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The effect of P2 receptor antagonists and ATPase inhibition on sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens

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1 Intracellular microelectrodes were used to record the transmembrane potential and excitatory junction potentials (e.j.p.s) produced by sympathetic nerve stimulation (1 Hz) in smooth muscle cells of the guinea-pig isolated vas deferens.

2 The symmetrical 3′-urea of 8-(benzamido)naphthalene-1,3,5-trisulphonic acid (NF023) produced a concentration-dependent inhibition of e.j.p. magnitude ($IC_{50} = 4.8 \times 10^{-6}$ M), but had no effect on the resting membrane potential of the smooth muscle cells.

3 Pyridoxal-5-phosphate (P-5-P) also depressed e.j.p. magnitude in a concentration-dependent manner, but was less potent than NF023 ($IC_{50} = 2.2 \times 10^{-5}$ M). At 10^{-4} M and above P-5-P significantly depolarized the smooth muscle cells.

4 The nucleoside triphosphatase inhibitor 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156) (5×10^{-5} M) significantly increased e.j.p. amplitude. ARL 67156 (10^{-4} M) further increased e.j.p. amplitude such that they often reached threshold for initiation of action potentials, causing muscle contraction and expulsion of the recording electrode.

5 After reduction of e.j.p.s by NF023 or P-5-P (both 10^{-5} M), subsequent co-addition of ARL 67156 (10^{-4} M) significantly increased their magnitude.

6 The overflow of endogenous ATP evoked by field stimulation of sympathetic nerves (8 Hz, 1 min) was measured by HPLC and flurometric detection. ARL 67156 (10^{-4} M) enhanced ATP overflow by almost 700% compared to control.

7 We conclude that for electrophysiological studies NF023 is preferable to other P2X receptor antagonists such as pyridoxalphosphate -6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin or P-5-P. Furthermore, breakdown of endogenous ATP by nucleoside triphosphatases is an important modulator of purinergic neurotransmission in the guinea-pig vas deferens.

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Abbreviations: α,β-meATP, α,β-methyleneATP; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; $ANAPP_3$, arylazidoaminopropionyl-ATP; ARL 671556, 6-N,N-diethyl-D-β,γ-dibromomethyleneATP; ATP, adenosine 5′-triphosphate; e.j.p.s, excitatory junction potentials; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-napthalenetrisulphonic acid) P-5-P, pyridoxal-5-phosphate; PPADS pyridoxalphosphate -6-azophenyl-2′,4′-disulphonic acid

Introduction

Considerable evidence has been accumulated to support the proposal by Burnstock (1972) that adenosine 5′-triphosphate (ATP) is an important neurotransmitter in a wide variety of tissues. In autonomic nerves innervating smooth muscle, ATP often acts as a cotransmitter with noradrenaline or acetylcholine (for reviews see Burnstock, 1990; Sneddon *et al*., 1996). For example, when ATP and noradrenaline are released as cotransmitters from sympathetic nerves in the guinea-pig vas deferens, ATP acts at $P2X_1$ receptors to elicit excitatory junction potentials (e.j.p.s), which in turn initiate action potentials and the predominantly purinergic, phasic component of the neurogenic contraction (Sneddon *et al*., 1982; 1996; Sneddon & Westfall, 1984). Noradrenaline does not contribute to e.j.p.s, but produces the smaller, predominantly noradrenergic, tonic phase of the contraction.

We have previously shown that e.j.p.s in guinea-pig vas deferens are blocked by $ANAPP_3$ (Sneddon *et al*., 1982), α,β-methyleneATP (α,β-meATP) (Sneddon & Burnstock, 1984), suramin (Sneddon, 1992) and PPADS (pyridoxalphosphate -6-azophenyl-2′,4′-disulphonic acid) (McLaren *et al*., 1994), but not by α-adrenoceptor antagonists. These studies showed that measuring e.j.p.s is a particularly useful method of monitoring $P2X_1$ receptor-mediated responses, since e.j.p.s are produced solely by ATP, whereas the mechanical responses to nerve stimulation are composed of two phases, each of which has some contribution from both transmitters (for details see Amobi & Smith, 1990).

Despite much research into P2X receptor antagonists, none of the available compounds is ideal, particularly for electrophysiological studies. For example, suramin and PPADS are two of the most popular agents, but suramin has low potency, is very slow to equilibrate (over 30 min) and is practically irreversible (Sneddon, 1992). PPADS is more

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potent and reversible, but we found that it not only blocked e.j.p.s but also produced substantial depolarization of smooth muscle cells in guinea-pig vas deferens (McLaren *et al*., 1994). The symmetrical 3′-urea of 8-(benzamido)naphthalene-1,3,5-trisulphonic acid, (NF023) and P-5-P (pyridoxal-5-phosphate) are two compounds which have been shown to produce selective antagonism of P2X receptor-mediated contractions of various smooth muscles, including vas deferens (Trezise *et al*., 1994; Bültmann *et al*., 1996; Lambrecht, 1996). Therefore, the first aim of this study was to examine the effects of these two compounds on membrane potential and e.j.p. magnitude in vas deferens to determine if their inhibitory properties at the electrophysiological level makes them more useful P2X receptor antagonists than the previously studied compounds.

The postjunctional actions of ATP are curtailed by its sequential degradation to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine by ectonucleotidases. The terminal phosphate group of ATP can be removed by ectonucleoside triphosphatases such as ectoATPase and apyrase. These are membrane bound enzymes that hydrolyse ATP on the outer surface of many cell types, including smooth muscle. (For reviews see Plesner, 1995; Zimmermann, 1996; 1999).

6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156) inhibits the breakdown of ATP with a pIC_{50} of 4.62 in human blood cells (Crack *et al*., 1995) and 5.1 in smooth muscle membrane vesicles isolated from the rat vas deferens (Khakh *et al*., 1995). Recently we showed that ARL 67156 also inhibits the ATPase activity released into the perfusate from guinea-pig vas deferens during sympathetic nerve stimulation (Todorov *et al*., 1997). ARL 67156 enhances contractions of the rabbit ear artery evoked by exogenous ATP, but not responses to the stable analogue α,β-meATP, (Crack *et al*., 1995). It also enhances neurogenic contractions of the guinea-pig vas deferens and those evoked by exogenous ATP, but not α,β-meATP (Westfall *et al*., 1996). This provided the first direct evidence that neurogenic, purinergic contractions are normally curtailed by the breakdown of ATP. Therefore, the second aim of this study was to use ARL 67156 to investigate the role of nucleoside triphosphatases in modulating purinergic e.j.p.s in the guinea-pig isolated vas deferens. (A preliminary account of these results has been published, Sneddon *et al*., 1997).

The overflow of endogenous ATP produced by sympathetic nerve stimulation in the guinea-pig vas deferens can be measured by HPLC and fluorometric detection (Todorov *et al*., 1996). If ARL 67156 enhances electrical and mechanical responses to neuronally released ATP by inhibiting the inactivation of the purine nucleotide, then it should enhance the overflow of ATP released during nerve stimulation. Therefore, the third aim of this study was to test whether the nerve evoked overflow of ATP from the vas deferens is enhanced by the same concentrations of ARL 67156 that modulate the actions of ATP as a neurotransmitter.

Methods

Electrophysiology

Albino male guinea-pigs (300–400 g) were killed with CO_2 and subsequent exsanguination. The vasa deferentia were dissected out, cleaned of connective tissue, mounted for recording, superfused at 2 ml min^{-1} with Krebs solution of the following composition (mM): NaCl 118, KCl 5.4, NaH_2PO_4 1.16, $NaHCO_3$ 25, $MgSO_4$ 1.16, $CaCl_2$ 2.5, glucose 11.1, bubbled with 95% O_2, 5% CO_2 and allowed to equilibrate at 35°C for 1 h.

Intracellular recording of the transmembrane potential was carried out using glass microelectrodes of 30–50 MΩ resistance. The signal was recorded on a storage oscilloscope (Tektronix) and a tape recorder (Racal) *via* a preamplifier (Cell Explorer 800, Dagan). Online computer analysis of the data was performed by the WCP software package by J. Dempster. Impalements were accepted if the resting potential maintained a stable level of at least −60 mV. E.j.p.s were evoked by field stimulation at 1 Hz, 0.2 ms pulse width and at a voltage lower than that necessary to initiate a contraction. Increasing concentrations of NF023 or P-5-P (10^{-7}–3×10^{-4} M) were applied in the superfusate. Initially, the lowest concentration was applied for 15 min before measurements of the e.j.p. magnitude and membrane potential were taken. Progressively higher concentrations were then administered and steady state e.j.p. magnitude and membrane potential recorded. ARL 67156 was added to the superfusate either alone or after inhibition of e.j.p.s by 15–30 min exposure to either NF023 or P-5-P.

ATP overflow

Male albino guinea-pigs (350–400 g) were killed by decapitation. The vasa deferentia were removed, cleaned of connective tissue and the lumen exposed by section along the longitudinal axis. Three tissues were loaded in a Brandel superfusion chamber (fluid volume ≈200 μl). Whatman 541 filters were cut to fit both ends of the chamber, which was then inserted vertically into a thermostatic block with two platinum 'screen' electrodes at either end. The tissues were superfused from bottom to top at 2 ml min^{-1} with Krebs solution (37°C) of the following composition (mM): NaCl 110, $NaHCO_3$ 24.8, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 5.6, bubbled with 95% O_2, 5% CO_2, and allowed to equilibrate for 45 min. Sympathetic nerves were stimulated by electrical field stimulation at 8 Hz with a pulse width of 0.1 ms and supramaximal voltage for 60 s.

The ATP content of superfusate samples was analysed as described previously (Todorov *et al*. 1996). Briefly, to measure the overflow of endogenous ATP, samples of the superfusate (approximately 320 μl) were collected in ice-cold test tubes at 10 s intervals, before, during and after sympathetic nerve stimulation. A control sample of the Krebs solution, which had not been in contact with the tissues was also prepared. Chloroacetaldehyde was then added to form the fluorescent 1 N^6-etheno derivative of ATP present in the samples. The ε-ATP was then separated on a gradient HPLC system, equipped with a Waters Resolve radial pack cartridge (8NV PH 4μ, 8 × 10 mm) and the amount of ATP quantified using a Shimadzu (RF 535) fluorescent monitor at an excitation wavelength of 230 nm and emission wavelength of 420 nm. The buffer solutions consisted of (1) 0.1 M phosphate buffer (pH 6.0) and (2) 75% 0.1 M phosphate buffer and 25% methanol. Identification of the ATP peak was achieved by comparison with the retention times of a known ε-ATP standard and the concentration was determined by the peak area per pmol relationship of known amounts of the standard. Standards were run with each set of samples. The results were normalized for volume and tissue weight and the data calculated as pmol mg^{-1}.

Statistics

Values in the text refer to mean ± s.e. mean or mean ± 95% confidence limits for IC_{50} values. Concentration-response curves were fitted to the data by logistic (Hill equation), non-

linear regression analysis (Graphpad Prism, San Diego, U.S.A). Statistical comparison of the results was tested by Student's *t*-test for paired or unpaired data, as appropriate. Differences were considered significant when $P<0.05$. All e.j.p. magnitude measurements were made during a continuous train of pulses after full facilitation had occurred. For each cell the e.j.p. magnitude is the mean of 10–20 individual e.j.p.s computed by a signal-averaging program.

Drugs

ARL 67156 was provided by Astra plc. NF023 was synthesized as described previously (Nickel *et al.*, 1986). P-5-P was obtained from Sigma. All drugs were dissolved in distilled water and kept frozen as stock solutions before dilution to the required concentration.

Results

Inhibition of e.j.p.s by NF023

The mean size of fully facilitated e.j.p.s recorded during a continuous train of stimulation at 1 Hz was 19.9 ± 1.1 mV ($n=14$). Cumulative addition of NF023 ($10^{-6}-10^{-4}$ M) caused a concentration-dependent reduction in the magnitude of the e.j.p.s and abolished them at the highest concentration used (Figures 1 and 3). The degree of inhibition reached equilibrium within 10 min of each drug addition. After 20 min washout of NF023 e.j.p.s recovered to 66% of their control value. The mean data gave an IC_{50} value of 4.8×10^{-6} M (95% confidence limits $=2.9\times10^{-6}-8.0\times10^{-6}$ M).

Figure 1 also shows that NF023 had no effect on the resting membrane potential of the smooth muscle cell, which remained around −68 mV throughout the experiment. The mean data confirmed the lack of effect of NF023 on resting membrane potential of the smooth muscle cells, (control; -67.1 ± 1.6 mV $n=16$; + NF023 (3×10^{-5} M); -65.6 ± 1.2 mV $n=10$)

Inhibition of e.j.p.s by P-5-P

In these experiments, the mean size of fully facilitated e.j.p.s recorded at 1 Hz was 18.0 ± 1.0 mV ($n=16$). Cumulative addition of P-5-P ($10^{-5}-10^{-3}$ M) caused a concentration-dependent reduction in e.j.p. magnitude and virtually abolished them at the highest concentration used (Figures 2

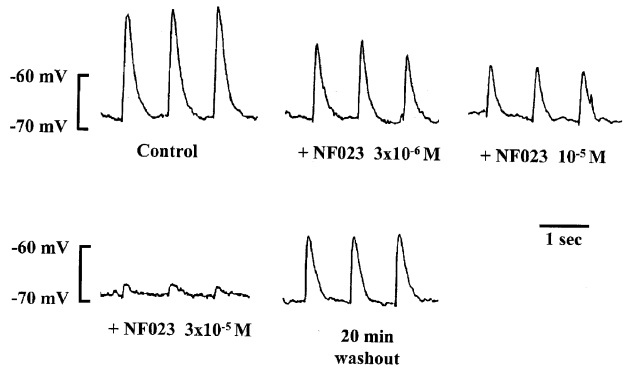

Figure 1 Effect of NF023 on e.j.p.s evoked at 1 Hz in a single cell of the guinea-pig isolated vas deferens. Control e.j.p.s of about 18 mV were reduced in size in a concentration-dependent manner by NF023 ($3\times10^{-6}-3\times10^{-5}$ M) applied in the superfusate. This effect was reversible on washout of the drug. There was no significant change in the resting membrane potential of the cell throughout the experiment.

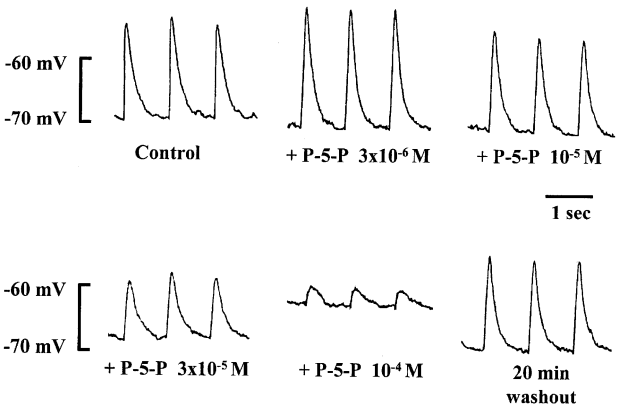

Figure 2 Effect of P-5-P on e.j.p.s evoked at 1 Hz in a single cell of the guinea-pig isolated vas deferens. Control e.j.p.s of about 16 mV were reduced in size in a concentration-dependent manner by P-5-P ($3\times10^{-6}-10^{-4}$ M) applied in the superfusate. P-5-P also depolarized the cell from −70 mV to −63 mV. Both effects were reversible on washout of the drug.

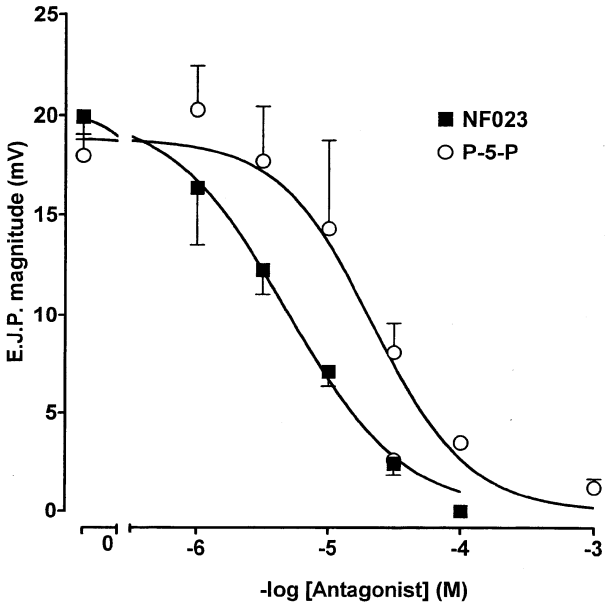

Figure 3 Inhibition of e.j.p.s by NF023 and P-5-P. The graph shows the mean amplitude of e.j.p.s evoked at 1 Hz in the absence and presence of a range of concentrations of each antagonist. Vertical lines indicate s.e.mean. The curves were fitted to the data by logistic (Hill equation), non-linear regression analysis. The data was pooled from recordings from 16 cells for P-5-P and 14 cells for NF023.

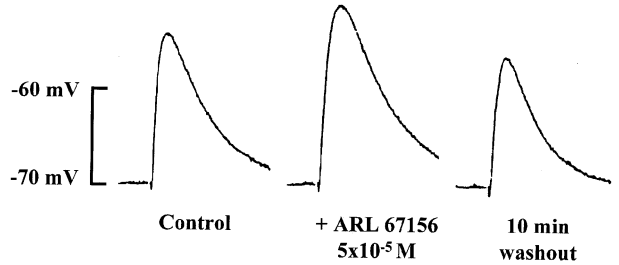

Figure 4 The effect of ARL 67156 on e.j.p.s in the guinea-pig isolated vas deferens. A signal averaging program was used to generate the mean of 20 e.j.p.s evoked at 1 Hz and recorded from the same cell, before, during and after addition of ARL 67156 (5×10^{-5} M). The potentiating effect of ARL 67156 reached equilibrium within 10 min, and was readily reversed on washout.

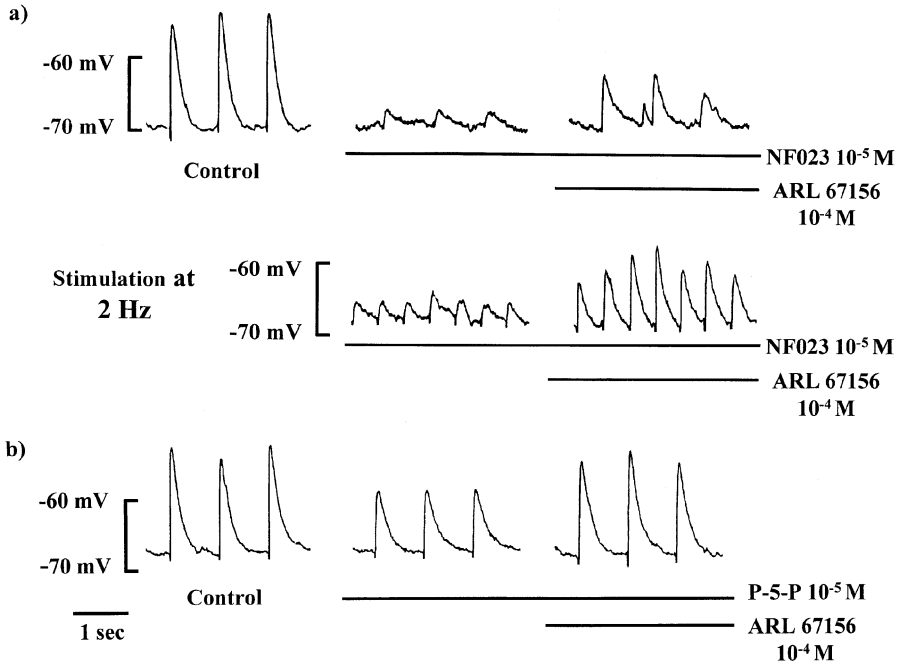

Figure 5 Effect of ARL 67156 on e.j.p.s recorded in the guinea-pig isolated vas deferens in the presence of NF023 or P-5-P. (a) Upper panel; control e.j.p.s evoked at 1 Hz and of about 18 mV amplitude were reduced to 2–3 mV by NF023 (10^{-5} M). Subsequent co-addition of ARL 67156 (10^{-4} M) partially reversed the effect of the P2 receptor antagonist. Lower panel; this shows a similar experiment where e.j.p.s were evoked at 2 Hz. NF023 (10^{-5} M) reduced the e.j.p. amplitude to 3–4 mV and co-addition of ARL 67156 (10^{-4} M) then enhanced e.j.p. amplitude to 8–12 mV. (b) The effect of ARL 67156 on e.j.p.s evoked at 1 Hz in the guinea-pig isolated vas deferens in the presence of P-5-P. Control e.j.p.s of 14–16 mV amplitude were reduced to about 8 mV by P-5-P (10^{-5} M). Subsequently, co-application of ARL 67156 (10^{-4} M) completely reversed the effect of the P2 receptor antagonist. Each trace shows sections of a continuous recording from a single cell.

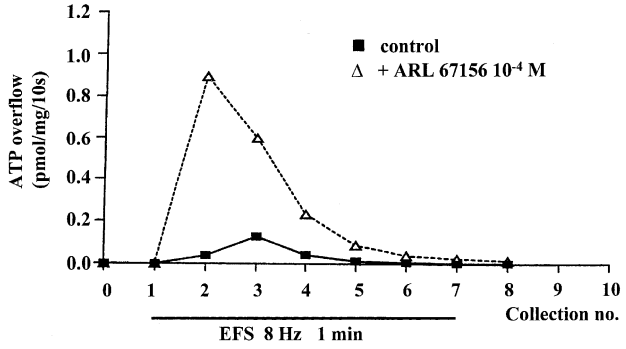

Figure 6 The effect of ARL 67156 on the overflow of ATP in the guinea-pig isolated vas deferens. Electrical field stimulation (EFS) of the sympathetic nerves at 8 Hz for 1 min is indicated by the black line below the graph. Samples were collected at 10 s intervals, as indicated by 'Collection no.' and the ATP content measured by HPLC and fluorescence detection. Under control conditions (solid line) only small amounts of ATP were present in the superfusate. However, in the presence of ARL 67156 (10^{-4} M) (dashed line) there was a substantial increase in the overflow of ATP. Each point on the graph is the mean overflow from 12 tissues measured in four separate experiments.

and 3). The degree of inhibition reached equilibrium within 10 min of each drug addition. After 20 min washout of P-5-P, e.j.p.s had recovered to 96% of their control value. The mean data gave an IC_{50} value of 2.2×10^{-5} M (95% confidence limits = $1.3 \times 10^{-5} - 3.6 \times 10^{-5}$ M).

Figure 2 also shows that at concentrations of 10^{-4} M and above, P-5-P depolarized the smooth muscle cells. The initial resting membrane potential in this experiment was −70 mV, which fell to −63 mV in the presence of P-5-P (10^{-4} M), then recovered to −71 mV on washout of the drug. The pooled data confirmed that P-5-P significantly depolarized the smooth muscle cells ($P<0.05$) (control; -71.3 ± 0.6 mV; + P-5-P (10^{-4} M), -66.1 ± 0.9 mV; + P-5-P (3×10^{-4} M), -64.1 ± 4.1 mV, $n=16$ each).

Enhancement of e.j.p.s by ARL 67156

Preliminary experiments indicated that at 2×10^{-5} M or less, ARL 67156 had no significant effect on the magnitude of e.j.p.s evoked at 1 Hz. Figure 4 shows that increasing the concentration of ARL 67156 to 5×10^{-5} M produced a small, but significant increase ($P<0.05$) in the magnitude of e.j.p.s, from a mean value of 13.0 ± 1.9 to 14.6 ± 2.1 mV ($n=6$). When the concentration of ARL 67156 was increased to 10^{-4} M, the e.j.p. magnitude was further increased within a few minutes, which often led to e.j.p.s reaching threshold for initiation of action potentials. The consequent muscle contraction caused expulsion of the recording microelectrode, preventing subsequent measurement of the effect of ARL 67156 on e.j.p. magnitude.

In order to avoid the problem of microelectrode expulsion, we examined the effect of ARL 67156 (10^{-4} M) after reducing the e.j.p. size by addition of NF023 or P-5-P. Under these conditions, continuous recordings could be made of the effect of ARL 67156 on e.j.p. magnitude. Figure 5a illustrates that after e.j.p.s evoked at 1 Hz were reduced from a control level of 18 to 2–3 mV by NF023 (10^{-5} M), subsequent co-application of ARL 67156 (10^{-4} M) approximately trebled the e.j.p. magnitude to 5–8 mV. When the stimulation frequency was increased to 2 Hz (lower panel,) the enhancing effect of ARL 67156 is even more obvious, with e.j.p.s increasing from 3–4 to 8–12 mV in the presence of ARL 67156. Similarly,

Figure 5b illustrates that after control e.j.p.s of 14–16 mV were reduced to about 8 mV by P-5-P (10^{-5} M), ARL 67156 (10^{-4} M) increased e.j.p. magnitude back to control level. The pooled data showed that under these conditions. ARL 67156 (10^{-4} M) significantly enhanced e.j.p.s at 1 Hz by an average of 168%, from 8.4 ± 2.4–14.0 ± 3.1 mV ($n=6$; $P<0.05$).

Enhancement of nerve-evoked ATP overflow by ARL 67156

In the absence of sympathetic nerve stimulation, the release of ATP into the superfusate bathing the vas deferens was negligible, as indicated by the pre-stimulation point (0) in Figure 6. Sympathetic nerve stimulation (8 Hz for 1 min) increased the overflow of ATP in a transient manner (Figure 6), consistent with our previous report (Todorov *et al*., 1996). Addition of ARL 67156 (10^{-4} M) to the solution superfusing the tissue, increased the peak overflow of ATP to 692% of the control value.

Discussion

Effects of antagonists

In the present study we found that NF023 produced a potent inhibition of e.j.p.s mediated by $P2X_1$ receptors in the guinea-pig isolated vas deferens. The inhibition was rapid in onset and reversible on washout. Furthermore, NF023 had no effect on the transmembrane potential of the smooth muscle cells. These properties indicate that NF023 is more useful as a P2X receptor antagonist compared with all compounds investigated previously.

In these experiments, NF023 inhibited the e.j.p.s with an IC_{50} of 4.8×10^{-6} M. This is similar to its potency in inhibiting contractions evoked by exogenous P2X receptor agonists in vascular (Urbanek *et al*., 1990; Lambrecht, 1996; Ziyal *et al*., 1997) and visceral (Bültmann *et al*., 1996; Lambrecht, 1996) smooth muscle preparations. NF023 competitively antagonized contractions elicited by α,β-meATP with pA_2 values of 5.68 in rabbit vas deferens (Lambrecht, 1996), 5.54 in rat mesenteric artery (Lambrecht, 1996) and 5.69 in rabbit saphenous artery (Ziyal *et al*., 1997). Similar concentrations of NF023 rapidly and reversibly antagonized the cloned $P2X_1$ receptor expressed in *Xenopus* oocytes, but higher concentrations were required to block $P2X_2$, $P2X_3$, and $P2X_4$ receptors (Soto *et al*., 1999). NF023 is also substantially less potent as an antagonist at P2Y receptors (Lambrecht, 1996; Ziyal *et al*., 1997; Harper *et al*., 1998). Thus, NF023 is selective in blocking the $P2X_1$ receptor compared to other P2 receptor subtypes.

P-5-P was less potent than NF023 and produced a depolarization of the smooth muscle cells at concentrations above 10^{-5} M. Therefore, it is not suitable for use as a selective purinoceptor antagonist in smooth muscle preparations. Interestingly, low concentrations of P-5-P produced a small increase in e.j.p. magnitude, although this was not statistically significant (see Figures 2 and 3). One possible explanation is that P-5-P has an inhibitory action on ecto-ATPase. However, this seems unlikely since we have found that P-5-P fails to inhibit significantly the activity of releasable ATPase from guinea-pig vas deferens at such low concentrations. (Westfall, T.D. *et al*., 2000 unpublished observations).

A number of compounds have previously been characterized as P2X receptor antagonists, but none of them is as suitable as NF023 for electrophysiological investigation of neurotransmission involving ATP. $ANAPP_3$ effectively blocked P2X receptors in the guinea-pig vas deferens, but also produced a large transient contraction and depolarization, thus preventing continuous microelectrode recording of e.j.p.s (Sneddon *et al*., 1982). It also required photo-activation for 20 min to produce covalent bonding of the drug to the receptors, which rendered its action irreversible. α,β-meATP produced selective desensitization of P2X receptors in the guinea-pig vas deferens and inhibition of e.j.p.s, but since this agent is a potent agonist, it also produced a large depolarization and contraction of the tissue, preventing continuous recording of e.j.p.s (Sneddon & Burnstock, 1984).

Suramin also blocked e.j.p.s in the guinea-pig vas deferens, but required high concentrations and at least 30 min equilibration time. This made continuous recording of its effects very difficult. It also had non-selective actions and was practically irreversible (Sneddon, 1992). More recently we found that the widely used P2X receptor antagonist PPADS was a potent inhibitor of e.j.p.s in the guinea-pig isolated vas deferens, but also depolarized the smooth muscle by about 12 mV (McLaren *et al*., 1994). This makes it difficult to assess accurately the effect of the PPADS on e.j.p. magnitude, as the electro-chemical driving force for the e.j.p.s declines in the presence of the antagonist. It also implies that PPADS has additional, non-selective effects on the muscle.

In the present study, we established that P-5-P also blocks e.j.p.s in the guinea-pig isolated vas deferens. However, P-5-P was less potent than NF023 and has the added disadvantage that it evokes concomitant membrane depolarization. Furthermore, a previous study reported that its antagonistic action at the cloned $P2X_1$ receptor is non-competitive (Evans *et al*., 1995). Thus, P-5-P does not appear to be useful as an antagonist at $P2X_1$ receptors.

Effect of ARL 67156

In this study, we demonstrated that ARL 67156 enhances the nerve stimulation-evoked overflow of ATP from the guinea-pig isolated vas deferens by almost 7 fold. The magnitude of the e.j.p.s was also greatly increased, but to a much lesser degree. Obviously, no direct comparison can be drawn between the two types of experiment since the electrophysiological studies are restricted to low stimulation frequencies, normally 1 Hz, whereas the overflow studies require stimulation of 8 Hz to obtain sufficient purine for reliable measurement. However, previous studies on the effect of ARL 67156 on neurogenic contractions were performed at stimulation frequencies from 1–8 Hz (Westfall *et al*., 1996). Here the degree of enhancement was greatest at the lower frequencies. Contractions at 1 Hz increased to 220% of control and those at 8 Hz to around 140%. This suggests that overflow of ATP and its effective concentration at the receptor are not directly proportional to each other.

We have shown previously that in the absence of ARL 67156, the main purine collected in the superfusate during sympathetic nerve stimulation is adenosine, suggesting that endogenously-released ATP is rapidly metabolized (Todorov *et al*., 1997). ARL 67156 inhibits the breakdown of ATP by nucleoside triphosphatases (Crack *et al*., 1995; Khakh *et al*., 1995; Todorov *et al*., 1997) and significantly enhances the contractile responses to nerve stimulation and exogenous ATP in the guinea-pig isolated vas deferens and urinary bladder (Westfall *et al*., 1996; 1997). Thus the present data are consistent with the suggestion that ARL 67156 potentiates the neurogenic contractions by inhibiting the breakdown of ATP in the synaptic cleft, leading to larger e.j.p.s, which in turn are

more likely to reach threshold for activation of action potentials and so initiation of muscle contraction (Westfall *et al*., 1996; 1997).

We have previously shown that exogenously applied adenosine receptor agonists can act prejunctionally to inhibit transmitter release in the isolated guinea-pig vas deferens, reducing the magnitude of e.j.p.s and neurogenic contractions (Sneddon *et al*., 1984). If ARL 67156 reduces the production of adenosine from ATP, then the observed increase in e.j.p. magnitude and ATP overflow produced by ARL 67156 could result from a reduction in adenosine-mediated negative feedback. At present we have no direct evidence to test this possibility. However, it seems unlikely since we have previously shown (Sneddon *et al*., 1984) that neurogenic contractions (2–8 Hz) in the guinea-pig vas deferens are not enhanced by adenosine receptor antagonists, suggesting that endogenous adenosine does not inhibit transmitter release.

In conclusion, this study has indicated that NF023 is the best available P2X receptor antagonist for electrophysiological investigation of ATP as a neurotransmitter. We have also demonstrated that enzymatic breakdown of ATP plays an important role in modulating ATP overflow, the size of e.j.p.s, and therefore the contractile responses to ATP as a neurotransmitter in the guinea-pig vas deferens.

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