition, human ET-1 plasma levels average 1 to 2 pg ml⁻¹ (Hartter & Woloszczuk, 1989; Stewart *et al.*, 1992), supporting a possible hormonal role. Receptors for ET-1 are probably membrane-bound proteins belonging to the GTP-coupled superfamily of receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Based on pharmacological principles, receptors for ET have been tentatively characterized into 2 main types: ET_A and ET_B (Masaki, 1991).

In this paper, we characterize pharmacologically the ET receptors in the bladder, examine the influence of ET on NANC transmission and compare the facilitation of the NANC responses with the potentiation of the direct muscular action of exogenous ATP and its analogues.

Methods

Bioassay

Adult male Sprague Dawley rats (250–300 g), raised in our Animal Reproduction Laboratories, were killed by cervical dislocation; the abdominal cavity was opened, the bladder was mechanically voided, excised from surrounding tissues, and mounted in a bath for the recording of isometric contractions as detailed by Acevedo & Contreras (1985). Unless otherwise specified, the composition of the incubation buffer was (mM): NaCl 118, KCl 5.4, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 23.8, glucose 11.1, atropine 0.001 and guanethidine 0.0034. To maintain pH close to 7.4, the buffer was bubbled with 95% O₂/5% CO₂. Only when specified, the bladder was everted; i.e., the mucosal side faced the superfusion media.

Tissues were initially adjusted to 1 g of basal tension which was maintained throughout the experimental procedure. Nerve-evoked contractions were elicited by transmural stimulation with electrical square pulses of 70 V, 1 ms duration, 0.15, 0.5, 1.5, 5, 15 Hz during 30 s, delivered by a Grass S44 stimulator. Such contractions were secondary to nerve activation as they were abolished by 200 nM tetrodotoxin.

Isometric contractions were recorded with force displacement transducers (FT 03C) connected to a Grass oscillograph. The incubation buffer was changed every 15 min during 1 h before the experiment.

Experimental protocols

Bladder tone Increasing concentrations of ET-1 ranging from 0.1 to 10 nM were applied in a non-cumulative manner at regular 15 min intervals. The peptides were incubated with the tissues for 5 min, unless otherwise stated and rinsed from the tissues when the maximal response had been achieved. At the end of each concentration-response experiment, four applications of 10 nM ET-1 were made. The increase in bladder tone was quantified as the grams (g) of tension generated by each peptide concentration. Similar protocols were performed with ET-2, ET-3, ET-C-terminal hexapeptide, the sarafotoxins, and a variety of ET-1 structural analogues. A separate preparation was used to examine the effect of each peptide; in all cases, the preparations were challenged, at the end of the experiment, with 10 nM ET-1 to assess tissue viability. Results are expressed either as the peptide concentration-response curves or as the peptide concentration required to increase bladder basal tension by 0.5 g (C_{0.5g}).

In a separate series of experiments, bladders were first incubated in normal buffer and challenged with 7.8 μM ATP and 1.5 Hz for 30 s. Thereafter, the preparations were incubated in Ca²⁺-free buffer containing 0.1 mM EGTA for

30 min before challenge with 7.8 μM ATP, nerve stimulation and 10 nM ET-1.

To ascertain whether the ET-1-induced motor activity was mediated in part by neuronal mechanisms, additional experiments were performed in separate preparations pretreated with 200 nM tetrodotoxin for 2 min prior to ET-1 applications.

Nerve-evoked contractions The effect of 0.01–30 nM ET-1, applied for 5 min prior to electrical stimulation, was examined on the nerve-evoked contractions caused by 30 s trains of pulses (0.15–15 Hz). Separate tissues were used to evaluate the effect of each ET-1 concentration on the frequency-response curve. A resting period of 15 min was allowed between the trains of pulses during which the preparations were washed. As a control, a frequency-contraction experiment was performed immediately before initiating tissue incubation with ET-1. The mean tension of the phasic and tonic components generated by the nerve-evoked contractions was assessed.

To examine the potency of ET-2, ET-3 and the ET-C-terminal hexapeptide, sarafotoxin S6b and S6c, the ET-1 structural analogues, as well as AGETB-9 and AGETB-89, concentration-response curves were performed in tissues stimulated with trains of electrical pulses of 5 Hz during 30 s. Each analogue was investigated on a separate preparation; however, all tissues were challenged with 10 nM ET-1 to confirm tissue viability. Results express the concentration of each peptide required to cause a 1 g increase in the tension of the phasic component of the 5 Hz stimuli.

Exogenous ATP To ascertain the effect of ET-1 on the contractile activity induced by exogenous ATP, non-cumulative ATP concentration-response experiments were performed before and after incubation with 1, 3 or 10 nM ET-1. A separate preparation was used to examine the influence of each ET-1 concentration; each preparation was used as its own control. Tissues were incubated with ET-1 for 5 min and challenged with ATP at 15 min intervals; each ATP application was followed by extensive tissue washing. As a further control for this experimental series, in a separate set of tissues, two successive ATP concentration-response experiments, spaced an hour apart, were performed in the same tissues; in the second, saline was added before ATP to test whether the tissues became supersensitive to ATP after 3 h of tissue incubation.

To ascertain the possible influence of neuronal connections on the ET-1-induced potentiation of the ATP responses, a control ATP concentration-response experiment was carried out before and after incubation with 200 nM tetrodotoxin 2 min before applications of 10 nM ET-1.

To assess the influence of the rise in basal tension caused by ET-1, an experiment was performed to compare the potency of ATP under basal tone and after the tone was mechanically increased by 1.3 g to mimic the increase in basal tension caused by 10 nM ET-1. In an additional series of experiments, tone was raised by application of 7.2 μM acetylcholine 30 s before ATP additions.

To evaluate the requirement of external Ca²⁺ on the ET-1-induced potentiation of the ATP motor responses, bladder were initially equilibrated in buffer containing 2.5 mM Ca²⁺, and challenged with 7.8 μM ATP and with 30 s trains of 1.5 Hz pulses to assess viability. Next, the preparations were incubated in Ca²⁺-free buffer plus 0.1 mM EGTA for 30 min prior to performing ATP and AMP-PNP concentration-response experiments. Immediately thereafter, tissues were incubated for 5 min with 10 nM ET-1 and the concentration-response protocols were repeated. Each tissue was used to test the effect of only one nucleotide. In some cases, at the end of the experiment, tissues were bathed in buffer containing 2.5 mM Ca²⁺ and challenged with 7.8 μM ATP and electrical pulses.

To examine the potency of ET analogues, including the

C-terminal hexapeptide, the sarafotoxins and ET_B receptor agonists, series of experiments were performed in tissues challenged with 7.8 μ M ATP prior to and following a 5 min exposure to 0.01–100 nM of the peptides. Results show either the concentration-response curves for each peptide or the peptide concentration required to increase by 1 g the ATP-induced contractile response (C_{1g}). For these protocols, each peptide was assayed in a separate tissue. In all cases, tissues were tested with 10 nM ET-1 to ensure tissue viability.

Characterization of the ET-1-induced potentiation of the exogenous ATP motor responses

Incubation time To investigate the duration of the incubation with ET-1 necessary to potentiate the exogenous ATP-induced motor responses, tissues were challenged with 7.8 μ M ATP before and during incubation with 10 nM ET-1 for 15 and 30 s or 1, 2, 3, 5 and 10 min. A separate preparation was used to study the effect of ET-1 on each incubation period. Upon completion of this protocol, preparations were discarded.

Time-course of tissue recovery from ET-1 effects To assess the duration of the potentiation caused by ET-1, separate tissues were incubated for 5 min with either 3 or 10 nM ET-1. The preparations were extensively washed and 15, 30 and 60 min thereafter, challenged with either 7.8 μ M ATP or a 30 s train of 1.5 Hz pulses. The tissues were extensively washed between stimuli. Each preparation was used to study one concentration of ET-1 in the presence of either ATP or electrical pulses.

Specificity of action In separate protocols, concentration-response experiments were performed with adenosine 5'-diphosphate (ADP), 2-methylthioadenosine triphosphate, and the slowly hydrolyzable nucleotides: 5'-adenylylimidodiphosphate (AMP-PNP), α,β -methyleneadenosine 5'-triphosphate (AMP-CPP), and β,δ -methyleneadenosine 5'-triphosphate (AMP-PCP) before and after a 5 min incubation with 10 nM ET-1. Furthermore, non-purine compounds such as acetylcholine, 5-hydroxytryptamine (5-HT), bradykinin, and prostaglandin F_{2 α} were examined following an identical protocol. (In the case of acetylcholine, no atropine was added to the incubation buffer). At the initiation of each of these protocols, tissues were challenged with 7.8 μ M ATP; at the end, ATP was tested in the presence of 10 nM ET-1 to ensure that the tissues were able to respond with the characteristic ET-1-induced potentiation. Each preparation was used to examine the effect of only one agonist. The concentrations of purine or non-purine agonists required to generate either 0.5 g of tension (ADP) or 2 g of tension (other agonists) was

calculated by interpolation from each concentration-response curve.

Pharmacological characterization of the endothelin receptor: studies with BE-18257B and FR 139317

BE-18257B To assess whether the ET-1-induced tone was antagonized by BE-18257B, tissues were incubated with either 0.3, 1, 3, or 7.5 μ M of this cyclic peptide for 20 min prior to the application of 10 nM ET-1. ATP concentration-response experiments were also performed, pretreating tissues for 5 min with 10 nM ET-1 plus the solvent and either 3 or 7.5 μ M BE-18257B plus 10 nM ET-1. A separate preparation was used to investigate each concentration of antagonist. Reversibility of blockade was tested 60 min after extensive tissue rinsing, challenging with 10 nM ET-1. Since the antagonist was dissolved in dimethylsulphoxide (DMSO), control experiments were performed to assess whether DMSO modified the potency of ATP and ET-1. For this purpose, ATP concentration-response curves were performed in untreated tissues, and bladders exposed to 100 μ l (46.9 μ M) DMSO. In addition, ATP determinations were performed in the presence of 3 or 7.5 μ M of the antagonist.

FR 139317 Similar protocols to those outlined for BE-18257B were performed; the antagonist concentrations were 0.01, 0.1, 1 and 10 μ M, and the linear tetrapeptide was dissolved in 50% DMSO and incubated with the tissues for 25 min. Control ATP concentration-response curves were performed in the absence and in the presence of either 50% DMSO, or 1 μ M FR 139317.

Statistical analysis

Computerized linear regression analysis of the concentration-response curves was used to interpolate the concentrations of ATP or ADP required to increase the muscular tension by 0.5 g ($C_{0.5g}$) as previously detailed by Donoso *et al.* (1992), or the concentration of the other agonists required to increase the bladder tension by 1 g (C_{1g}) or 2 g (C_{2g}). Likewise, regression analysis was used to derive other values interpolated from concentration-response curves. Since in the majority of the cases each tissue served as its own control, Student's paired *t* test was used to establish the significance of the peptide effect. For multiple comparisons with a single control, Dunnett's *t* tables were used. To study the statistical significance of the shifts in the concentration-response curves, co-variance analysis was used. Significance was set at a *P* value less than 0.05.

Table 1 Endothelin-1 (ET-1)-induced facilitation of NANC neurotransmission

Hz	Phasic contractile component (g)			
	Control	+ 1 nM ET-1	+ 3 nM ET-1	+ 10 nM ET-1
0.15	0.7 $\pm$ 0.2 (21)	1.4 $\pm$ 0.2 (6)	1.9 $\pm$ 0.4* (6)	2.0 $\pm$ 0.2** (9)
0.5	0.8 $\pm$ 0.1 (21)	1.3 $\pm$ 0.2* (6)	2.1 $\pm$ 0.4** (5)	2.6 $\pm$ 0.1** (9)
1.5	1.5 $\pm$ 0.2 (15)	2.0 $\pm$ 0.2 (9)	2.2 $\pm$ 0.2* (9)	2.9 $\pm$ 0.3** (9)
5.0	2.9 $\pm$ 0.3 (18)	3.8 $\pm$ 0.3 (6)	3.8 $\pm$ 0.3 (6)	4.2 $\pm$ 0.2* (6)
15.0	3.8 $\pm$ 0.3 (16)	ND	ND	5.3 $\pm$ 0.6 (9)
Hz	Tonic contractile component (g)			
	Control	+ 1 nM ET-1	+ 3 nM ET-1	+ 10 nM ET-1
0.5	0.7 $\pm$ 0.1 (16)	1.1 $\pm$ 0.2 (6)	1.7 $\pm$ 0.3* (5)	1.8 $\pm$ 0.3** (6)
1.5	0.8 $\pm$ 0.1 (15)	1.3 $\pm$ 0.1* (6)	3.8 $\pm$ 0.2** (9)	3.4 $\pm$ 0.3** (9)
5.0	0.8 $\pm$ 0.1 (18)	1.3 $\pm$ 0.2 (6)	1.3 $\pm$ 0.2 (6)	1.5 $\pm$ 0.2 (6)
15.0	0.8 $\pm$ 0.1 (16)	ND	ND	1.5 $\pm$ 0.1** (9)

Values are given $\pm$ s.e.mean.

P* < 0.05, *P* < 0.01, Dunnett's *t* tables for multiple comparisons with a single control.

ND, not determined.

Peptides and drug sources

Human endothelin-1 (ET-1), [Trp(For)²¹]-hET-1, [Met(O)⁷,Trp(For)²¹]-hET-1, [Cys(ACM)^{1,15}]-hET-1, [Cys(ACM)^{1,15},Trp(For)²¹]-hET-1, [Cys(ACM)^{1,15},Cys(SH)^{3,11}, Trp(For)²¹]-hET-1, AGETB-9 (Succ⁸-[Glu⁹,Ala^{11,15}]-ET-1(8-21)) and AGETB-89 (Succ⁸-[Asp⁸,Glu⁹,Ala^{11,15}]-ET-1(7-21)) were all synthesized at INRS-Santé, Québec, Canada. Endothelin-2 (ET-2), endothelin-3 (ET-3), sarafotoxin S6b, sarafotoxin S6c, ET-C-terminal hexapeptide and the endothelin receptor antagonist, BE-18257B (cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp)) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). Adenosine 5'-triphosphate (ATP, disodium salt), adenosine 5'-diphosphate (ADP), 5'-adenylylimidodiphosphate (AMP-PNP), α,β -methyleneadenosine 5'-triphosphate (AMP-CPP), β,δ -methyleneadenosine 5'-triphosphate (AMP-PCP), acetylcholine, 5-HT(serotonin), prostaglandin F_{2a}, bradykinin, dimethylsulphoxide (DMSO), and tetrodotoxin were purchased from Sigma Chemical Co., MO, U.S.A. 2-Methylthioadenosine triphosphate tetrasodium salt (2-Me-S-ATP) was purchased from RBI (Natick, Mass, U.S.A.). FR 139317 ((R)-2-[(R)-2-[[1-(hexahydro-1H-azepinyl)carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1H-indolyl)]propionyl]amino-3-(2-pyridyl)propionic acid) was kindly provided by Prof. L. Edvinsson, Lund University, Sweden.

Results

Effects of ET-1

Basal tension ET-1 increased bladder tone with a minimal effective concentration of 3 nM (Figure 1). The peak tension was generated within 60–90 s; tension declined slowly thereafter reaching basal tone within 10 min. Repeated applica-

Figure 1 Endothelin-1 (ET-1)-induced increase in bladder tone. (a) Concentration-dependence; (b) tension developed by 4 successive applications of 10 nM ET-1 spaced 15 min apart followed by extensive rinsing between additions. Columns indicate mean values with s.e.mean. Numbers indicate the preparations examined (*n*). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

tions of 10 nM ET-1, developed less tension; the fourth application was inactive (Figure 1). The duration of the contractions was likewise shortened. In the absence of external Ca²⁺, the 10 nM ET-1-induced increase in basal tension was abolished (*n* = 6, *P* < 0.001). Eversion of the bladder, abolished the 10 nM ET-1-induced contractions (*n* = 6, *P* < 0.01).

The contractile response to 10 nM ET-1 was not significantly modified by tetrodotoxin (1.56 ± 0.33 g (*n* = 3) as compared to 1.1 ± 0.08 g (*n* = 26) in untreated preparations). The toxin did not alter the rate of ET-1 desensitization following repeated ET-1 applications.

Facilitation of contractile responses evoked by both nerve stimulation and exogenous ATP ET-1 potentiated, in a concentration-dependent fashion, the motor activity induced either by transmural stimulation or by exogenous ATP (Table 1 and Figure 2). The potentiation of ATP contractions was not decreased by tetrodotoxin. The control ATP C_{0.5g} in one particular series of experiments was 0.76 ± 0.39 μ M, which decreased to 0.022 ± 0.004 μ M (*n* = 3) in the presence of 10 nM ET-1 plus the toxin, reflecting a 34.5 fold potentiation. Eversion of the bladder resulted in a total loss of the ATP-induced motor activity.

Repeated applications of ATP did not sensitize the tissues to the nucleotides; the ATP C_{0.5g} calculated from the first concentration-response curve was 2.33 ± 0.7 μ M, and that from a second successive curve was 2.19 ± 0.6 μ M, (*n* = 6, *F*(1,81) = 0.49, *P* > 0.05).

Mechanically increasing the bladder tone did not modify significantly the potency of ATP. The C_{0.5g} was 3.6 ± 1.7 μ M versus 2.1 ± 0.9 μ M (*n* = 3) obtained when, in the same tissues, the tone was artificially increased. Likewise, precontraction of the bladder with acetylcholine, did not significantly modify the potency of ATP (*F*(1,57) = 0.072, *P* > 0.05).

Characterization of ET-1-induced activity

Concentration-dependency

Potentiation of nerve-evoked contractions ET-1 potentiated the phasic and tonic components of the contractions evoked by 0.15–15 Hz nerve stimulation. Applications of 1 nM ET-1 increased, between 20–80%, the tension generated by both components independently of the frequency of stimulation; larger concentrations of ET-1 produced proportional incre-

Figure 2 Effect of endothelin-1 (ET-1) on the concentration-response curves to ATP and 5'-adenylylimidodiphosphate (AMP-PNP): influence of external Ca²⁺. ATP and AMP-PNP concentration-response experiments were performed in buffer containing 2.5 mM Ca²⁺ (a) and in Ca²⁺-free media with 0.1 mM EGTA (b) in the absence (●) and in the presence of either 1 (□), 3 (Δ) or 10 nM ET-1 (○). Symbols indicate mean values $\pm$ s.e.mean. The number of separate tissues used in the determination of each concentration-response curve is indicated in parentheses.

Table 2 Specificity of the 10 nM endothelin-1 (ET-1)-induced potentiation of purinoceptor responses

Purinoceptor agonists		<i>C</i> _{2g} tension ^a (μM)		Ratio
		Without ET-1	Plus 10 nM ET-1	
ATP ^b	(10)	2.13 ± 0.4	0.1 ± 0.04**	21.3
ADP ^b	(3)	12.05 ± 1.95	2.73 ± 0.12**	4.4
2-Me-S-ATP	(3)	7.63 ± 2.59	1.96 ± 0.98*	3.8
AMP-CPP	(5)	0.36 ± 0.06	0.11 ± 0.02**	3.3
AMP-PCP	(3)	3.86 ± 1.06	1.33 ± 0.59*	2.9
AMP-PNP	(5)	2.37 ± 0.54	0.73 ± 0.32*	3.2

Non-purinoceptor agonists		<i>C</i> _{2g} tension ^a (μM)		Ratio
		Without ET-1	Plus 10 nM ET-1	
Acetylcholine	(6)	0.48 ± 0.23	0.53 ± 0.14	0.9
5-HT	(5)	3.73 ± 1.4	3.16 ± 1.7	1.2
PGF _{2α}	(5)	7.1 ± 2.3	3.1 ± 1.8	2.3
Bradykinin	(4)	0.41 ± 0.27	0.28 ± 0.18	1.46

Values are given ± s.e.mean.

^aRefers to the concentration of agonists required to increase the bladder tension by 2 g; ^bin the particular case of ATP and ADP it refers to the concentration required to increase the bladder tension by 0.5 g.

P* < 0.05; *P* < 0.01. Student's *t* test.

For abbreviations, see text.

ments in the phasic component while the tonic phase was maximal at 3 nM (Table 1).

Potentiation of exogenous ATP and AMP-PNP-evoked contractions; influence of extracellular Ca²⁺ Incubations with 1, 3 or 10 nM ET-1 caused a non-parallel, leftward displacement of the ATP concentration-response curves, increasing proportionally the maximal ATP contractile response (Figure 2). The *F* values when comparing these curves with their respective controls were (1,192) = 68.8, *P* < 0.005; (1,185) = 157.8, *P* < 0.005; and (1,220) = 249.6, *P* < 0.005 respectively. In contrast to the rather shallow ATP concentration-response curve, AMP-PNP, produced a significantly steeper curve (*F*(1,174) = 39.2, *P* < 0.005). In the presence of 10 nM ET-1, the AMP-PNP concentration-response curve was shifted 3 fold to the left, in a parallel fashion (*F*(1,45) = 8.2, *P* < 0.025, Figure 2, Table 2). In everted bladders, the con-

Figure 3 Time-course of the endothelin-1 (ET-1)-induced ATP potentiation. (a) ET-1 (10 nM)-induced potentiation of the 7.8 μM ATP contractions after 5 min of tissue incubation. (W = washout); (b) time-course of the facilitation following incubation with 10 nM ET-1. Solid column: tension generated by a set of 30 applications of 7.8 μM ATP. Symbols indicate mean tension ± s.e.mean generated by 7.8 μM ATP following various incubations with ET-1. At least 4 separate experiments were performed at each incubation time. **P* < 0.05; ***P* < 0.01.

Figure 4 Recovery of endothelin-1 (ET-1)-induced potentiation of the contractions caused by ATP or nerve stimulation. (a) Contractions caused by 7.8 μM ATP alone (solid column), or following a 5 min incubation with either 3 (open column) or 10 nM ET-1 (stippled column) were rinsed and re-challenged with 7.8 μM ATP, 15, 30 and 60 min later (*n* = 3 in each case). (b) Phasic component of the contractions evoked by trains of 1.5 Hz for 30 s in the absence (solid column) and in the presence of a 5 min incubation with either 3 (open column) or 10 nM ET-1 (stippled column). Following incubations, preparations were rinsed and electrically stimulated 15, 30 and 60 min later (*n* = 3 per condition). Symbols indicate the mean tension ± s.e.mean. Numbers inside columns refer to the experiments performed in separate preparations.

tractile activity of AMP-PNP was abolished; priming with 10 nM ET-1 did not potentiate its motor action ($n = 3$).

The motor activity of both ATP and AMP-PNP was significantly reduced ($P < 0.005$) following bladder incubations in Ca^{2+} -free medium. However, 10 nM ET-1 significantly potentiated the contractile action of ATP ($F(1,63) = 25.2$, $P < 0.005$) and AMP-PNP ($F(1,45) = 38.4$, $P < 0.005$) (see Figure 2). Upon addition of Ca^{2+} to the incubation buffer, a partial restoration of the ATP, nerve and ET-1-induced motor activity was observed.

Time-course Within the first minute of 10 nM ET-1 addition, the contractile effect of $7.8 \mu\text{M}$ ATP increased by 50%. However, the maximal potentiation required about 5 min (Figure 3). In contrast to the ET-1-induced increase in basal tone, this effect of ET-1 was long-lasting and resistant to washing. The duration of the potentiation was dependent on the ET-1 concentration; tissues pretreated with 3 nM ET-1 required about 30 min of tissue washing to restore control levels of contraction to $7.8 \mu\text{M}$ ATP or nerve stimulation. With 10 nM ET-1 the potentiation to both stimuli was maintained for at least 60 min, in spite of extensive washing (Figures 3 and 4).

Specificity

Purinoreceptor agonists ET-1 selectively potentiated the motor activity evoked by purinoreceptor agonists, without altering the potency of acetylcholine, 5-HT, prostaglandin $\text{F}_{2\alpha}$ or bradykinin (Table 2). While 10 nM ET-1 displaced the ATP concentration-response curve 21 fold to the left, only a 3–4 fold shift was seen with ADP, 2-Me-S-ATP and the slowly hydrolyzable ATP analogues (Table 2). In the case of the latter nucleotides, the ET-1-induced shift was parallel. In the presence of ET-1, the maximal response of ADP was increased.

Characterization of the ET receptor

ET-1 structural analogues ET-2, but not ET-3, mimicked the actions of ET-1. The potency of ET-2 as a contractile agent was 10 fold greater than ET-1, whereas as a poten-

tiator of either ATP or electrical stimulation it was 4 and 1.5 fold more effective than ET-1 respectively. ET-3 and the ET-C-terminal hexapeptide were inactive up to 100 nM. Sarafotoxin S6b was 2–5 times less potent than ET-1; sarafotoxin S6c was inactive (up to 100 nM). The ET_B receptor agonists, AGETB-9 and AGETB-89 were inactive. These results are summarized in Table 3 and Figure 5.

Figure 5 Potency of endothelin-1 (ET-1), ET-2, ET-3, and the sarafotoxins S6b and S6c. Increase in bladder tension (a), and difference in tension caused by the phasic component of the contraction elicited by trains of 5 Hz for 30 s (b), and in the tension generated by $7.8 \mu\text{M}$ ATP (c) prior to and after a 5 min incubation with varying concentrations of ET-1 (●), ET-2 (■), ET-3 (▲), sarafotoxin S6b (□), and sarafotoxin S6c (△). Symbols represent mean values $\pm$ s.e.mean. ($n = 6$, except for sarafotoxin S6c, $n = 3$).

Table 3 Potency of endothelin-1 (ET-1) (nM) and its natural and synthetic derivatives (nM), in contracting the rat bladder detrusor and potentiating NANC nerve evoked contractions and exogenous ATP motor activity

	Basal tension ($\text{C}_{0.5\text{g}}$)	5 Hz (phasic) ($\text{C}_{1\text{g}}$)	ATP ($7.8 \mu\text{M}$) ($\text{C}_{1\text{g}}$)
ET-1	6.2 ± 1.0 (6)	4.1 ± 0.9 (6)	2.9 ± 1.2 (6)
ET-2	0.6 ± 0.2 (6)	2.7 ± 0.5 (6)	0.7 ± 0.3 (6)
ET-3	$> 100^e$ (6)	$> 100^e$ (6)	$> 100^e$ (6)
C-Ter	$> 100^e$ (3)	$> 100^e$ (3)	$> 100^e$ (3)
S6b	29.8 ± 6.9 (6)	7.7 ± 2.6 (6)	10.8 ± 2.2 (6)
S6c	$> 100^e$ (3)	$> 100^e$ (3)	$> 100^e$ (3)
1	15.1 ± 4.5 (6)	23.1 ± 8.0 (6)	9.0 ± 2.4 (6)
2	5.9 ± 2.0 (3)	7.4 ± 1.2 (3)	1.0 ± 0.2 (3)
3	$> 100^e$ (6)	$> 100^e$ (6)	$> 100^e$ (6)
4	$> 100^e$ (6)	$> 100^e$ (6)	$> 100^e$ (6)
5	$> 100^e$ (6)	$> 100^e$ (6)	$> 100^e$ (6)
6	$> 100^e$ (3)	$> 100^e$ (3)	$> 100^e$ (3)
7	$> 100^e$ (3)	$> 100^e$ (3)	$> 100^e$ (3)

Values are given $\pm$ s.e.mean.

$\text{C}_{0.5\text{g}}$ refers to the concentration of peptide required to increase tension by 0.5 g; $\text{C}_{1\text{g}}$ refers to the concentration of peptide required to increase tension by 1 g.

^eInactive in concentrations up to 100 nM.

S6b = Sarafotoxin S6b.

S6c = Sarafotoxin S6c.

1 = [Trp(For)²¹]-ET-1.

2 = [Met (0)⁷, Trp(For)²¹]-hET-1.

3 = [Cys(ACM)^{1,15}]-hET-1.

4 = [Cys(ACM)^{1,15}, Trp(For)²¹]-hET-1.

5 = [Cys(ACM)^{1,15}, Cys(SH)^{3,11}, Trp(For)²¹]-hET-1.

6 = AGETB-9.

7 = AGETB-89.

Formyl tryptophan-21 ET-1, was 2 to 6 fold less potent than ET-1; oxidation of methionine-7, together with formylation of tryptophan-21, resulted in a peptide with about equal potency to ET-1 on bladder tone, 3 fold greater potency on the ATP contractions, and 2 fold less potency on nerve-evoked contractions (Table 3). Cleavage of one or both ET-1 disulphide bonds (1,15 or 1,15 and 3,11), resulted in a loss of activity up to 100 nM (Table 3).

Studies with ET_A antagonists BE-18257B: this cyclic peptide was devoid of ET-like activity but reduced, in a concentration-dependent fashion, both the ET-1-induced increase in basal tension and the potentiation of the ATP-induced contractions. The antagonist was apparently more potent against the ET-1-induced increase in basal tension than the potentiation of the ATP contractions (Figure 6a). Whereas 0.3 μ M BE-18257B caused a significant reduction in the increase in basal tension, 7.5 μ M was required to reduce the potentiation of ATP ($F(1,21) = 3.8$, $P < 0.1$; Figure 6a). The antagonism was slowly reversible; at least 30 min of washout was required to recover either the increase in basal tension or the ATP potentiation induced by ET-1. Neither DMSO nor BE-18257B *per se* modified the potency of ATP (data not shown).

FR 139317: as with BE-18257B, this linear tetrapeptide was devoid of ET-like activity but antagonized in a concentration-dependent fashion, both actions of ET-1. This compound was at least 10 fold more potent than BE-18257B as an ET_A antagonist; its potency against the ET-1-induced increase in bladder basal tension was larger than that required to antagonize the ATP potentiation. In fact, whereas 0.1 μ M almost abolished the 10 nM ET-1-induced increase in basal tension, a 10 fold larger concentration was required to cause a significant ($F(1,24) = 6.17$, $P < 0.025$) reduction in the potentiation of ATP, while 10 μ M completely antagonized the potentiation ($F(1,49) = 16.8$, $P < 0.005$; Figure 6b). Neither DMSO nor 1 μ M FR 139317 modified *per se* the potency of ATP.

Figure 6 Antagonism of endothelin-1 (ET-1) activity by BE-18257B and FR 139317. (a) BE 18257B blockade of the increase in basal tension caused by 10 nM ET-1 (left panel), and the ATP potentiation (right panel). ATP concentration-response curves were performed either alone (●); in the presence of 10 nM ET-1 (○), or 10 nM ET-1 plus either 3 (Δ) or 7.5 (□) μ M BE-18257B. (b) FR 139317 antagonism of the 10 nM ET-1-induced increase in bladder tension (left panel), and ATP potentiation (right panel). ATP concentration-response curves were performed either alone (●); in the presence of 10 nM ET-1 (○), or 10 nM ET-1 plus 0.1 μ M FR (Δ); 10 nM ET-1 plus 1 μ M FR (□), and 10 nM ET-1 plus 10 μ M FR (◇). Columns show mean values $\pm$ s.e.mean. Numbers indicate the separate preparations examined. * $P < 0.05$; ** $P < 0.01$. Symbols indicate mean values $\pm$ s.e.mean.

Discussion

The most interesting finding of this investigation is the selectivity of the ET-1-induced potentiation of the contractions induced by exogenous ATP and related analogues, an effect paralleled by the facilitation of the NANC nerve-evoked responses. In the vas deferens, ET-1 also facilitates neurotransmission, potentiating ATP, but not the noradrenaline-induced contractions (Donoso *et al.*, 1992). The ATP receptors in the bladder are P_{2x} in nature, as indicated by the order of potency of several nucleotides: AMP-CPP > AMP-PCP > AMP-PNP > ATP > 2-Me-S-ATP > ADP (Acevedo & Contreras, 1985; Donoso *et al.*, unpublished observations). The similarity between the facilitation of the nerve-evoked contractions and that elicited by exogenous ATP suggests that the ET-1-induced effect involves, at least in part, the participation of endogenous ATP as a bladder transmitter. Furthermore, the facilitation was unaltered by tetrodotoxin, which may imply that intra bladder neurones do not participate in this effect, therefore suggesting a postjunctional site of action for ET-1.

Although the interaction involves essentially P_{2x}-purinoceptors, not all of the ATP analogues were potentiated to the same degree by ET-1. Part of this difference could be due to the finding of Acevedo *et al.* (1992) that adenosine blocks ATP responses. It is possible that adenosine-derived from ATP metabolism may limit the magnitude of the ATP or ADP contractions, flattening their concentration-response curves. In addition, this discrepancy may only be apparent, since the curves of the non-hydrolyzable ATP analogues did not reach the maximal response and the magnitude of the contraction used to quantify the potentiation induced by ET-1 is markedly larger than used in the case of ATP. Furthermore, ET-1 may also inhibit ecto ATPases explaining the lesser potentiation of the non-hydrolyzable ATP analogues.

It is well documented that almost all ET-1 contractions, independent of the smooth muscle, are sluggish and relatively persistent. In the human, rabbit or rat bladder detrusor muscle, the ET-1-induced contractions are slow in developing maximal effect (Maggi *et al.*, 1989a; 1990; Lecci *et al.*, 1991) and are sustained. The present results show that both the ET-1-induced rise in bladder tension and the potentiation of the ATP responses, develop maximally within minutes; however, the potentiation of ATP is extraordinarily persistent, much more so than the ET-1-induced contractions. In addition, our findings demonstrate that the duration of the ATP potentiation is concentration-dependent; the lower the concentration of ET-1, the faster the tissue recovers its original excitability. It is plausible that within physiological concentrations, ET does not produce persistent effects and that the persistence of the effects obtained in pharmacological assays is of doubtful physiological relevance. The persistence of ET-1 activity imposes a serious restriction in evaluating experiments where multiple exposures to ET-1 are required. With regard to the mechanisms advanced to explain the persistence of ET, some investigators invoke a slow rate of dissociation from the ET receptors (Hemsén *et al.*, 1990; Traish *et al.*, 1992), while others propose that ET-1 triggers a late, post-receptor signalling event (Marsault *et al.*, 1991) and a complex process of receptor internalization followed by externalization (Marsault *et al.*, 1993).

The increase in basal tone is not obligatory for the potentiation of the ATP responses. Furthermore, the ET-1-induced rise in tone appears to involve mechanisms different from those underlying the potentiation of NANC transmission or exogenous ATP responses. Several findings support this contention: (1) the concentration of ET-1 required to potentiate either the ATP or the nerve elicited contractions is 3 to 10 fold lower than that required to increase significantly the bladder tone; (2) the time required to achieve the maximal increase in basal tension caused by 10 nM ET-1 is less than that required to cause the maximal ATP facilitation; (3) while

the mechanism leading to the rise in bladder tone rapidly desensitizes, that of the ATP potentiation does not; (4) the motor action of ET-1 is dependent on extracellular Ca^{2+} , while the facilitation of the ATP contractions is attained even in the absence of external Ca^{2+} , suggesting it requires an intracellular pool of Ca^{2+} (Salas *et al.*, 1992); (5) the different rank order of potency of some ET-1 derivatives in raising the bladder tone and potentiating ATP or nerve responses; (6) both ET_A receptor antagonists are more potent against the ET-1-induced rise in tone than the potentiation of ATP.

Several arguments substantiate our hypothesis that ET_A receptor(s) are involved in both the increase in basal tension and the facilitation of purinoceptor mechanisms. First, the order of potency of the natural ETs: $\text{ET-2} > \text{ET-1} \gg \text{ET-3}$; the C-terminal hexapeptide, which has an apparent selectivity for the ET_B receptor (Maggi *et al.*, 1989b,c), was inactive. Secondly, whereas sarafotoxin S6b is less potent than ET-1, sarafotoxin S6c is completely inactive within the range of concentrations examined. Similar findings were reported by Maggi *et al.* (1990) with regard to the increase in bladder tension caused by ET-1. Thirdly, ET-1 analogues with one or no disulphide bonds were inactive. In contrast, these peptides have intrinsic activity for the ET_B receptor and were used as pharmacological tools by Germain *et al.* (1993) to describe an ET_B receptor population in the rat trachea. Fourthly, both ET-1 actions were antagonized by compounds with apparent selectivity for ET_A receptors. In agreement with Ihara *et al.* (1991a), the present results also indicate that BE-18257B is a weak and slowly reversible antagonist. Furthermore, FR 139317, a selective ET_A receptor antagonist (Aramori *et al.*, 1993), blocked both ET-1 effects albeit showing marked differences in potency. As in the case of BE-18257B, a considerably lower concentration of antagonist was required to block the ET-1-induced increase in basal tension. The finding that $10 \mu\text{M}$ completely blocked the potentiation of ATP, suggests that this action is ET_A receptor mediated. In support of the interpretation of ET_A receptors in the bladder, Garcia-Pascual *et al.* (1990) and Traish *et al.* (1992) demonstrated ET binding sites in the rabbit bladder smooth muscle. One of the ET binding sites described by Traish *et al.* (1992) has a pharmacological profile with all the characteristics of an ET_A receptor.

It remains to be established whether the same ET_A receptor underlies the increase in bladder tension and the potentiation of the ATP contractions. It is possible that either the bladder contains subtypes of an ET_A receptor or that this receptor might be coupled to different intracellular transduction mechanisms. Traish *et al.* (1992) demonstrated two ET binding sites in the rabbit bladder, only one of which was properly characterized pharmacologically. A more detailed classification of these sites, using various pharmacological and molecular biology markers will help to elucidate this problem. It is clear however, that the Ca^{2+} requirement for each effect as well as the sensitivity of the two ET_A receptor antagonists used to block these effects are different (Salas *et al.*, 1992). The implication(s) of these latter findings is being further investigated.

Several hypothetical models may account for the ET-induced potentiation of ATP (Figure 7). An ET_A receptor may sensitize the bladder smooth muscle to ATP, acting distal to the receptor. For example, ET facilitates the release of intracellular Ca^{2+} with a slow equilibrium with the extracellular cation in contrast to the Ca^{2+} pool mobilized by ATP (Figure 7a). In this connection, Chau *et al.* (1993) recently suggested that both ET and ATP hydrolyze a separate pool of polyphosphoinositides in neuroblastoma-glioma hybrid cells resulting in additive responses due to the release of separate pools of Ca^{2+} . Alternatively, an ET_A membrane receptor may 'cross-talk' with a P_{2X} -purinoceptor increasing its affinity and/or modifying its ionophore function (Figure 7b). It is possible that these two mechanisms might operate simultaneously. Although less likely, ET may interact directly at an allosteric regulatory site on the P_{2X} -purinoceptor, in-

Figure 7 Hypothetical model diagrams: the bladder is endowed with ET_A receptor responsible for the rise in tone. This site is linked, directly or indirectly to Ca^{2+} channels. The bladder contains, in addition, another ET_A binding site that sensitizes the detrusor to actions triggered by ATP acting on P_{2X} -purinoceptors. Three models are proposed to explain the mechanism of the ET-1-induced selective ATP potentiation, an effect which is independent of the rise in basal tone. (a) ET_A receptor activation sensitizes the muscle to ATP, via a mechanism distal to the P_{2X} -purinoceptor. This mechanism entails the release of independent second messengers which as an end result amplifies the motor action of ATP. (b) The ET_A receptor 'cross-talks' with P_{2X} -purinoceptor, favouring purinergic responses. (c) The ET_A receptor is allosterically localized in the P_{2X} -purinoceptor.

creasing its affinity or altering the kinetics of its ionophore function (Figure 7c). With regard to the increase in bladder tension caused by the ETs, the ET_A receptor involved could be coupled to a calcium channel mainly dependent on the influx of extracellular Ca^{2+} (Figure 7).

These ET_A receptors could be localized in the same or in different bladder smooth muscle cell populations. Ihara *et al.* (1991b) demonstrated that vascular smooth muscle cells express both ET_A and ET_B receptors, while Donoso *et al.* (1992) demonstrated marked differences in the ET-1 responses between the epididymal and prostatic segments of the rat vas deferens. Obviously, neither the available techniques nor current knowledge on ET-1 and ATP pharmacodynamics are sufficient to allow resolution of this question (Van Renterghem *et al.*, 1988; Marsden *et al.*, 1989; Highsmith *et al.*, 1992).

The possible physiological implications of ET-1 in the bladder, given that the epithelial and non vascular smooth muscle cells of this organ synthesize ET-1 (Saenz de Tejada *et al.*, 1992), are of paramount interest. The present results support the hypothesis that ET-1 may participate in the control of bladder tone by acting as an autocrine hormone. Furthermore, we propose that the autocrine role of ET may include the facilitation of NANC transmission.

Since ET-1 is also known to enhance the purinergic motor component of sympathetic neurotransmission in the rat vas

deferens (Donoso *et al.*, 1992), our working hypothesis suggests that ET-1 plays a role as a local modulator of the purinergic component of neurotransmission. The mechanism involved in this interaction warrants further experimentation.

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