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European Journal of Pharmacology 272 (1995) 31–38

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Endothelin-induced facilitation of sympathetic neurotransmission to the rat vas deferens: effects of suramin

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Received 9 June 1994; revised MS received 28 September 1994; accepted 7 October 1994

Abstract

Experiments were conducted to elucidate the mechanisms of action of endothelins in facilitating neurotransmission to the rat isolated vas deferens. Endothelin-1 and endothelin-3 potentiated field stimulation-induced contractions and those evoked by ATP and α,β-methylene ATP. Responses to noradrenaline were unaffected. The *C*-terminal hexapeptide, endothelin-(16–21) was without effect on neurotransmission. The facilitation by endothelin-1 of responses to trains of stimulation (10 Hz for 10 s) was absent in the presence of the P_2-purinoceptor antagonist, suramin, in concentrations which antagonised the contractile effects of α,β-methylene ATP, but not those of noradrenaline. Suramin did not affect 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (Bay K 8644)-induced potentiation of contractions in response to field stimulation. These results support the hypothesis that endothelin-induced facilitation of sympathetic neurotransmission to the vas deferens is due to potentiation of the postjunctional effects of the co-transmitter, ATP, acting at P_{2X}-purinoceptors, and indicate that this effect is mediated through actions at endothelin receptors that are not of the ET_B-subtype.

Keywords: α,β-Methylene ATP; Endothelin; Phosphoramidon; Suramin; Sympathetic co-transmission; Vas deferens, rat

1. Introduction

Peptides of the endothelin family exert potent contractile activity in various vascular and non-vascular smooth muscles (Yanagisawa et al., 1988; Maggi et al., 1989a). Endothelins are also believed to modulate neuroeffector transmission through interaction at specific endothelin receptors distributed in peripheral tissues (Davenport et al., 1989; Hoyer et al., 1989) and in the central nervous system (Jones et al., 1989). Endothelins facilitate field stimulation-evoked contractions in the rat vas deferens (Maggi et al., 1989a; Wiklund et al., 1990, Télémaque and D'Orléans-Juste, 1991). There are conflicting observations, however, on the nature of neuromodulatory actions of endothelins in the rat vas deferens: endothelin-1 has been reported to inhibit (Wiklund et al., 1990) or not to modify (Donoso et al., 1992) the electrically evoked overflow of tritium after preincubation of the tissue with [^{3}H]noradrenaline. It has been proposed that endothelin-1 facilitates neurotransmission in the rat vas deferens by potentiating the effects of ATP released from sympathetic nerve terminals (Wiklund et al., 1990; Donoso et al., 1992). These observations prompted us to investigate further the effects of endothelin-1, endothelin-3 and the *C*-terminal hexapeptide, endothelin-(16–21) (Maggi et al., 1989b) on responses of the rat vas deferens to single pulses and trains of electrical field stimulation as well as on responses to exogenous ATP, α,β-methylene ATP and noradrenaline. The P_{2X}-purinoceptor antagonist, suramin, was employed in this investigation.

2. Materials and methods

2.1. Tissue preparation and contraction studies

Male Wistar rats (280–330 g) were decapitated and the vasa deferentia rapidly removed and bisected transversely into prostatic and epididymal segments. Seg-

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SSDI 0014-2999(94)00624-5

ments of rat vas deferens were suspended vertically under 1 g tension (Ventura and Pennefather, 1994) in 4-ml siliconised organ baths, containing warm (37°C), oxygenated (5% CO_2 in O_2) Krebs Henseleit solution (pH 7.4) of the following composition (mM): NaCl 118.1, KCl 4.87, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4$ 0.5, $CaCl_2$ 2.5. Prostatic segments were field-stimulated using two parallel platinum electrodes connected to a Grass S88 stimulator. Electrical field stimulation (1 ms pulse width, dial voltage 60 V) was applied either as single pulses at 0.1 Hz or trains of pulses at 10 Hz for 10 s. Epididymal segments were unstimulated. Preparations were allowed to equilibrate for 60 min, with bath medium changes every 15 min, before the start of experiment. Isometric contractions were recorded with FT03C force displacement transducers and displayed on a Macintosh SE/30 computer using MacLab Chart 3.3 recording system.

2.2. Experimental protocols

2.2.1. Effect of endothelins on contractions induced by exogenous noradrenaline, ATP or α,β-methylene ATP in the epididymal segment of the rat vas deferens

Postjunctional effects of endothelins were assessed in preparations of the epididymal segment of the rat vas deferens. Contractile activities of exogenous noradrenaline, ATP or α,β-methylene ATP were studied before and 10 min after exposure to endothelins (1 and 10 nM). Cumulative concentration-response curves to noradrenaline (0.01–30 μM) were established at 30 min intervals. Discrete concentration-response curves to ATP (0.01–1 μM) and α,β-methylene ATP (0.1 and 1 μM) were constructed; the agonist exposure period was 30 s with a 15 min dose-cycle.

2.2.2. Effect of endothelins on contractile responses to single pulse field stimulation at 0.1 Hz and on the basal force in the prostatic segment of the rat vas deferens

Concentration-response curves to endothelin-1, endothelin-3 (both at concentrations ranging from 1 to 300 nM) and endothelin-(16–21) (10 nM–3 μM) were constructed cumulatively with a progression ratio of half a log unit, the next concentration being added to the bath when the effects of the preceding one had reached a plateau 6–10 min later. Only one concentration-response curve was established per tissue.

2.2.3. Effect of phosphoramidon on the actions of endothelins

Tissues were exposed to the neutral endopeptidase inhibitor, phosphoramidon (10 μM), 30 min prior to construction of concentration-response curves for endothelin-1 or endothelin-3.

2.2.4. Effect of endothelins and Bay K 8644 on contractile responses evoked by trains of field stimulation at 10 Hz for 10 s in the prostatic segment of the rat vas deferens

After obtaining at least two reproducible control contractile responses to trains of field stimulation (1 ms, dial voltage 60 V, 10 Hz for 10 s at 20 min intervals), tissues were exposed to endothelin-1, endothelin-3 or Bay K 8644 (1–10 nM) 10 min before subsequent stimulation.

2.2.5. Effect of suramin on the actions of endothelin-1 and Bay K 8644

Tissues were preincubated with suramin (100 μM) for 1 h before examination of the effects of endothelin-1 or Bay K 8644 on contractile responses to trains of field stimulation at 10 Hz for 10 s and to those of exogenous noradrenaline and α,β-methylene ATP.

2.3. Data and statistical analysis

Areas under the force-time curve of the biphasic contractile response to trains of field stimulation were determined separately over the first 1.5 s for the initial phase and over the last 8.5 s for the secondary phase of the stimulus train. Since purinergic and adrenergic mechanisms contribute to both phases of the response to trains of stimuli (Major et al., 1989; Mallard et al., 1992a; Ventura and Pennefather, 1994), the total field stimulation-induced response was assessed by measuring area under the force-time curve of the contractile response over the total duration of stimuli (10 s).

Data are presented as mean values ± standard error of the mean (S.E.M.) of at least three separate experiments. Computerised linear regression analysis was used to determine statistical significance of the shifts in the concentration-response curves caused by the different experimental conditions studied. In experiments involving trains of field stimulation, ATP and α,β-methylene ATP, the Student's *t*-test for paired and unpaired data was applied as appropriate, and P values < 0.05 were taken as significant.

2.4. Drugs

Drugs used were: endothelin-1 (human, porcine) and endothelin-3 (human, rat) (Auspep), endothelin-(16–21) (gift from Chiron Mimotopes, Australia), ATP disodium, α,β-methylene ATP lithium, (−)-arterenol bitartrate (noradrenaline), phosphoramidon (Sigma), suramin (gift from Bayer, Australia) and Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester) (Research Biochemical, Natick, USA). Stock solutions of peptides (10 μM), prepared in distilled water, were

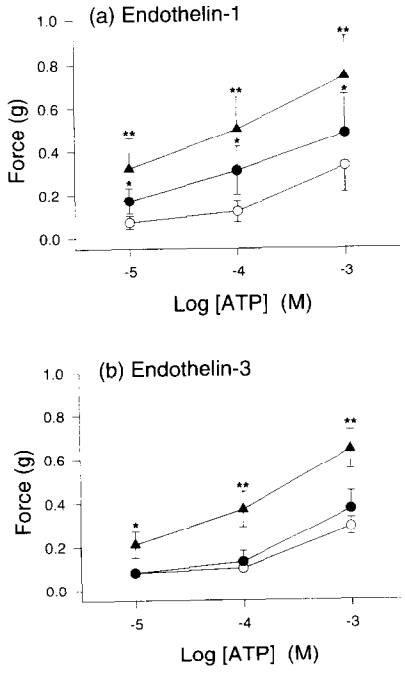

Fig. 1. Effects of (a) endothelin-1 and (b) endothelin-3 at varying concentrations (0 nM, open circles; 1 nM, filled circles; 10 nM, filled triangles) on contractions induced by ATP in the prostatic segment of the rat vas deferens. Each point represents the mean ± S.E.M. of four to five experiments. * $P < 0.05$ and * * $P < 0.01$ indicate significant differences between control (in the absence of endothelins) and the corresponding value after pretreatment with endothelins.

aliquoted and stored at −15°C and thawed only once prior to use. Noradrenaline was dissolved and diluted in a catecholamine diluent (mM: NaCl 154.0, NaH_2PO_4 1.2 and ascorbic acid 0.2). Phosphoramidon was dissolved in 0.01 M HCl; 1 mM aliquots were stored at −15°C until use. Bay K 8644 was prepared as 1 mM stock solutions in ethanol and diluted in distilled water. To protect from light, solutions of Bay K 8644 were kept in dark containers. All other drugs were dissolved and diluted in distilled water.

3. Results

3.1. Effect of endothelins on contractions induced by exogenous noradrenaline, ATP or α,β-methylene ATP in the epididymal segment of the rat vas deferens

Both endothelins (at 10 nM) induced contractile effects in the epididymal segment of the rat vas deferens (endothelin-1: 0.27 ± 0.05 g; $n = 10$ and endothelin-3: 0.17 ± 0.05 g; $n = 15$). Endothelin-1 and endothelin-3 (1 and 10 nM) concentration dependently augmented contractile responses evoked by ATP (Fig. 1) and α,β-methylene ATP (Fig. 2), while log concentration-response curves to noradrenaline were unaffected (Fig. 3).

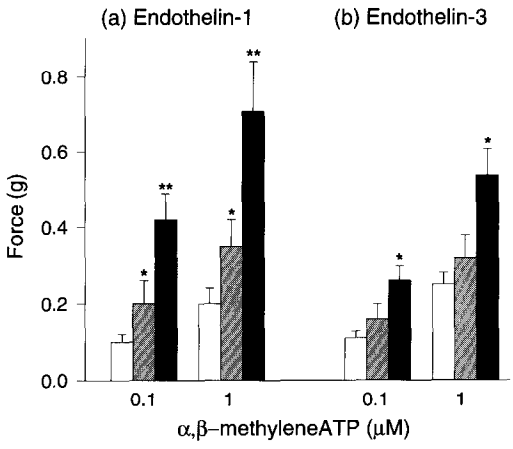

Fig. 2. Effects of (a) endothelin-1 and (b) endothelin-3 at varying concentrations (0 nM, open bars; 1 nM, hatched bars; 10 nM, filled bars) on contractions induced by α,β-methylene ATP in the epididymal segment of the rat vas deferens. Each bar represents the mean ± S.E.M. of four to five experiments. * $P < 0.05$ and * * $P < 0.01$ indicate significant differences between control (in the absence of endothelins) and the corresponding value after pretreatment with endothelins.

3.2. Effect of endothelins on contractile responses to single pulse field stimulation at 0.1 Hz and on the basal force in the prostatic segment of the rat vas deferens

Single pulse electrical field stimulation at 0.1 Hz evoked regular twitch-like contractions (1.39 ± 0.18 g;

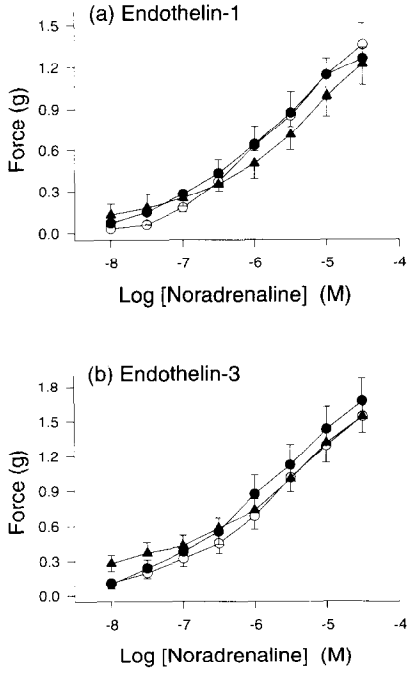

Fig. 3. Effects of (a) endothelin-1 and (b) endothelin-3 at varying concentrations (0 nM, open circles; 1 nM, filled circles; 10 nM, filled triangles) on the cumulative concentration-contractile response curves to noradrenaline in the epididymal segment of the rat vas deferens. Each point represents the mean ± S.E.M. of five to six experiments.

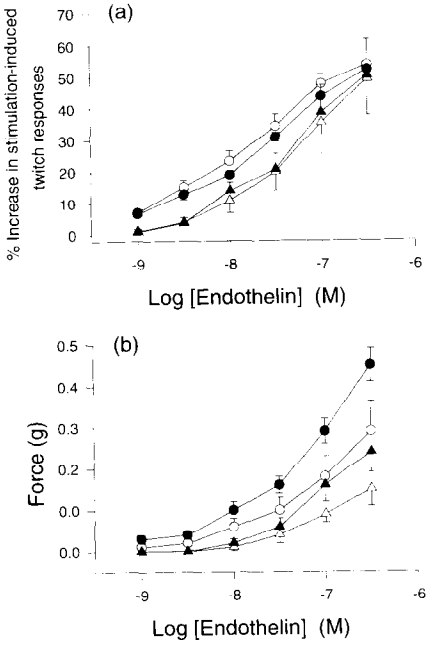

Fig. 4. (a) The potentiating effects of endothelin-1 (circles) and endothelin-3 (triangles) on single pulse field stimulation (1 ms, dial voltage 60 V, 0.1 Hz)-induced twitches and (b) the corresponding increases in basal force in the prostatic segment of the rat vas deferens. Log concentration-response curves were constructed in the absence (open symbols) and presence (filled symbols) of 10 μM phosphoramidon. Each point represents the mean ± S.E.M. of five to six experiments.

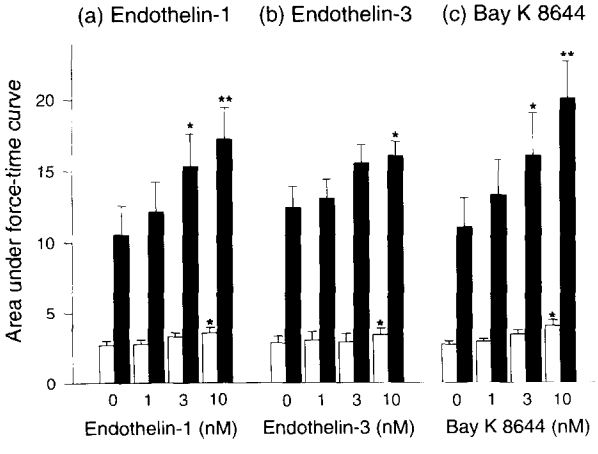

Fig. 5. Effects of varying concentrations of (a) endothelin-1, (b) endothelin-3 and (c) Bay K 8644 on the initial and secondary phases of the contractile response to trains of field stimulation (1 ms, dial voltage 60 V, 10 Hz for 10 s) applied to the prostatic segment of the rat vas deferens. The area under the force-time curve for the initial and secondary phases was determined by measuring responses over the first 1.5 s (open bars) and the last 8.5 s (filled bars) of the stimulus train, respectively. Each bar represents the mean ± S.E.M. of four to seven experiments. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences between values in the absence of endothelins or Bay K 8644 and corresponding values after pretreatment with endothelins or Bay K 8644.

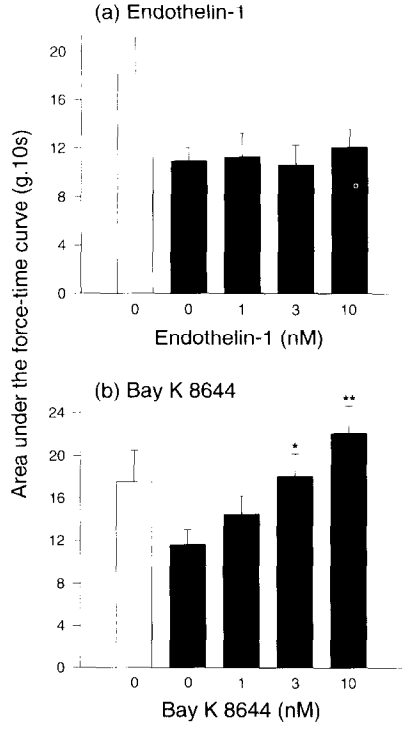

Fig. 6. The effects of (a) endothelin-1 and (b) Bay K 8644 on responses to trains of field stimulation (1 ms, dial voltage 60 V, 10 Hz for 10 s) applied to the prostatic segment of the rat vas deferens in the presence (filled bars) of 100 μM suramin. The open bars show comparable responses to trains of stimuli before the addition of either suramin or agonist. Total field stimulation-induced response was determined by measuring the area under the force-time curve of the contractile response over the 10-s period of trains of stimuli. Each bar represents the mean ± S.E.M. of four to six experiments. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences between values in the absence of endothelin-1 or Bay K8644. and corresponding values after pretreatment with endothelin-1 or Bay K8644.

$n = 11$) in the prostatic segment of the rat vas deferens. Both endothelin-1 and endothelin-3 (1–300 nM) concentration dependently enhanced responses evoked by single pulse field stimulation (Fig. 4a), in addition to exerting slight but significant increases in basal tone at higher concentrations (Fig. 4b). Endothelin-1 was approximately 3-fold more potent than endothelin-3 in potentiating responses to field stimulation and in causing increases in basal force; potency ratios are 3.24 with 95% confidence limits: 1.55, 7.19, 54 d.f. and 3.25 with 95% confidence limits: 1.64, 6.92, 56 d.f., respectively. Both peptides evoked similar enhancement in the highest concentration applied (300 nM). The enhancing actions of the peptides were long lasting and resistant to tissue washout. In contrast, contractile responses induced by endothelin-1 or endothelin-3 were completely reversible after washing. Endothelin-(16–21), at concentrations ranging from 10 nM to 30 μM, was without effect on field stimulation-induced contractions or basal force in the rat vas deferens ($n = 4$, data not shown).

3.3. Effect of phosphoramidon on the actions of endothelins

Phosphoramidon (10 μM) was without effect on resting twitch-like contractile responses to single pulse field stimulation at 0.1 Hz. In the presence of phosphoramidon, log concentration-contractile response curves to endothelin-1 and endothelin-3 were shifted 2.76 (95% confidence limits: 1.59, 5.19; 31 d.f.)-fold and 2.66 (95% confidence limits: 1.14, 9.32; 27 d.f.)-fold respectively to the left (Fig. 4b), whereas endothelin-induced potentiation of field stimulation-induced twitch responses were not significantly affected (Fig. 4a).

3.4. Effect of endothelins and Bay K 8644 on contractile responses evoked by trains of field stimulation at 10 Hz for 10 s in the prostatic segment of the rat vas deferens

As previously reported (Swedin, 1971; Amobi and Smith, 1987), trains of field stimulation applied to the rat vas deferens evoked biphasic contractile responses that comprise an initial fast twitch followed by a slow tonic contraction. The initial twitch occurred within the first 1.5 s of the stimulus train. The tonic phase, slowly developing during stimulation, persisted until the end of the stimulus train. In vehicle control experiments, trains of pulses elicited reproducible contractile responses at 20-min intervals for up to 100 min. Both the early phase and the late plateau of the response to trains of pulses were enhanced in the presence of endothelin-1, endothelin-3 or Bay K 8644 (Fig. 5).

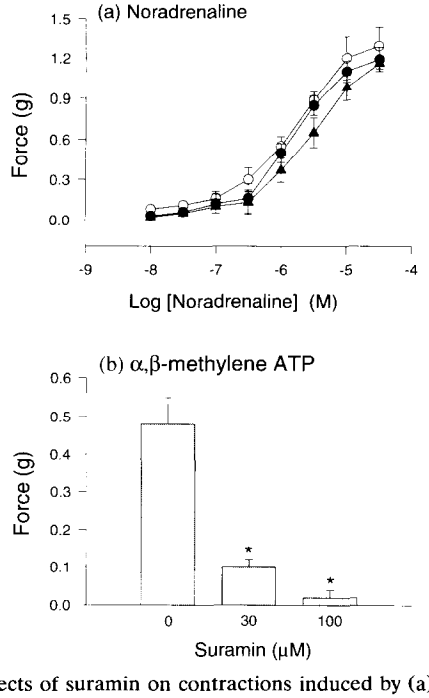

Fig. 7. Effects of suramin on contractions induced by (a) noradrenaline and (b) α,β-methylene ATP in the prostatic segment of the rat vas deferens. In (a), suramin (30 μM, filled circles; 100 μM, filled triangles) was without effect on the log concentration-response curves to noradrenaline. In contrast, in (b), α,β-methylene ATP (1 μM)-induced contractile responses were markedly reduced in the presence of suramin. Data are presented as means ± S.E.M. of three experiments. * $P < 0.01$ indicate significant differences between control (in the absence of suramin) and corresponding value after pretreatment with suramin.

3.5. Effect of suramin on the actions of endothelin-1 and Bay K 8644

Areas under the force-time curve of the first and second phase of the contractile response to trains of pulses (10 Hz for 10 s) applied to the prostatic segment of the rat vas deferens were reduced by 45% and 52%, respectively after pretreatment with 100 μM suramin. The total field stimulation-induced response in the absence and presence of suramin is shown in Fig. 6. Suramin had negligible effect on the endothelin-1-induced increases in basal tone in the prostatic segment of the rat vas deferens. The field stimulation-induced contractions were unaffected by endothelin-1 in the presence of 100 μM suramin (Fig. 6a). In contrast, suramin was without effect on Bay K 8644-induced potentiation of the contractions evoked by trains of stimuli (Fig. 6b). Suramin, at concentrations used in this series of experiments, antagonised contractions induced by α,β-methylene ATP but not those by noradrenaline (Fig. 7).

4. Discussion

In the present study endothelin-1 and endothelin-3, in nanomolar concentrations, were found to potentiate the contractile responses of the prostatic segment of the rat vas deferens evoked by single pulses of electrical field stimulation. They also potentiated both phases of the response of this segment of the vas deferens to trains of field stimulation. A novel finding was that the effects of endothelin-1 were absent in the presence of the P_2-purinoceptor antagonist, suramin (Dunn and Blakeley, 1988; Mallard et al., 1992b). These observations, together with our finding that the endothelins potentiated the effects of ATP and α,β-methylene ATP but not those of noradrenaline on the epididymal segment of the vas deferens, extend and confirm earlier studies of Wiklund et al. (1990) and of Donoso et al. (1992) that indicated that the facilitation of sympathetic neurotransmission in the rat vas deferens by endothelins is due to a selective enhancement of the postjunctional effects of ATP released on field stimulation. They indicate that the potentiation is effected at the P_{2X}-purinoceptor.

An alternative, simpler explanation for the enhancement of twitch responses of the prostatic segment of

the rat vas deferens to field stimulation by endothelin-1 and endothelin-3 would be that these peptides cause a simultaneous increase in basal force, which amplifies the responses to field stimulation. In recent studies, Télémaque and D'Orléans-Juste (1991) and Warner et al. (1993) reported that peptides of the endothelin/sarafotoxin family, although potentiating responses to field stimulation, were without effect on basal force. In the present study, however, reports that endothelin-1 and endothelin-3 can cause contraction of the smooth muscle of the prostatic segment of the vas deferens (Donoso et al., 1992; Eglezos et al., 1993) were confirmed. Endothelin-1 was approximately 3 times more potent than endothelin-3 in producing these effects; the threshold concentrations were only slightly higher than those facilitating neurotransmission. Nevertheless, in our hands the enhancing effects of endothelins on neurotransmission to the rat vas deferens had a slow onset, were long lasting and resistant to thorough tissue washing, whereas the direct effects on basal force were rapid in onset and readily reversed by washing. Similar results have previously been reported by Donoso et al. (1992) who found that removal of calcium from, or addition of nifedepine to, the bathing medium reduced the direct effect on basal force, but was without effect on endothelin-induced potentiation of ATP responses. These authors did not comment on the effects of these procedures on the response to field stimulation.

In our experiments we also examined the effects of the neutral endopeptidase inhibitor, phosphoramidon, on responses of the vas deferens to endothelin-1 and endothelin-3. In a concentration of 10 μM it enhanced the effects of both peptides to cause an increase in basal force, but was without effect on the enhancement these peptides produced of the response to single pulse field stimulation. This differential influence of phosphoramidon on the two types of response was unexpected, but may indicate that these are mediated by different receptor subtypes. In addition to preventing the breakdown of endothelins by neutral endopeptidase (Vijayaraghavan et al., 1990), and the conversion of big endothelin-1 to endothelin-1 (Okada et al., 1990), phosphoramidon has recently been shown to increase endothelin binding site numbers in cultured Swiss 3T3 fibroblasts (Wu-Wong et al., 1993). Compared to endothelin-1, endothelin-3 is a relatively poor substrate for neutral endopeptidase (Vijayaraghavan et al., 1990), in our experiments, however, phosphoramidon potentiated both endothelin-1 and endothelin-3 to a similar extent. Thus it is possible that an action to increase in receptor number, rather than to inhibit neutral endopeptidase, may be the major mechanism by which phosphoramidon potentiated the effects of endothelins on basal force. For this explanation to be plausible, however, it must be assumed that such an effect can occur quickly and that the numbers of those receptor subtypes mediating twitch enhancement are, in contrast to those mediating increases in basal force, not response limiting.

Strong evidence suggesting that the receptor subtype leading to twitch enhancement may differ from that mediating effects on contractile force comes firstly from our finding and those of Wiklund et al. (1990) and Donoso et al. (1992) that endothelins do not potentiate the effects of exogenous noradrenaline, and secondly from the observations of the latter workers that calcium removal and nifedepine abolished the effects of endothelins on basal tone but not the effect to potentiate the actions of ATP. In addition to potentiating the twitch response of the rat vas deferens to single pulse field stimulation, endothelin-1 and endothelin-3 potentiated both components of the biphasic response of the prostatic half of the rat vas deferens evoked by trains of electrical field stimulation at 10 Hz. Both ATP and noradrenaline are now thought to contribute to both components of the biphasic contractions of the vas deferens in response to trains of pulses (Major et al., 1989; Mallard et al., 1992a; Ventura and Pennefather, 1994). The ability of endothelin-1 to potentiate both components of the biphasic response to trains of pulses was absent in the presence of the P_{2X}-purinoceptor antagonist, suramin. In the dosage regime used (100 μM for 1 h), suramin was effective in blocking α,β-methylene-ATP-induced contractions, but had no effect on noradrenaline-induced contractions. Thus we propose that its effect to inhibit endothelin-induced enhancement of the twitch response observed in our experiments was due to a selective action to block the postjunctional effects of ATP released by field stimulation.

Another tool used in these experiments, as in earlier experiments of Shoji and Goto (1990), was the dihydropyridine, Bay K 8644, which is an L-type Ca^{2+}-channel opener. This also induced enhancement of both phases of the biphasic response to trains of field stimulation. In contrast to endothelin-1, however, its effects were not modified by suramin. This observation provides further evidence that the concentration of suramin used in this study is acting selectively at P_2-purinoceptors rather than non-selectively at L-type Ca^{2+}-channels.

Although the effects of endothelins on co-transmitter release remain to be established, our experiments, taken together, afford strong support to the original suggestion of Wiklund et al. (1990) that the mechanism by which endothelins facilitate neurotransmission to the rat vas deferens is by selective enhancement of the postjunctional effects of ATP. In addtion to the facilitatory action of endothelins in co-transmission to the vas deferens, the presence of endothelin-like immunoreactivity in the epithelial cells and the intersti-

tial space of the rat epididymis (Wong et al., 1991) suggests a biological significance for endogenous endothelins in the regulation of male reproduction.

An additional feature of the present study was our use of endothelin-(16–21). This *C*-terminal hexapeptide has been reported, by Maggi et al. (1989a,b), to act as a full agonist at ET_B-receptors present in the prostatic portion of the rat vas deferens. In our experiments it was without effect on either basal force or neurotransmission. While this inactivity might be due in part to its rapid breakdown by peptidases, since inhibitors of these enzymes were not used in our experiments with this peptide, it is of interest that the stable and selective ET_B-receptor agonist, sarafotoxin S6c (Williams et al., 1991) has also been shown to be inactive in this tissue (Eglezos et al., 1993; Warner et al., 1993). These findings support suggestions that receptors other than the ET_B-subtype mediate the potentiation of field stimulation-induced twitches in the rat vas deferens. While our experiments did not directly address the nature of the receptor subtype mediating twitch enhancement in the vas deferens, our findings that endothelin-1 was more potent than endothelin-3, and that endothelin-(16–21) was inactive, are consistent with the studies of Ihara et al. (1992), indicating that the endothelin receptor subtype involved may be of the ET_A-subtype or, as recently proposed by Eglezos et al. (1993), may be of a novel subtype.

Acknowledgements

We are grateful to Chiron Mimotopes and to Bayer for gifts of endothelin-(16–21) and suramin, respectively. This study was funded by the Australian Research Council Grant (Small) to W.A.K.L. and J.N.P. This work had the prior approval of the Monash Standing Committee on Ethics in Animal Experimentation.

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