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Blockade by 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS) of P_{2X}-purinoceptors in rat vas deferens

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1 The possibility of an antagonist effect of 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS) at P_{2X}-purinoceptors was studied in rat vas deferens.

2 DIDS reduced contractions elicited by α,β-methylene ATP 3 μM, IC_{50} 1.6 μM, but did not change contractions elicited by K^+ 35 mM. DIDS 3.2 μM slightly shifted the concentration-response curve of α,β-methylene ATP to the right and reduced the maximum. DIDS 10 μM markedly decreased and DIDS 32 μM abolished contractions over the entire range of the α,β-methylene ATP concentration-response curve. DIDS 32 μM also abolished contractions elicited by ATP but did not change contractions elicited by noradrenaline. The antagonist effect of DIDS was only slowly reversible.

3 The presence of either suramin 320 μM or α,β-methylene ATP 10 μM during the exposure to DIDS protected the tissue from the long-lasting blocking effect of DIDS.

4 4,4′-Diisothiocyanatodihydrostilbene-2,2′-disulphonate (H_2DIDS) was equipotent with DIDS whereas several analogues in which one or both of the isothiocyanate residues were replaced were less effective or without effect against α,β-methylene ATP.

5 DIDS attenuated the purinergic component of neurogenic contractions elicited by electrical field stimulation, IC_{50} 3.9 μM, but did not change the adrenergic component.

6 It is concluded that DIDS causes a selective, long-lasting, non-equilibrium blockade of P_{2X}-purinoceptors in rat vas deferens. Due to this effect it also selectively blocks the purinergic component of neurogenic contractions.

Keywords: Rat vas deferens; DIDS; α,β-methylene ATP; P_{2X}-purinoceptor; P_2-purinoceptor antagonists; co-transmission; purinergic transmission

Introduction

4,4′-Diisothiocyanatostilbene-2,2′-disulphonate (DIDS), commonly used as an anion transport inhibitor (see Cabantchik & Greger, 1992), blocks a number of presumably purinoceptor-mediated responses, namely ADP-induced bovine platelet aggregation (Kitagawa *et al.*, 1983), ATP-induced ion fluxes in murine erythroleukemia cells (Chahwala & Cantley, 1984), the ATP-induced entry of calcium into rat parotid acinar cells (McMillian *et al.*, 1988; Soltoff *et al.*, 1993), the ATP-induced cation current in smooth muscle cells of the rabbit ear artery (Amédée *et al.*, 1990), ATP-induced currents in chick skeletal muscle (Thomas *et al.*, 1991), the ATP-induced acidification of the cytosol of rat cardiac cells (Pucéat *et al.*, 1991), an ATP-activated Cl^- current in human airway epithelial cells (Stutts *et al.*, 1992), and nucleotide-induced contractions of the guinea-pig trachea (Fedan *et al.*, 1993). Most of these effects of DIDS have been attributed to blockade of anion transport mechanisms or Cl^- channels. In rat parotid acinar cells, however, DIDS also decreased the binding of [^{32}P]-ATP (McMillian *et al.*, 1988); moreover, purinoceptor ligands protected the cells from the irreversible effect of DIDS, indicating that DIDS blocked the (P_{2Z}) purinoceptor rather than the subsequent transduction mechanism (Soltoff *et al.*, 1993; see Cusack, 1993, for a review on P_2-purinoceptors).

The possibility of a blockade by DIDS of P_{2X}-purinoceptors has not been investigated. We used rat vas deferens to examine this possibility. In rat vas deferens, P_{2X}-purinoceptors mediate contractions to the prototypic P_{2X}-purinoceptor agonist, α,β-methylene ATP (Burnstock & Kennedy, 1985), as well as the purinergic component of neurogenic contractions (Mallard *et al.*, 1992) and part of the response to exogenous ATP (Bültmann & Starke, 1994).

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Methods

Male Wistar rats (240–300 g) were decapitated and the vasa deferentia removed and cleaned of adherent tissue. The medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Contraction

Whole vasa deferentia or (experiments with α,β-methylene ATP) prostatic thirds were suspended vertically in a 5.7 ml organ bath. The lower end was fixed and the upper end attached to an isometric force transducer (K30, Hugo Sachs Elektronik, Hugstetten, Germany) under an initial tension of 9.8 mN. The medium was replaced every 15 min (occasionally 25 min). Tissues relaxed to about 3 mN during a 60 min equilibration period. This final resting tension remained constant for the rest of the experiments. The tension was recorded on a Graphtec thermal pen recorder (Ettlingen, Germany).

Contractions were elicited by α,β-methylene ATP, high K^+, ATP, noradrenaline or electrical field stimulation, of which only one was tested on a single preparation. Unless stated otherwise, agonists and high K^+ were washed out immediately after contractions had peaked. High K^+ was added as 35 mM KCl, the final K^+ concentration was therefore 40.7 mM, without osmotic compensation. Field stimulation (single pulses, 0.3 ms pulse width, 100 mA) was applied via platinum electrodes located at the top and the bottom of the organ bath (Stimulator II, Hugo Sachs Elektronik). Where reasonable, concentration-response data were analysed by logistic curve fitting to the weighted mean contraction values using equation No. 25 of Waud (1976) and non-linear regression. The calculation yielded the maximal

effect and the IC_{50} or EC_{50}, i.e. the concentration producing 50% of the maximum.

Tritium overflow

Experiments with [^{3}H]-noradrenaline were carried out as described (Bültmann *et al.*, 1993b). Briefly, slices of the prostatic portion of the vas deferens were preincubated with [^{3}H]-noradrenaline and then superfused with medium of the above composition. The stimulation periods (S_1 to S_6) consisted of 50 pulses per 5 Hz. Drugs or solvent were added after S_3. For evaluation of their effects on basal tritium efflux, ratios were calculated of efflux in the 2 min before S_4 over efflux in the 2 min before S_1. For evaluation of effects on stimulation-evoked tritium overflow, the sum of the overflow elicited by S_1 to S_3 (S_{1-3}) and the sum of the overflow elicited by S_4 to S_6 (S_{4-6}) was formed and ratios S_{4-6}/S_{1-3} were then calculated.

Materials

Adenosine 5'-triphosphate disodium salt (ATP), α,β-methylene ATP lithium salt, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid disodium salt (DIDS), (−)-noradrenaline bi-(+)-tartrate (Sigma, Deisenhofen, Germany), 4,4'-diazidostilbene-2,2'-disulphonic acid disodium salt, 4,4'-dinitrostilbene-2,2'-disulphonic acid disodium salt, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid disodium salt (SITS; Aldrich, Steinheim, Germany), 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonic acid disodium salt (H_2DIDS; Molecular Probes, Eugene, U.S.A.) and suramin (Bayer, Wuppertal, Germany) were dissolved in distilled water. KCl for high K^+ was dissolved in medium. 4,4'-Diaminostilbene-2,2'-disulphonic acid (Aldrich) was dissolved in water with addition of twice the molar amount of sodium hydroxide. Solutions of stilbenes and suramin were prepared freshly before each experiment. Drug solutions were added to the organ bath in aliquots not exceeding 100 μl.

Statistics

The arithmetic mean ± s.e.mean (for IC_{50} or EC_{50} values of fitted curves the s.e. as defined by Waud, 1976) is given throughout.

Differences between means were tested for significance by the Mann–Whitney test. Differences between fitted curves were tested according to p. 371 of Motulsky & Ransnas (1987). Differences with error probabilities <0.05 were taken to be statistically significant.

Results

Contraction

In initial experiments, α,β-methylene ATP was given every 60 min at the same concentration, 3 μM. It elicited rapid transient contractions of 9.9 ± 0.3 mN ($n = 54$; first addition; see Figure 3 below). The contractions increased upon repeated addition in the presence of solvent (by 68 ± 5% at 6th addition; $n = 5$). Increasing concentrations of DIDS, H_2DIDS and SITS reduced and finally abolished the contractions, with IC_{50} values of 1.6 ± 0.3, 2.0 ± 0.3 and 8.9 ± 1.0 μM, respectively (Figure 1). 4,4'-Diazidostilbene-2,2'-disulphonate and 4,4'-dinitrostilbene-2,2'-disulphonate reduced contractions elicited by α,β-methylene ATP 3 μM only at very high concentrations. 4,4'-Diaminostilbene-2,2'-disulphonate was without effect at concentrations of up to 1 mM (Figure 1). The interaction of DIDS with K^+ 35 mM was studied with the same protocol. DIDS (0.32–32 μM) did not alter contractions elicited by high K^+ ($n = 4$ and 5 for solvent and DIDS, respectively).

The effect of DIDS on the concentration-response curves of α,β-methylene ATP, ATP and noradrenaline was then determined. Increasing concentrations of α,β-methylene ATP elicited increasing contraction with an EC_{50} of 3.5 ± 0.4 μM ($n = 15$; all first concentration-response curves pooled). A second concentration-response curve after addition of solvent was very similar to the first one (EC_{50} 3.6 ± 0.6 μM, $n = 4$; (○) in Figure 2a). DIDS 3.2 μM shifted the concentration-response curve of α,β-methylene ATP slightly to the right (EC_{50} 8.5 ± 1.0 μM; $P<0.05$) and decreased the maximum (by 33%; $P<0.01$). DIDS 10 and 32 μM markedly depressed and abolished, respectively, contractions over the entire range of α,β-methylene ATP concentrations (Figure 2a).

Increasing concentrations of ATP and noradrenaline likewise elicited increasing contractions. For both agonists, a second concentration-response curve after addition of solvent could be superimposed on the first one ($n = 4$ each). DIDS 32 μM abolished contractions elicited by ATP (Figure 2b) but did not alter contractions elicited by noradrenaline (Figure 2c).

The antagonism of DIDS against α,β-methylene ATP was only slowly reversible. The blockade by DIDS 32 μM of the

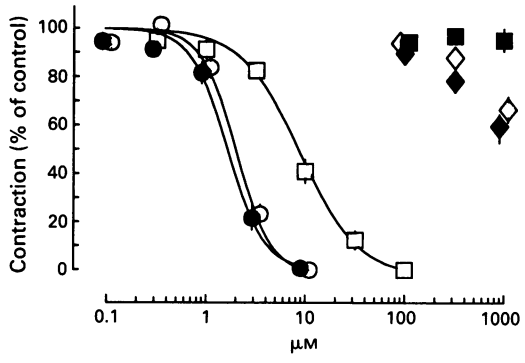

Figure 1 Effect of DIDS and related compounds on contractions elicited by α,β-methylene ATP. α,β-Methylene ATP 3 μM was added to the bath at 60 min intervals and washed out immediately after the contraction had peaked. DIDS (●), H_2DIDS (○), SITS (□), 4,4'-diazidostilbene-2,2'-disulphonate (◆), 4,4'-dinitrostilbene-2,2'-disulphonate (◇) or 4,4'-diaminostilbene-2,2'-disulphonate (■) was added at increasing concentrations immediately after the first and all following responses to α,β-methylene ATP. Abscissae: antagonist concentration. Ordinates show contraction as a percentage of first, pre-antagonist, contraction, corrected for any change observed in controls (solvent). Means ± s.e.mean from 3 to 5 experiments.

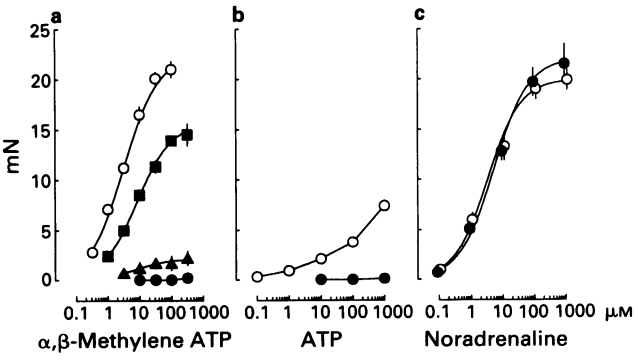

Figure 2 Effect of DIDS on concentration-response curves of (a) α,β-methylene ATP, (b) ATP and (c) noradrenaline. Increasing concentrations of antagonists were added every 30 min and washed out immediately after the contraction had peaked. Two concentration-response curves for the agonist studied were determined in each tissue. Solvent or DIDS was added to the medium after completion of the first curve and the second curve was determined 60 min later. Abscissae: agonist concentration. Ordinates show contractions (mN) in second curves in the presence of solvent (○) or DIDS 3.2 (■), 10 (▲) or 32 μM (●). Means ± s.e.mean from 3 to 5 experiments.

effect of α,β-methylene ATP 3 μM, for example, initially complete, still amounted to 58% after 3 h of washout (application of α,β-methylene ATP every 60 min, of DIDS for 60 min; $n = 3$).

A long-lasting, non-equilibrium blockade of P_{2X}-purinoceptors might explain the non-competitive character of the antagonism of DIDS against α,β-methylene ATP (Figure 2a). Receptor protection experiments were performed to examine this possibility. α,β-Methylene ATP 3 μM was administered twice, with an interval of 130 min. In controls, the second contraction slightly exceeded the first one (by 20 ± 2%; $n = 6$). Compared with (and corrected for) these controls, incubation with DIDS 32 μM for 60 min followed by 60 min washout reduced the subsequent response to α,β-methylene ATP 3 μM by 77 ± 2% ($P < 0.01$; $n = 5$; Figure 3a). Suramin 320 μM and α,β-methylene ATP 10 μM, the protecting drugs, were applied immediately after the first addition of α,β-methylene ATP 3 μM for 70 min and then washed out for 60 min. After exposure to suramin 320 μM or α,β-methylene ATP 10 μM alone, the second contraction elicited by α,β-methylene ATP 3 μM again slightly exceeded the first one (by 4 ± 6 and 33 ± 4%, respectively; $n = 4$ each). Compared to (and corrected for) the 'suramin alone' group, incubation with DIDS 32 μM in the presence of suramin 320 μM reduced the subsequent response to α,β-methylene ATP 3 μM by 22 ± 6% ($P < 0.05$; $n = 4$; Figure 3b), much less than the 77% reduction produced by DIDS alone. Presence of α,β-methylene ATP 10 μM afforded even better protection: exposure to DIDS 32 μM in the presence of α,β-methylene ATP 10 μM did not lead to a significant decrease of the subsequent response to α,β-methylene ATP 3 μM (12 ± 4% reduction compared to and corrected for the 'α,β-methylene ATP 10 μM alone' group; $n = 4$; $P > 0.05$; Figure 3c).

Electrical stimulation of vasa deferentia with single pulses every 60 min elicited biphasic contractions (Figure 4) which remained approximately constant in solvent controls ($n = 4$). DIDS reduced the rapid (purinergic) phase in a concentration-dependent manner but did not alter the slow (adrenergic) phase (Figure 4). For further analysis, purinergic and adrenergic phases were isolated by prazosin and suramin, respectively (Bültmann *et al.*, 1993a). In the presence of prazosin 0.3 μM, contractions amounted to 9.1 ± 0.6 mN (first contraction; $n = 10$) and remained approximately constant upon repeated stimulation in solvent controls ($n = 5$). DIDS progressively reduced and eventually abolished these purinergic contractions, IC_{50} 3.9 ± 0.6 μM (Figure 5). In the presence of suramin 300 μM, contractions amounted to 12.8 ± 1.5 mN (first contraction; $n = 8$) and again remained approximately constant in controls ($n = 4$). DIDS did not alter the adrenergic contractions (Figure 5).

Tritium overflow

Experiments with [^{3}H]-noradrenaline were carried out in order to examine whether DIDS, like suramin (Kurz *et al.*,

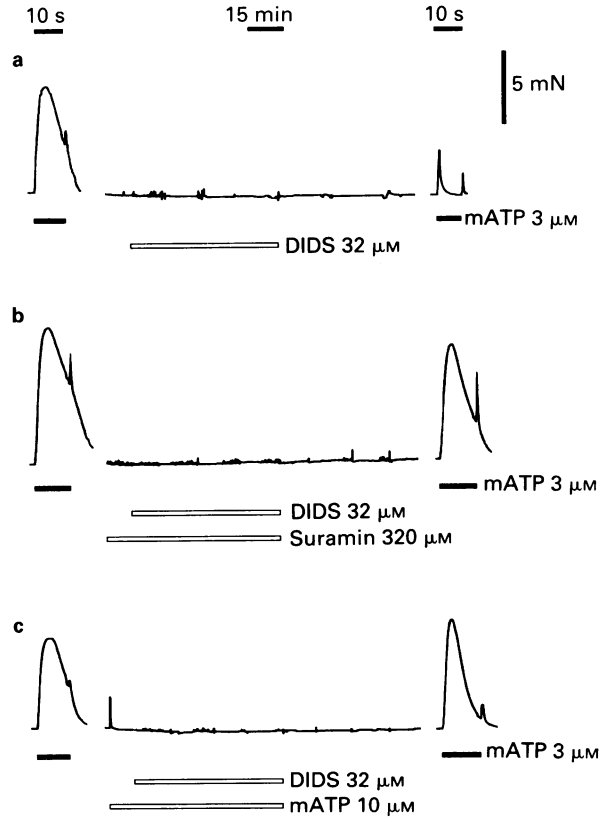

Figure 3 Protection by suramin and α,β-methylene ATP (mATP) against the long-lasting blocking effect of DIDS. α,β-Methylene ATP 3 μM was added twice, at an interval of 130 min. DIDS 32 μM was administered about 11 min after the first addition of α,β-methylene ATP 3 μM and left in the bath for 60 min. DIDS was administered either alone (a) or in the presence of suramin 320 μM (b) or α,β-methylene ATP 10 μM (c) which were given immediately after the first response to α,β-methylene ATP 3 μM and washed out together with the latter. Note different time calibrations for middle tracings on the one hand and left- and right-hand tracings on the other. Due to desensitization, α,β-methylene ATP 10 μM elicited only a very small contraction (c). Representative tracings from 4 or 5 experiments.

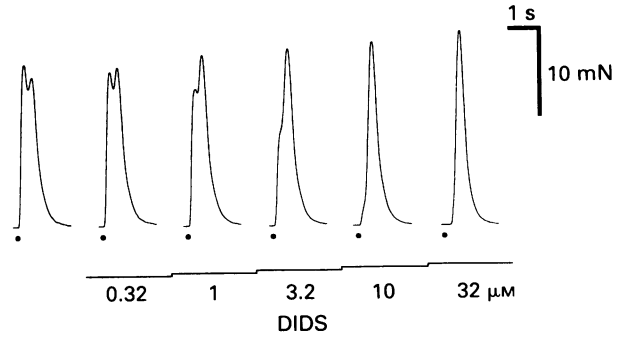

Figure 4 Effect of DIDS on neurogenic contractions. Tissues were electrically stimulated by single pulses every 60 min (dots). DIDS was added at increasing concentrations immediately after the first and all following stimulations. Representative tracings from 6 experiments.

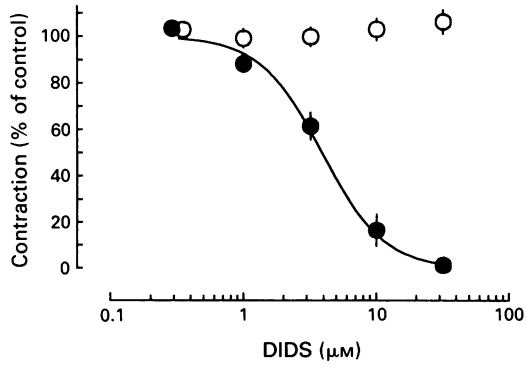

Figure 5 Effect of DIDS on purinergic and adrenergic components of neurogenic contractions. The medium contained either prazosin 0.3 μM (●) or suramin 300 μM (○) from the beginning. Tissues were electrically stimulated by single pulses every 60 min. DIDS was added at increasing concentrations immediately after the first and all following stimulations. Abscissae: DIDS concentration. Ordinates show contraction as a percentage of the first, pre-DIDS, contraction, corrected for any change observed in controls (solvent). Means ± s.e.mean from 4 or 5 experiments.

1993), affected sympathetic transmitter release. There were 6 stimulation periods (S_1 to S_6). Solvent, suramin or DIDS was added after S_3, 64 min before S_4, and kept for the remainder of the experiment. The fractional rate of tritium efflux immediately before S_1 was $0.00155 \pm 0.00003\ min^{-1}$. The overflow evoked by S_1 to S_3 (sum of S_1, S_2 and S_3) amounted to $0.168 \pm 0.007\%$ of tissue tritium ($n = 23$). In solvent controls, the evoked overflow remained approximately constant ($S_{4-6}/S_{1-3} = 1.06 \pm 0.06$; $n = 8$). Suramin 320 μM increased the evoked overflow ($S_{4-6}/S_{1-3} = 1.35 \pm 0.03$; $n = 7$; $P < 0.005$). DIDS 32 μM, in contrast, caused no change ($S_{4-6}/S_{1-3} = 0.94 \pm 0.05$; $n = 8$; $P > 0.05$). Neither suramin nor DIDS changed the basal outflow of tritium.

Discussion

DIDS reduced contractions elicited by α,β-methylene ATP in a concentration-dependent manner (Figures 1 and 2a). Contractions elicited by high K^+ or (Figure 2c) noradrenaline, in contrast, were not changed, thus raising the possibility that the inhibition was due to P_{2X}-purinoceptor blockade.

The blockade produced by DIDS was long-lasting. This at best slowly reversible, non-equilibrium type of effect also showed up in the non-competitive change of the concentration-response curve of α,β-methylene ATP (Figure 2a). The persistent blockade was prevented when the tissue was exposed to DIDS in the presence of either suramin or α,β-methylene ATP (Figure 3). Suramin acts on sites in addition to P_2-purinoceptors (Voogd *et al.*, 1993), and the desensitization produced by α,β-methylene ATP may lead to changes other than those of the P_{2X}-purinoceptor. However, the fact that both compounds protected the tissue from the long-lasting effect of DIDS supports the view that DIDS blocked the P_{2X}-purinoceptor, specifically, its nucleotide binding site.

The IC_{50} value of DIDS against α,β-methylene ATP 3 μM was 1.6 μM. DIDS was, hence, more potent than suramin (IC_{50} 10.1 μM) and about equipotent with pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; IC_{50} 2.1 μM) when the latter were tested against α,β-methylene ATP with the same protocol (Bültmann & Starke, 1994). It should be noted that DIDS blocked the rat vas deferens P_{2X}-purinoceptor with about the same potency with which it irreversibly blocks the human red blood cell Cl^- exchanger, the anion transporter most sensitive to DIDS (IC_{50} 2 μM; Table 4 of Cabantchik & Greger, 1992).

Several of the present findings resemble results obtained in rat parotid acinar cells where ATP, by activation of P_{2Z}-purinoceptors, stimulated the uptake of $^{45}Ca^{2+}$ (Soltoff *et al.*, 1993). As in the rat vas deferens, the effect of ATP on rat parotid cells was blocked by DIDS, the blockade (tested against ATP instead of α,β-methylene ATP) persisted despite washout, and in protection experiments the blockade was prevented by P_2-purinoceptor ligands. Moreover, as in the present results, H_2DIDS was about equipotent with DIDS, DIDS was about 3 times more potent that SITS, and 4,4'-dinitrostilbene-2,2'-disulphonate had no effect, in rat parotid cells (Soltoff *et al.*, 1993). Therefore, at P_{2X}-purinoceptors as well as at P_{2Z}-purinoceptors, isothiocyanate residues may be decisive for the blockade produced by stilbene disulphonates, as suggested by Soltoff *et al.* (1993). In further support of the suggestion, two additional non-isothiocyanato stilbene disulphonates, 4,4'-diazidostilbene-2,2'-disulphonate and 4,4'-diaminostilbene-2,2'-disulphonate, were at best weak blocking agents. DIDS was more potent at rat vas deferens P_{2X}-purinoceptors (IC_{50} 1.6 μM against α,β-methylene ATP 3 μM) than at rat parotid P_{2Z}-purinoceptors (IC_{50} about 35 μM against ATP 300 μM; Soltoff *et al.*, 1993). This was not due to the longer exposure periods used in the present study (60 min as compared to 10 or 20 min in rat parotid cells), since DIDS also was more potent in rat vas deferens when the exposure per concentration was reduced to 10 min (IC_{50} 6.6 μM against α,β-methylene ATP 3 μM; R. Bültmann, unpublished data).

As well as against exogenous contraction-producing agents, DIDS also acted selectively against the components of neurogenic contractions: the purinergic, P_{2X}-receptor-mediated (Mallard *et al.*, 1992) component was decreased with an IC_{50} (3.9 μM) similar to that found against α,β-methylene ATP, whereas the adrenergic component was unchanged. The pattern of antagonism confirms postganglionic sympathetic noradrenaline-ATP co-transmission in rat vas deferens (see Burnstock, 1990; von Kügelgen & Starke, 1991). Suramin increased the evoked overflow of tritium, and hence the release of noradrenaline, from tissues preincubated with [^{3}H]-noradrenaline. The increase has been demonstrated previously and explained by the interruption of a negative feedback mediated by prejunctional P_{2Y}-like autoreceptors (Kurz *et al.*, 1993; von Kügelgen *et al.*, 1993; 1994). DIDS did not share this effect with suramin and, hence, apparently does not block the prejunctional receptors (see also Fuder & Muth, 1993).

Whereas α,β-methylene ATP and the purinergic transmitter contract the rat vas deferens exclusively through P_{2X}-purinoceptors, exogenous ATP seems to act through three receptors: P_{2X}, P_{2Y} and a non-P_{2X}-non-P_{2Y}-receptor; suramin blocks the P_{2X}- and P_{2Y}-, PPADS the P_{2X}- and the non-P_{2X}-non-P_{2Y}-receptor (Bültmann & Starke, 1994). The complete suppression by DIDS of the effect of ATP (Figure 2b) suggests that DIDS may block all three receptors.

An interaction of DIDS with ATP in guinea-pig vas deferens has been mentioned in a review article: DIDS 100 μM caused only a moderate depression of the amplitude of contractions induced by ATP 0.1 μM–10 mM and in addition prolonged the contractions (Fedan & Lamport, 1990). The reason may be the brief duration, 15 min, of the exposure to DIDS (Fedan & Lamport, 1990). A species difference seems unlikely: incubation of the guinea-pig vas deferens with DIDS 1–32 μM for 60 min per concentration progressively reduced and finally abolished contractions elicited by α,β-methylene ATP 3 μM as well as the purinergic component of neurogenic contractions, in accord with the results on rat vas deferens (R. Bültmann, unpublished data).

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