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The mechanisms of enhancement and inhibition of field stimulation responses of guinea-pig vas deferens by prostacyclin analogues

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1 In the guinea-pig isolated vas deferens preparation bathed in Tyrode's solution, the prostacyclin analogues, cicaprost, TEI-9063, iloprost, taprostene and benzodioxane-prostacyclin, enhanced twitch responses to submaximal electrical field stimulation (20%-EFS). The high potency of cicaprost (EC_{150} = 1.3 nM) and the relative potencies of the analogues (equi-effective molar ratios = 1.0, 0.85, 1.6, 17 and 82, respectively) suggest the involvement of a prostacyclin (IP-) receptor.

2 Maximum enhancement induced by cicaprost in 2.5 mM K^+ Krebs-Henseleit solution was similar to that in Tyrode solution (2.7 mM K^+), but was progressively reduced as the K^+ concentration was increased to 3.9, 5.9 and 11.9 mM. There was also a greater tendency for the other prostacyclin analogues to inhibit EFS responses in 5.9 mM standard K^+ Krebs-Henseleit solution; this may be attributed to their agonist actions on presynaptic EP_3-receptors resulting in inhibition of transmitter release.

3 The EFS enhancing action of cicaprost was not affected by the α_1-adrenoceptor antagonist prazosin (100 and 1000 nM). Cicaprost (20 and 200 nM) did not affect contractile responses of the vas deferens to either ATP (5 μM) or α,β-methylene ATP (1 μM) in the presence of tetrodotoxin (TTX, 100 nM). In addition, enhancement by cicaprost of responses to higher concentrations of ATP (30 and 300 μM) in the absence of TTX, as shown previously by others, was not seen. Prostaglandin E_2 (PGE_2, 10 nM) and another prostacyclin analogue TEI-3356 (20 nM) enhanced purinoceptor agonist responses. Unexpectedly, TTX (0.1 and 1 μM) partially inhibited contractions elicited by 10–1000 μM ATP; contractions elicited by 1–3 μM ATP were unaffected. Further studies are required to establish whether a pre- or post-synaptic mechanism is involved.

4 In a separate series of experiments, cicaprost (5–250 nM), TEI-9063 (3–300 nM), 4-aminopyridine (10–100 μM) and tetraethylammonium (100–1000 μM) enhanced both 20%-EFS responses and the accompanying overflow of noradrenaline to a similar extent. In further experiments with the EP_1-receptor antagonist AH 6809, TEI-3356 (1.0–100 nM) and the EP_3-receptor agonist, sulprostone (0.1–1.0 nM) inhibited both maximal EFS responses and noradrenaline overflow, thus confirming previous reports of the high activity of TEI-3356 at the EP_3-receptor. Cicaprost had no significant effect on noradrenaline overflow at 10 and 100 nM, but produced a modest inhibition at 640 nM.

5 In conclusion, our studies show that prostacyclin analogues (particularly TEI-3356) can inhibit EFS responses of the guinea-pig vas deferens by acting as agonists at presynaptic EP_3-receptors. Prostacyclin analogues (particularly cicaprost and TEI-9063) can also enhance EFS responses through activation of IP-receptors. The mechanism of the enhancement has not been rigorously established but from our results we favour a presynaptic action to increase transmitter release.

Keywords: Guinea-pig vas deferens; prostanoid IP-receptors; prostacyclin analogues; cicaprost; EP_3-receptors; noradrenaline release; K^+-channel blockers; P_{2X}-receptors; tetrodotoxin

Introduction

In the guinea-pig vas deferens, prostaglandin E_2 (PGE_2) suppresses transmitter release resulting from sympathetic nerve stimulation. This has been demonstrated for noradrenaline (NA) (Stjärne, 1973; Hedqvist, 1974), and later for the co-transmitter adenosine 5′-triphosphate (ATP) when the greater postsynaptic release of ATP was suppressed by a combination of the α_1-adrenoceptor antagonist prazosin and the P_{2X}-purinoceptor antagonist suramin (Driessen & Starke, 1994, and references therein). Electrophysiological studies have indicated that PGE_2 inhibits the entry of Ca^{2+} into the sympathetic varicosity (Ito & Tajima, 1979). The prostanoid EP-receptor involved has been characterized as an EP_3 subtype (see Coleman *et al*., 1994 for a review of prostanoid receptors), on the basis of the relative potencies of PGE analogues in inhibiting twitch responses to maximal electrical field stimulation (EFS) (Coleman *et al*., 1987; Lawrence *et al*., 1992). Sulprostone, which is highly potent on the vas deferens (IC_{50} = 0.2 nM), was used as the standard EP_3 agonist in the present study.

Maximal responses to EFS of the guinea-pig vas deferens are also inhibited by stable analogues of prostacyclin, such as carbacyclin (IC_{50} value = 110 nM), iloprost (160 nM) and cicaprost (~1000 nM) (Lawrence *et al*., 1992). It seems likely that these analogues behave as low potency EP_3 agonists in the vas deferens, since they are usually much more potent on prostacyclin (IP-) receptor preparations. However, an IP agonist action may have been responsible for the enhancement of maximal EFS responses (2–25%) that was seen with lower concentrations of cicaprost (5–100 nM) (Jones, 1993). Similar effects were not obtained with carbacyclin and iloprost, possibly because they have lower IP/EP_3 specificities than cicaprost; these analogues are known to have lower IP/EP_1 agonist specificities than cicaprost (Dong *et al*., 1986; Lawrence *et al*., 1992). Since cicaprost did not affect direct contractile responses to exogenous NA, it was tentatively proposed that cicaprost activates presynaptic IP-receptors to enhance transmitter release (Jones, 1993). However, McKay and Poyser (1995) subsequently found that cicaprost (1–100 nM) enhanced the

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contractile action of ATP, but not that of NA, on the vas deferens. These observations are highly relevant since ATP is the transmitter mainly responsible for contractions of the vas deferens elicited by brief sympathetic nerve stimulation (Fedan *et al*., 1981; Sneddon & Westfall, 1984; Kirkpatrick & Burnstock, 1987). McKay and Poyser proposed that the IP-receptors are situated postsynaptically; this would imply an interaction of the IP agonist with the purinergic, but not the noradrenergic, effector system of the vas deferens muscle cell.

In this paper we present data on the excitatory and inhibitory actions of a number of prostacyclin analogues on the guinea-pig vas deferens, with a view of determining the underlying mechanisms. Of particular significance is our failure to observe enhancement of direct purinoceptor-mediated responses by cicaprost.

Methods

Guinea-pig isolated vas deferens preparations

Male Dunkin-Hartley guinea-pigs, weighing 400–450 g, were killed by stunning and exsanguination. Both vasa deferentia were excised and the loose connective tissue removed. Prostatic or epididymal sections (15 mm) were cut and suspended between two stainless steel electrodes under an initial tension of 1.5 g. Tension was recorded with Grass FT03 force-displacement transducers linked to a MacLab data acquisition system (Chart programme, acquisition rate 40 Hz). The bathing fluids used were Tyrode's solution (NaCl 136.9, KCl 2.68, $MgCl_2$ 1.03, $CaCl_2$ 1.77, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, glucose 5.05 mM) and standard Krebs-Henseleit solution (NaCl 118.4, KCl 4.69, $MgSO_4$ 1.18, $CaCl_2$ 2.52, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 10.6 mM), aerated with 95% O_2/5% CO_2; the K^+ concentration was modified by decreasing or increasing the amount of KCl present.

Experimental protocols

All preparations were allowed about 30 min to equilibrate before application of maximal EFS (1 s period of 1 ms square-wave pulses at 60 V and 10 Hz) every 30 s for 30 min. In 20%-EFS studies, the voltage was reduced to give twitch response 20% of maximum. The mean initial 20%-EFS response for each drug test lay between 18 and 25% of maximum (grand mean = 20.7%). Prostanoid doses were added cumulatively and only one compound was tested on each preparation.

For the direct purinoceptor agonist studies in the presence of tetrodotoxin (TTX), maximal EFS responses were first abolished by 100 nM TTX; EFS was stopped and the organ bath washed out; after immediate readdition of TTX, a dose of α,β-methylene ATP (1 μM) or ATP (5 μM) was added and washed out a few seconds after the contraction had begun to wane; this latter procedure was repeated at 7 min intervals. In the case of experiments without TTX, EFS was stopped, the preparation washed and the first dose of ATP given 15 min later. Test drugs were added to the organ bath 3 min before addition of the purinoceptor agonist.

Measurement of noradrenaline overflow

Noradrenaline overflow from the vas deferens was measured by the method of Bourreau (1996). Each preparation consisted of the proximal and distal sections of one vas tied side by side and suspended in Tyrode solution containing indomethacin (10 μM), hydrocortisone (40 μM), desipramine (1 μM) and rauwolscine (10 μM). The EP_1-receptor antagonist AH 6809 (1 μM) was also present throughout the maximal EFS experiments. Muscle tension was recorded as described earlier. During four identical collection periods (S1–S4), separated by 40 min wash periods, bathing fluid was collected after 24 min without field stimulation (basal release), and 4 min after a 20 min period of field stimulation (stimulated release). Increasing concentrations of test compound were added at the beginning of the field stimulation periods in S2, S3 and S4.

3,4-Dihydroxybenzylamine (3.0 ng, internal standard) and 1 ml Tris buffer were added to the collected bathing fluid, which was then shaken with 50 mg acid-washed alumina for about 10 min. The alumina was allowed to settle, the supernatant discarded, and the alumina washed with distilled water and aspirated to dryness. Distilled water (1 ml) was added to the alumina and the slurry was transferred to a microfilter tube. The alumina was centrifuged at 2000 *g* for 1 min. After mixing 0.12 ml 0.1 M perchloric acid with the dry alumina, the acidic extract was then collected into a new receiver tube by centrifuging at 2000 *g* for 30 s. NA in the extract was assayed chromatographically by passing the effluent from a Bio-Rad AS-100 high performance liquid chromatography (h.p.l.c.) column eluted at 0.8 ml min^{-1} with methanol-water 1 : 10, containing 230 mM acetic acid, 50 mM sodium acetate, 0.5 mM EDTA and 0.17 mM sodium octylsulphate, through an electrochemical detector (Bioanalytical System LC 4C). NA release (stimulated minus basal) was expressed as pg ml^{-1} mg^{-1} dry tissue weight.

Data analysis

For the EFS studies, the mean of the 4 twitch responses immediately preceding the first prostanoid dose was taken as the control measurement (100%); comparisons were made with the means of the last 4 twitch responses during each prostanoid application. For the purinoceptor agonist studies, each response was compared to the mean (= 100%) of the responses to applications 3, 4 and 5 of the purinoceptor agonist.

In each NA overflow experiment, twitch tension and NA overflow (stimulated minus basal) obtained during S2, S3 and S4 were expressed relative to S1 (= 100%). Statistical analyses were performed on absolute values of twitch tension and NA overflow by Repeated Measures Two-Factor ANOVA (4 dose/5 treatment levels for 20%-EFS and 4 dose/4 treatment levels for maximal EFS) by use of SuperANOVA software (Abacus Concepts Inc., CA). Planned contracts (Glass & Hopkins, 1984) were used to compare S1 means with S2, S3 and S4 means. ATP log concentration-response curves were analysed similarly (7 dose/2 treatment levels).

Drugs

The following prostanoids were gifts: cicaprost, iloprost and sulprostone from Schering AG, Berlin, Germany; TEI-9603 ($\Delta^{6(6a)}$-6a-carba-17α,20-dimethyl PGI_1) and TEI-3356 ($\Delta^{6(6a)}$-6a-carba-14a-homo-15β-methyl PGI_1) from Teijin Pharmaceuticals (Tokyo, Japan), taprostene (sodium salt) from Grunenthal GmbH (Germany); benzodioxane-prostacyclin ([1**R**,2**S**,3a**R**,9a**S**]-2-hydroxyl-1-[3-hydroxy-1-octenyl]-2,3,3a,9a-tetrahydro-1H-cyclo-penta[b][1.4]benzodioxin-5-yl]oxyacetic acid) from Shionogi Research Laboratories (Osaka, Japan). AH 6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) was a gift from Glaxo Wellcome (U.K.). 4-Aminopyridine, desipramine hydrochloride, 3,4-dihydroxybenzylamine, hydrocortisone, noradrenaline bitartrate and tetrodotoxin were obtained from Sigma Chemical Co. (U.S.A.), tetraethylammonium chloride from E. Merck (Germany); PPADS (pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium) and rauwolscine hydrochloride from Research Biochemicals Inc. (U.S.A.) and suramin sodium from Sapphire Bioscience (Australia).

Results

Both epididymal and prostatic portions of each vas deferens were used and, although absolute strengths of contractions (to EFS, P_{2X} agonists, etc.) were greater in the former, normalized responses to enhancing or inhibitory agents were very similar on both portions. Consequently, each data set represents ex-

periments on equal numbers of prostatic and epididymal preparations, unless otherwise stated.

Optimization of enhancement of EFS responses and estimation of relative potencies of prostacyclin analogues

Initial experiments were conducted with the vas deferens bathed in standard Krebs-Henseleit solution aerated with 95% O_2/5% CO_2. Reduction of the EFS voltage to give progressively smaller twitch reponses resulted in greater enhancement by cicaprost, measured on a percentage basis. However, twitch responses less than 15% of maximum were sometimes erratic and it was decided to use 20% maximal EFS (20%-EFS) responses routinely. The enhancing action of cicaprost showed good consistency between different preparations; threshold responses were induced by 1 nM cicaprost and maximum enhancement of about 75% above control was obtained at 20–40 nM (Figure 1a). However, it was disappointing to find that a second IP-receptor agonist, taprostene (10–400 nM) (Flohé *et al*., 1983; Michel & Seipp, 1990), induced only a slight enhancement, and a third IP agonist, benzodioxane-prostacyclin (BDP, 10–400 nM) (Mori & Takechi, 1990), produced only inhibition (Figure 1a). As expected, the EP_3 agonist sulprostone was a highly potent inhibitor of 20%-EFS responses (IC_{50} = 0.07 nM) (Figure 1a).

At this stage in the study, it was found by chance that bathing the vas deferens in Tyrode's solution (95% O_2/5% CO_2) resulted in much greater enhancement of 20%-EFS responses by cicaprost, with a maximum response about 200% above control (Figure 1b). Initial maximal EFS responses were similar in Krebs-Henseleit (5.60 ± 0.27 g, n = 22) and Tyrode's (5.57 ± 0.48 g, n = 14) solutions. The isocarbacyclin TEI-9063 (Negishi *et al*., 1991) was marginally more potent than cicaprost, with a similar log concentration-response profile (data not shown). Iloprost, taprostene and BDP were successively less potent enhancers and had lower maximum responses than cicaprost, and with the exception of taprostene, showed bell-shaped log concentration-response curves. The remaining prostacyclin analogue examined, TEI-3356 (0.3–70 nM), never showed any potentiating action and, indeed, was a moderately potent inhibitor of 20% EFS responses (IC_{50} = 12 nM) (Figure 1b). Sulprostone was a slightly less potent inhibitor in Tyrode's solution (IC_{50} = 0.25 nM) compared to when in Krebs-Henseleit solution.

Effect of K^+ concentration on cicaprost enhancement

In view of the different responses to prostacyclin analogues in standard Krebs-Henseleit and Tyrode solutions, the effect of modifying the K^+ concentration of Krebs-Henseleit solution on the enhancing activity of cicaprost was investigated. As shown in Figure 1c, enhancing activity diminished as the K^+ concentration was increased from 2.5 to 5.9 mM. With 11.9 mM K^+ in the bathing solution, 20%-EFS contractions were erratic and, although cicaprost had no obvious effect at the lower cicaprost concentrations and some inhibition with

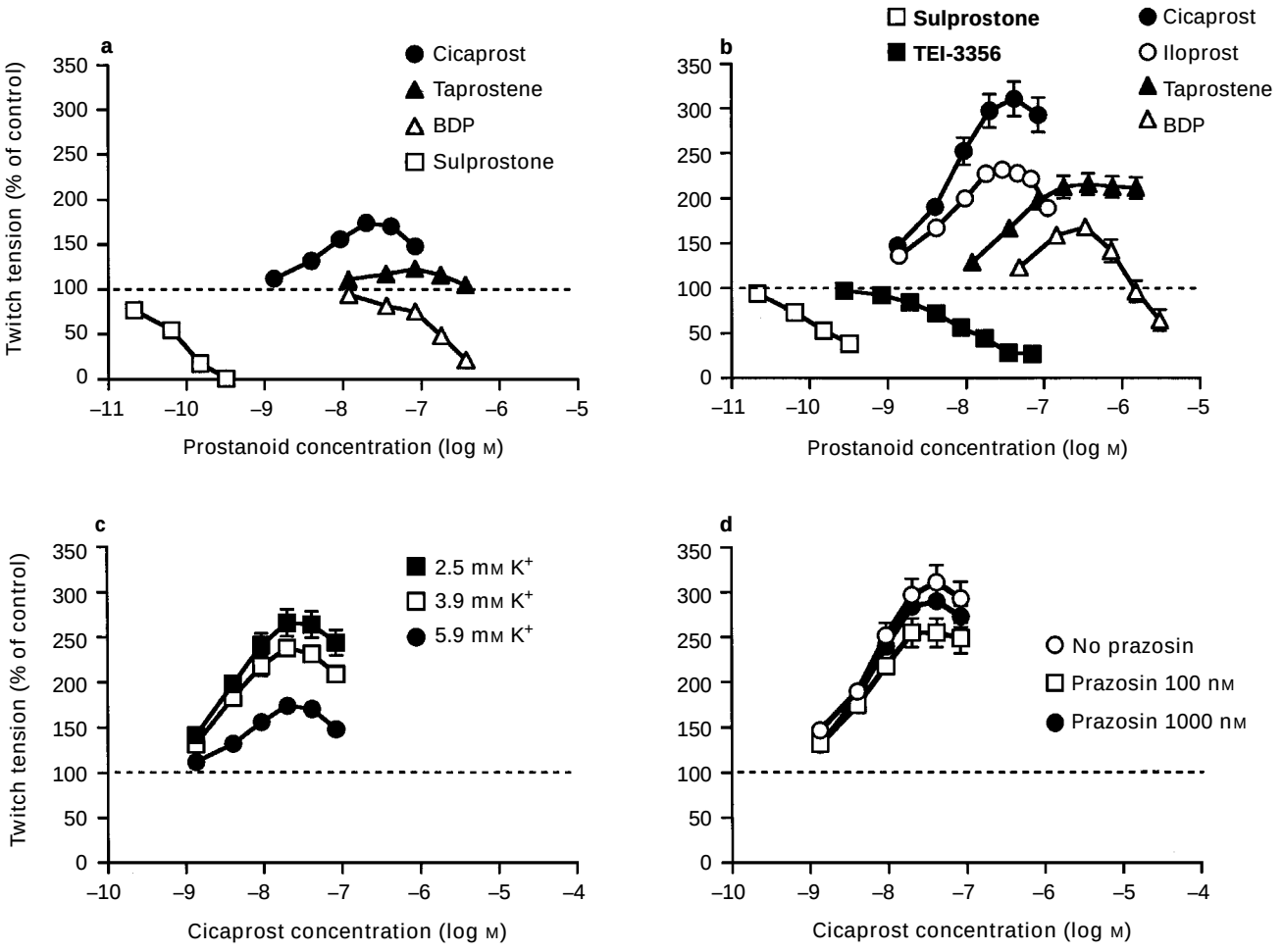

Figure 1 Effects of prostanoids on 20%-EFS contractions of guinea-pig vas deferens: Log concentration-response curves for (a) cicaprost (n = 4), taprostene (n = 10) benzodioxane-prostacyclin (BDP, n = 4) and sulprostone (n = 6) in standard (5.9 mM K^+) Krebs-Henseleit solution; (b) sulprostone (n = 6), TEI-3356 (n = 6), cicaprost (n = 10), iloprost (n = 16), taprostene (n = 10) and BDP (n = 4) in Tyrode solution; (c) cicaprost in Krebs-Henseleit solutions containing 2.5 (n = 12), 3.9 (n = 10) and 5.9 (standard) (n = 22) mM K^+; (d) cicaprost acting alone and in the presence of 100 nM and 1000 nM prazosin (all n = 6). Values are means and vertical lines show s.e.mean; error bars when not shown fall within the limits of the symbol.

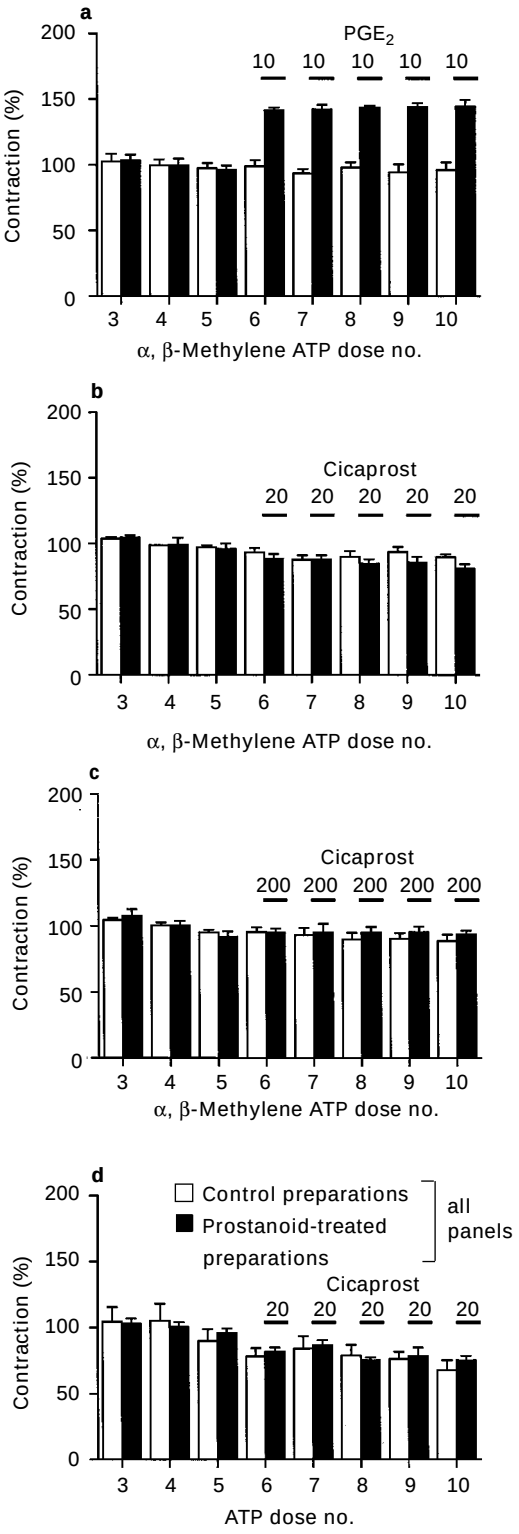

Figure 2 Guinea-pig vas deferens: effects of (a) PGE_2 and (b and c) cicaprost on contractions elicited by consecutive doses of 1 μM α,β-methylene ATP, and (d) cicaprost on contractions elicited by 5 μM ATP (prostanoid concentrations in nM). TTX (100 nM) was present in all tests. In each panel, contraction is expressed relative to the mean response for doses 3, 4 and 5 (=100%); error bars indicate s.e.mean (all $n=4$).

the two highest concentrations, we felt that the data were not reliable enough to be published. Initial maximal EFS responses were 6.65 ± 0.36, 5.00 ± 0.23, 5.60 ± 0.27 and 8.08 ± 0.61 g for the Krebs-Henseleit solutions with increasing K^+. The cicaprost log concentration-response curve obtained in Tyrode solution (2.7 mM K^+) was similar to that obtained in 2.5 mM K^+ Krebs-Henseleit solution. All subsequent experiments were performed with Tyrode solution.

Effects of prazosin on cicaprost enhancement

Cicaprost still produced marked enhancement of 20%-EFS responses in the presence of the α_1-adrenoceptor antagonist prazosin at 100 nM and 1 μM (Figure 1d).

Effect of prostanoids on contractile responses to purinoceptor agonists

Initially, the effects of prostanoids were examined on the transient contractions of the vas deferens induced by the selective P_{2X}-receptor agonist, α,β-methylene ATP (α,β-meATP, 1 μM). TTX (100 nM) was included in the bathing fluid to diminish potential stimulant effects on neuronal elements in the preparation. The second α,β-meATP response was usually about 75% of the first response, but afer that only a slow decline was seen. PGE_2 (10 nM), added 3 min before the α,β-meATP dose, potentiated the response and this effect was well maintained for 35 min (Figure 2a). TEI-3356 (20 nM) behaved similarly, producing about 30% enhancement. In contrast, cicaprost at 20 and 200 nM did not potentiate the contractions to α,β-meATP (Figure 2b,c). The P_{2X}-receptor antagonists PPADS (10 and 30 μM) and suramin (30 and 100 μM) inhibited the responses to α,β-meATP by 61 and 80% and by 79 and 97%, respectively (calculated with respect to α,β-meATP doses 7/8 and 9/10 on control preparations). 4-Aminopyridine (4-AP, 100 μM) only slightly enhanced the responses to α, β-meATP (13%), whereas $BaCl_2$ (100 μM) produced 70% enhancement (data not shown). By use of a similar protocol, contractions to 5 μM ATP (about 75% of 1 μM α,β-meATP contractions) were not affected by cicaprost at 20 and 200 nM (Figure 2d).

Following the study of McKay and Poyser (1995) showing enhancement of ATP contractions by cicaprost, we decided to perform further experiments with conditions closer to their conditions of high ATP concentration (140–360 μM) in the absence of TTX. Tachyphylaxis to the high doses of ATP was minimized by first obtaining reproducible contractions to 5 μM ATP challenges. Cicaprost at 20 nM did not enhance contractions to 300 μM ATP on either epididymal or prostatic preparations (Figure 3a,b). However, since the response to 300 μM ATP was quite large and the log concentration-response curve showed a tendency to plateau in that concentration range (Figure 4), we felt that the conditions used may not have been ideal for demonstrating enhancement. Consequently we performed another series of experiments with 30 μM ATP (TTX absent), which gave a response of about 50% of that elicited by 1000 μM ATP and similar to that obtained by McKay and Poyser (35–55%). Again, we could not detect any enhancement by cicaprost (20 and 200 nM), whereas 10 nM PGE_2 showed a distinct enhancement (Figure 3c,d).

At the time of these experiments, it was found that TTX had an inhibitory effect on contractile responses of the vas deferens to ATP. In the 300 μM ATP experiments, 100 nM TTX produced about 50% inhibition on the epididymal preparations and the effect was readily reversible (Figure 3a). A similar profile was obtained with 1 μM TTX on the prostatic preparations (Figure 3b). Subsequently, the effect of 1 μM TTX on the concentration-response relationship for ATP was determined (Figure 4). There appeared to be two components to the ATP curve, with the less sensitive component being TTX-sensitive.

Effect of prostanoids on EFS-induced noradrenaline release

In a separate series of experiments, 'double vas preparations' were set up in Tyrode solution for recording of muscle tension

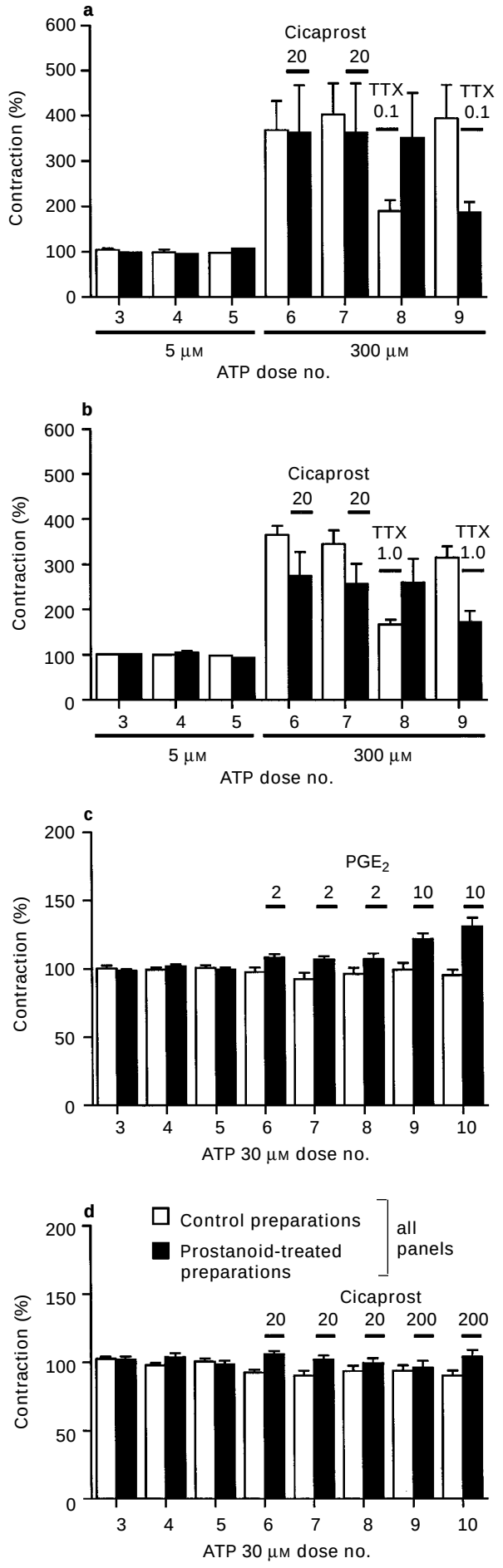

Figure 3 Guinea-pig vas deferens: effects of cicaprost on contractions of (a) epididymal and (b) prostatic preparations ($n=4$) elicited by 300 μM ATP, and the effects of PGE_2 (c) and cicaprost (d) on contractions of epididymal/prostatic preparations ($n=10$) elicited by 30 μM ATP (prostanoid concentrations in nM). Also shown in (a) and (b) are the rapidly reversible partial inhibition of ATP contractions by TTX (μM). In each panel, contraction is expressed relative to the mean of ATP doses 3, 4 and 5 (=100%); error bars indicate s.e.mean.

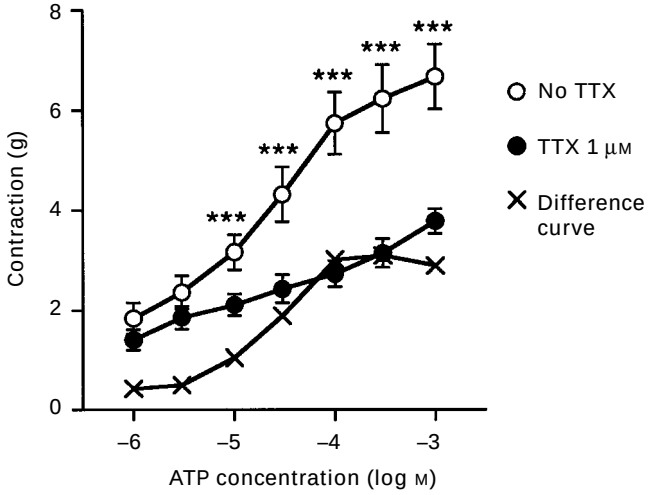

Figure 4 Log concentration-response curves for contraction of guinea-pig vas deferens by ATP in the absence and presence of TTX (1 μM); the difference curve shows the TTX-sensitive component. Values are means and vertical lines show s.e.mean ($n=7$). Data were analysed by 2-Factor ANOVA. ***$P<0.001$ for absence versus presence of TTX.

and collection of bathing fluid for measurement of NA overflow; prostanoid biosynthesis was inhibited by indomethacin (10 μM), NA uptake inhibited by hydrocortisone (40 μM) and desipramine (1 μM), and α_2-adrenoceptors blocked by rauwolscine (10 μM). NA release (stimulated minus basal) corresponding to four consecutive periods of EFS (S1–S4) was determined by an h.p.l.c./electrochemical method. Increasing concentrations of test drug were applied during the S2, S3 and S4 EFS periods and results are expressed as percentage of the corresponding S1 values.

In experiments involving 20%-EFS responses (Figure 5a,c), there was a trend towards increased NA release during the second and third periods in the control group, but this did not reach statistical significance (S1 vs S2, $P=0.074$; S1 vs S3, $P=0.077$; S1 vs S4, $P=0.68$). Cicaprost (5–250 nM), TEI-9063 (3–300 nM), 4-AP (10–100 μM) and TEA (100–1000 μM) significantly enhanced NA release, and this correlated well with their abilities to enhance the twitch response.

In experiments involving maximal EFS responses (Figure 5b,d), the EP_1-receptor antagonist AH 6809 (1 μM) was present throughout. Sulprostone (0.1–1.0 nM) and TEI-3356 (1–100 nM) inhibited NA release, in parallel with their abilities to inhibit twitch responses. Cicaprost at 10 and 80 nM had no effect on either NA release or twitch responses. However, 640 nM cicaprost significantly reduced both NA release (S1 vs S4, $P<0.02$) and twitch responses (S1 vs S4, $P<0.02$).

Discussion

Characterization of prostanoid receptors in the guinea-pig vas deferens

The differing profiles of the prostacyclin analogues under investigation are explainable on the basis of different agonist

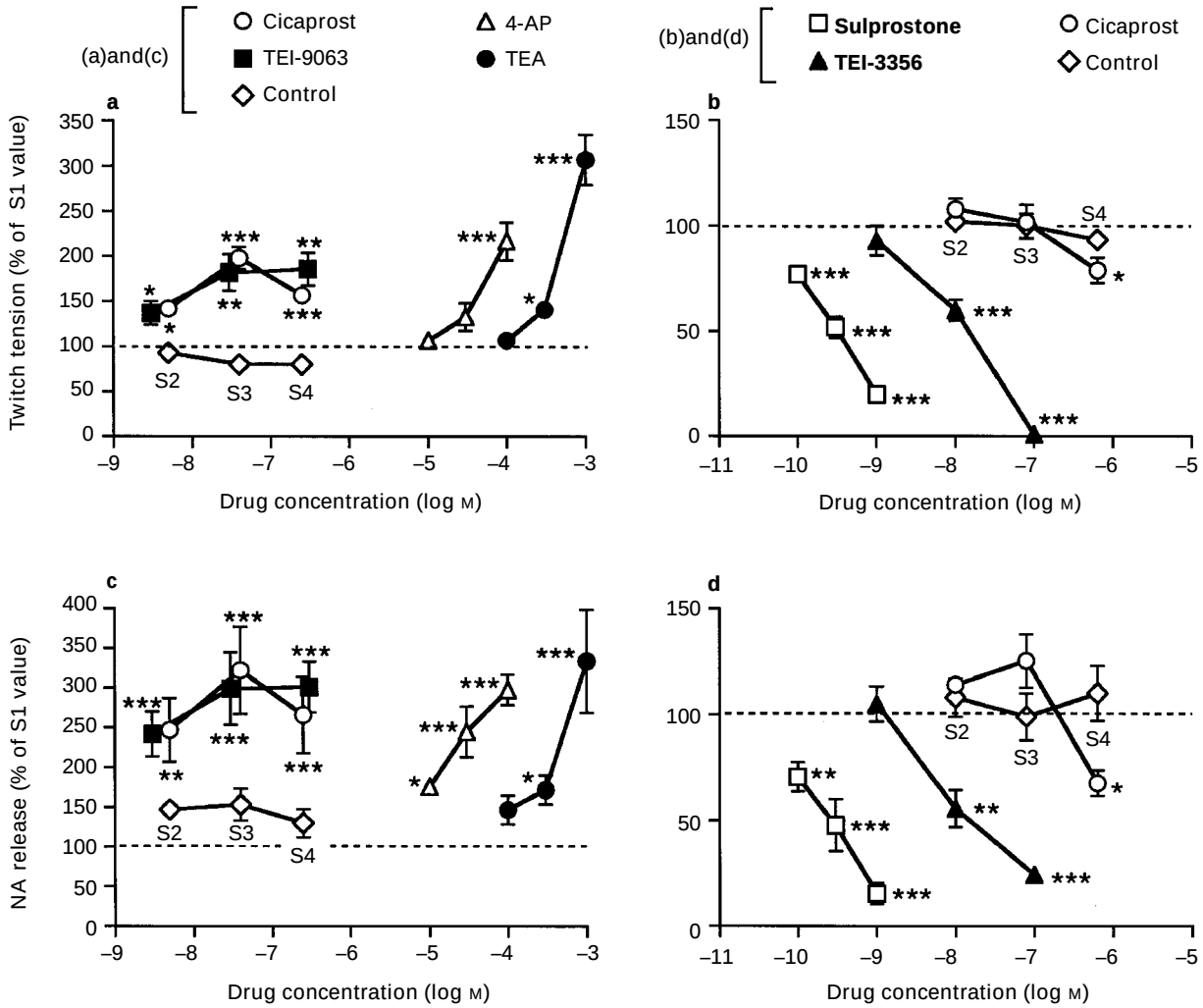

Figure 5 Effects of prostanoids and K^+ channel blockers on contractions and associated NA release elicited by 20%- (a and c) and maximal (b and d) EFS in guinea-pig vas deferens. Values are expressed relative to S1 (=100%) and show means with vertical lines indicating s.e.means ($n=6$). In each panel, control group values are aligned with the cicaprost values only. Statistical comparisons with S1 values by 2-Factor ANOVA: $*P<0.05$, $**P<0.01$, $***P<0.001$.

potencies at excitatory and inhibitory prostanoid receptors within the vas deferens. Thus we suggest that cicaprost, which showed the greatest enhancement of electrically-evoked contractions, acts predominantly at IP-receptors. Its activity in the low nanomolar concentration range is in agreement with its high potency in many other IP-receptor systems (Skuballa *et al*., 1986; Stürzebecher *et al*., 1986; 1988; Dong *et al*., 1986; Armstrong *et al*., 1989). The measurement of the relative EFS-enhancing potencies of the other analogues is made difficult by their lower maxima and also their tendency to produce inhibition of EFS responses with increasing concentration. However, using the concentration required to increase 20%-EFS responses by 50% (EC_{150}) as a measure of potency, we found that their relative potencies are in good agreement with those obtained for other IP-receptor preparations (Table 1). The remaining analogue TEI-3356 is a low potency IP-agonist on the human pulmonary artery (Jones *et al*., 1997) (Table 1) and this would fit with its apparent lack of EFS enhancing activity.

Inhibition of EFS responses by the prostacyclin analogues is likely to be due to activation of EP_3-receptors. TEI-3356 produced a monophasic inhibition curve and was about 50 fold less potent than sulprostone. From our previous studies (Lawrence *et al*., 1992), this would give TEI-3356 an EP_3 agonist potency only 5–10 times lower than that of PGE_2. TEI-3356 has been shown to have high affinity ($IC_{50}\sim0.5$ nM) for mouse EP_3-receptors expressed in Chinese hamster ovary (CHO) cells, whereas for expressed mouse EP_1- and EP_2-receptors its IC_{50}s were about 1 and 10 μM respectively (Negishi *et al*., 1994). The other prostacyclin analogues would appear to be much less potent as EP_3 agonists. On the rat vena cava, iloprost inhibits EFS-induced NA release with an IC_{40} value of 1350 nM, compared with an IC_{40} of 14 nM for sulprostone (Molderings *et al*., 1992). By analogy, in the guinea-pig vas deferens, which is much more sensitive to EP_3 agonist action than the rat vena cava, the reversal of EFS enhancing action of iloprost above 50 nM is consistent with an EP_3 agonist potency some two orders of magnitude less than sulprostone. The binding IC_{50} for iloprost in CHO cells expressing a mouse EP_3-receptor isoform ($EP_{3\gamma}$) was about 300 nM (Irie *et al*., 1993). At concentrations of 100 nM and above, cicaprost also probably has EP_3 agonist activity. However, at the present time it appears to be the best standard IP-receptor agonist available.

Location of prostanoid receptors

There seems little doubt that the inhibitory EP_3-receptors in the vas deferens are situated on the sympathetic varicosities. Thus there was a good correlation between inhibition of maximal EFS response and inhibition of NA overflow for both sulprostone and TEI-3356. Although there is no evidence for an EP_1-receptor system in the guinea-pig vas deferens, the high EP_1 agonist potency of some prostacyclin analogues (Dong *et al*., 1986; Lawrence *et al*., 1992) and of the standard EP_3 agonist sulprostone (Coleman *et al*., 1987) prompted us to conduct these experiments in the presence of the EP_1-receptor antagonist AH 6809 (Coleman *et al*., 1985).

Table 1 Comparison of potencies of prostacyclin analogues on IP-receptor preparations

	Equi-effective molar ratios			*IC_{50}* (nM)		
Prostanoid	*Guinea-pig vas deferens*	*Human pulmonary artery*	*Human platelet membranes*	*Human platelets (PRP)*	*Rabbit platelets (PRP)*	*Mouse mastocytoma P-815 cells*
Cicaprost	1.0	1.0	1.0	0.64	–	–
TEI-9063	0.85	0.71	0.13	–	–	40
Iloprost	1.6	2.4	0.54	0.84	13.5	100
Taprostene	17	23	6.5	17	–	–
BDP	82	380	72	–	1350	–
TEI-3356	–*	2370**	138	210	–	–

Guinea-pig vas deferens: enhancement of 20% EFS responses; cicaprost $EC_{150} = 1.3$ nM; *inhibition of EFS responses only (data from this study). Human pulmonary artery: relaxation of phenylephrine-induced tone; cicaprost $IC_{50} = 0.6$ nM; **IP agonist potency likely to be underestimated owing to opposing EP_3 contractile activity (Jones *et al*., 1997). Human platelet membranes: competition for [^{3}H]-iloprost binding; cicaprost $K_i = 22$ nM (Jones *et al*., 1997). Human platelet-rich plasma (PRP): inhibition of aggregation induced by ADP (Skuballa *et al*., 1986; Jones, unpublished observations for TEI-3356) or arachidonic acid (Flohé *et al*., 1983). Rabbit platelet-rich plasma: inhibition of aggregation induced by ADP (Lidbury *et al*., 1989; Mori & Takechi, 1990). Mouse mastocytoma P-815 cells: inhibition of thrombin-induced increase in $[Ca^{2+}]_i$ (Negishi *et al*., 1991).

The location of the proposed IP-receptor within the vas deferens is open to debate. McKay and Poyser (1995) concluded that it is postsynaptic, on the basis that cicaprost enhanced contractions elicited by ATP over the same concentration range that it enhanced EFS responses. Thus, in the absence of TTX, cicaprost enhanced ATP (140–360 μM) contractions by 12% at 2.7 nM, 55% at 27 nM and 73% at 109 nM. This enhancement of ATP contractions is highly relevant since purinergic transmission is the major component of brief EFS responses, and we have also shown that EFS enhancement of cicaprost is unchanged by high concentrations of the α_1-blocker prazosin. In agreement with our findings (Jones, 1993), McKay and Poyser found that NA contractions were not enhanced by cicaprost; consequently their 'postsynaptic hypothesis' requires that activation of IP-receptors specifically augments the purinergic contractile system. However, we were unable to obtain enhancement of purinergic contractions by cicaprost. This was the case with low concentrations of ATP (5 μM) and α,β-meATP (1 μM) in the presence of TTX, where activation of P_{2X}-receptors is the dominant postsynaptic mechanism (see later discussion). Also, in the absence of TTX, we saw no enhancement of contraction induced by either 30 μM ATP, which matches the contraction size obtained by McKay and Poyser, or 300 μM ATP, which is within their concentration range. The authenticity of our cicaprost solutions is not in doubt, since regular testing of these solutions on the human isolated pulmonary artery elicited relaxation at subnanomolar concentrations. At this time, we are unable to account for the differences between our findings and those of McKay and Poyser.

PGE_2 enhanced purinoceptor agonist contractions of the vas deferens, an effect that has been obtained previously (Ellis & Burnstock, 1990; McKay & Poyser, 1995). TEI-3356 also enhanced α,β-meATP contractions, which when taken together with the enhancement of ATP contractions by sulprostone found by McKay and Poyser (1995) would support their proposal for the presence of postsynaptic EP_3-receptors. The postsynaptic EP system is about 10 times less sensitive than the presynaptic EP_3 system (threshold effects for PGE_2 being elicited at 0.1–0.3 nM and 1–3 nM, respectively) and we might perhaps expect EFS inhibition for PGE_2 to reverse as its concentration is increased. This has been seen in some studies (Dreissen & Starke, 1994), whereas monophasic EFS inhibition curves were obtained in others (Lawrence *et al*., 1992; McKay & Poyser, 1995).

As McKay and Poyser (1995) have pointed out, the measurement of ATP release from the vas deferens should help to define the mechanism of the EFS enhancing action of IP agonists. We have experimented with both luciferin-luciferase and fluorescence derivatization h.p.l.c. assays for ATP, but the amounts of ATP collected under conditions where cicaprost enhancement is most apparent (low intensity, low frequency EFS) were too low to be reliably measured. We had realised from the beginning that the development of a sound ATP assay would not be an easy task and had consequently attempted to define an optimum experimental protocol by first measuring NA release with an assay developed by one of us for studying transmitter function in the rat vas deferens (Bourreau, 1996). As reference agents, we used 4-AP and TEA, which by blocking neuronal K^+ channels prolong the action potential, increasing intraneuronal Ca^{2+} and enhancing transmitter release (Johns *et al*., 1976; Stjärne *et al*., 1989; Allgaier *et al*., 1993; Docherty & Brady, 1995). 4-AP enhanced both NA overflow and EFS responses, while having little effect on α,β-meATP-induced contractions, indicating a predominantly presynaptic action. Importantly, our results show that IP-receptor agonists increase NA release over the same concentration range and under the same conditions that they enhance 20%-EFS contractions. This strongly supports a presynaptic location of IP-receptors in the guinea-pig vas deferens.

Interaction of TTX and purinoceptor agonists

TTX was originally included in the purinoceptor agonist experiments in an attempt to restrict the interaction of cicaprost and the purinoceptor agonist to smooth muscle cells of the vas. We were concerned that the purinoceptor agonist could also have a stimulant action on sympathetic neuronal elements and if this action were to be enhanced (presynaptically) by cicaprost, the mechanism would be misinterpreted as a postsynaptic IP action. Of course, the presence of TTX does not entirely preclude neuronal activation since Ca^{2+} entry into the varicosities can still occur in the absence of functional Na^+-channels (Kirpekar & Prat, 1978). However, our finding that ATP contractions are partially inhibited by TTX caused us to consider another possibility: ATP may act at two postsynaptic purinoceptors, one of which is linked to a Na^+-channel that is blocked by TTX. It has been proposed previously that ATP induces contraction of the guinea-pig vas deferens by two distinct mechanisms (Fedan & Lamport, 1990, and references therein). The more sensitive mechanism (0.1–3 μM ATP) appears to involve the classical P_{2X}-receptor, being blocked by the specific P_{2X}-receptor photoaffinity antagonist $ANAPP_3$. The less sensitive mechanism (3–10,000 μM ATP) is responsible for the more prolonged component of the ATP response and is blocked by ATP-2′,3′-dialdehyde (P-ATP). In addition, it has been recently found that PPADS and suramin block contractile responses of the guinea-pig vas deferens to the ATP analogues AP_4A and AP_5A, whereas only PPADS was effective against (300 μM) ATP (Westfall *et al*., 1996). The relationships of these P-ATP-sensitive, suramin-insensitive and TTX-sensitive purinergic mechanisms is not clear and further studies are required. However, it is of interest that action potentials that are highly sensitive to TTX ($IC_{50} \sim 10$ nM) can be elicited in

several isolated smooth muscle cells, including those of guinea-pig ureter (Muraki *et al*., 1991) and mouse vas deferens (Holman *et al*., 1995). The function of the proposed Na^+-channels may be to accelerate the generation of action potentials by triggering the more slowly activating Ca^{2+} current (Muraki *et al*., 1991).

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