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Adenosine 5′-(2-Fluorodiphosphate) is not a Selective P_{2Y} Purinoceptor Agonist in the Rabbit Jugular Vein

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ABSTRACT

The relaxant properties of the putative selective P_{2Y} agonist adenosine 5′-(2-fluorodiphosphate) (ADP-β-F) and its structural analog adenosine 5′-(2-thiodiphosphate) have been investigated in the rabbit precontracted jugular vein preparation. In tissues with intact endothelium, ADP-β-F produced a multiphasic agonist concentration/effect curve made up of two vasorelaxant components which were kinetically and pharmacologically distinct. The higher potency phase (p[A_{50}] 5.58 ± 0.13), characterized by slow, tonic responses, was retained after endothelial denudation and blocked by the selective P_1 purinoceptor antagonist 8-sulphophenyltheophylline. The lower potency phase (p[A_{50}] 3.98 ± 0.07), characterized by fast, phasic responses, was abolished by endothelial denudation and is presumed to be mediated at P_{2Y} purinoceptors. By contrast, the agonist concentration/effect curve to adenosine 5′-(2-dithiophosphate) in endothelium-intact tissues appear monophasic and was unaffected by 8-sulphophenyltheophylline (p[A_{50}]6.86 ± 0.12), although endothelial denudation revealed a secondary P_1-induced relaxant component (p[A_{50}] 5.73 ± 0.20). This study demonstrates that in the rabbit jugular vein, relaxant responses to ADP-β-F are mediated primarily by activation of P_1 purinoceptors, and it is, therefore, invalid to regard ADP-β-F as a selective probe for P_{2Y} purinoceptors, whereas adenosine 5′-(2-thiodiphosphate) does show some selectivity for this receptor.

The classification of P_2 purinoceptors is limited by the absence of selective, competitive antagonists and has, therefore, relied heavily on determination of the relative order of agonist potencies (Burnstock and Kennedy, 1985). However, few agonists have been described that are both pharmacologically selective and resistant to degradation by ectonucleotidases. Conventionally, the P_{2Y} subclass has been defined by a potency order where 2-MeSATP is significantly more potent than ATP. However, 2-MeSATP is known to be readily degraded (Welford *et al.*, 1987), and so, dependence on 2-MeSATP alone for positive identification of P_{2Y} purinoceptors may be unreliable. Other selective agonists would clearly be desirable.

A recent report (Hourani *et al.* 1988) identifies ADP-β-F as a specific and relatively stable P_{2Y} purinoceptor agonist in guinea pig *Taenia coli.* As such, ADP-β-F would be the first compound of this type and would represent an important tool for characterizing P_2 purinoceptors. Indeed, one recent review of purinoceptor subtypes has highlighted the apparent utility of ADP-β-F for this purpose (Kennedy, 1990).

We have attempted to verify the selectivity of ADP-β-F using a vascular preparation, the rabbit isolated jugular vein. In this preparation, ADP is known to cause endothelium-dependent relaxations (Komori *et al.*, 1990). P_{2Y} purinoceptor agonists cause endothelial-dependent relaxation of vascular smooth muscle by release of endothelium-derived relaxing factor and/or prostacyclin (Gordon, 1986). A comparison has been made with ADP-β-S, a stable structural analog also reported to have P_{2Y} agonist properties (Burnstock *et al.*, 1984) and recently used as a radiologand for this receptor subtype (Cooper *et al.*, 1989). Preliminary accounts of parts of this study have been presented to the British Pharmacological Society (Wood *et al.*, 1989) and at the New York Academy of Sciences Conference on The Biological Actions of Extracellular ATP, Philadelphia (Wood *et al.*, 1990).

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Methods

Tissue and protocol. Rings (3–5 mm) of external jugular vein from male New Zealand White rabbits were prepared for organ bath studies as described elsewhere (Leff *et al.*, 1987). Experiments were performed in Krebs' solution containing 2.8×10^{-6} M indomethacin at 37°C in 95% O_2/5% CO_2, and changes in tension generated were measured isometrically. Some tissues were denuded of endothelium by gentle abrasion with a scored cannula.

Rings were contracted with the thromboxane mimetic U46619 (10^{-8} M), and the functional integrity of the endothelium was checked by monitoring relaxation to acetylcholine (10^{-6} M). After washing and

ABBREVIATIONS: ADP-β-F, adenosine 5′-(2-fluorodiphosphate); ADP-β-S, adenosine 5′-(2-thiodiphosphate); 8-SPT, 8-sulphophenyltheophylline; 2-MeSATP, 2-methylthio-D-ATP; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; E/[A], agonist concentration-effect relationship; NECA, 5′-(N-ethylcarboxamido)-adenosine.

recontraction with U46619, a single E/[A] relaxation curve was constructed to either ADP-β-F (3×10^{-7} to 10^{-3} M) or ADP-β-S (10^{-8} to 10^{-4} M). Using a separate tissue for each E/[A] curve, relaxant responses to ADP-β-F and ADP-β-S were determined in endothelium-intact or -denuded preparations in the presence or absence of the selective P_1 purinoceptor antagonist 8-SPT (3×10^{-4} M). This concentration of 8-SPT produced a 100-fold rightward shift of the curve to the selective P_1 purinoceptor agonist NECA in a denuded preparation (mean dose ratio 99.02 ± 18.8, S.E., $n = 7$). This was consistent with published values of the pA_2 for 8-SPT (Collis *et al.*, 1987; Kenakin and Beek, 1987). The same dose of 8-SPT produced no shift of a curve to 2-MeSATP.

All E/[A] curves were constructed cumulatively. It is to be noted (see fig. 1) that endothelium-dependent effects were not well sustained, possibly calling into question the use of cumulative techniques. However, a preliminary experiment comparing curves obtained to 2-MeSATP by single sequential and cumulative applications indicated no significant difference in either the maximum or $[A_{50}]$ of the curves.

Relaxant responses were measured as a percentage of the U46619-induced contraction and expressed graphically in the form of E/log[A] curves for each experimental condition. Negative log molar agonist concentrations producing 50% of maximum response ($p[A_{50}]$ values) were calculated as the index of agonist potency by logistic curve fitting (Leff *et al.*, 1987). In order to support the analysis of multiphasic E/[A] curves, the data generated were compared with computer simulations of the "one agonist:two receptor" theoretical model of Furchgott (1981).

Drugs. ADP-β-F was synthesised by D. Cox in the Department of Medicinal Chemistry, Fisons, Loughborough, U.K. ADP-β-S was purchased from Boehringer Mannheim (Mannheim, F.R.G.); 8-SPT was purchased from Research Biochemicals Inc. (St. Albans, U.K.); and acetylcholine, indomethacin and U46619 were purchased from Sigma Chemical Co. (St. Louis, M.O.). Indomethacin was dissolved initially in 10% Na_2CO_3, and all other drugs were dissolved in distilled water.

Results

In endothelium-intact tissues, ADP-β-F produced a multiphasic E/[A] curve which appeared to consist of two vasorelaxant components. Figure 1a illustrates that responses were slow and tonic over the concentration range 3×10^{-7} to 3×10^{-5} M, but fast and phasic between 10^{-4} and 10^{-3} M. In intact tissues in the presence of 8-SPT (fig. 1b), the higher potency phase was abolished, revealing the apparently monophasic lower potency component, which had its threshold around 3×10^{-5} M. The converse result was obtained upon endothelial denudation (fig. 1c), with ADP-β-F producing slow, tonic relaxant responses with a threshold around 3×10^{-7} M, which became maximal at 10^{-4} M. On some occasions, higher concentrations of ADP-β-F were examined [as illustrated by fig. 1, where 3×10^{-4} (two occasions) and 10^{-3} M (one occasion) were used], revealing virtually complete abolition of the phasic component. Mean data from these studies ($n = 4$–6) are shown in figure 2. In denuded tissues, relaxant responses to ADP-β-F ($p[A_{50}]$ 5.58 ± 0.13, S.E., $n = 6$) were virtually abolished by 8-SPT, indicating that they were due to activation of P_1 purinoceptors. The lower potency endothelium-dependent component, fully defined only in the presence of 8-SPT, had a $p[A_{50}]$ of 3.98 ± 0.07 (S.E., $n = 4$). A computer simulation of the "one-agonist:two-receptor model" (Furchgott, 1981) (fig. 3b) shows good agreement with E/[A] curves constructed from the experimental data (fig. 3a), thus increasing the confidence with which the endothelium-dependent component can be described quantitatively.

The E/[A] curve to ADP-β-S, by contrast, appeared monophasic, consisting of fast, phasic relaxant responses over the range 3×10^{-8} to 10^{-5} M. These responses were unaffected by 8-SPT (fig. 4) and were apparently of endothelial origin ($p[A_{50}]$ 6.86 ± 0.12, S.E., $n = 5$). However, removal of endothelium did reveal slow, tonic relaxations occurring over a slightly higher concentration range ($p[A_{50}]$ 5.73 ± 0.20, S.E., $n = 4$) that were blocked by 8-SPT.

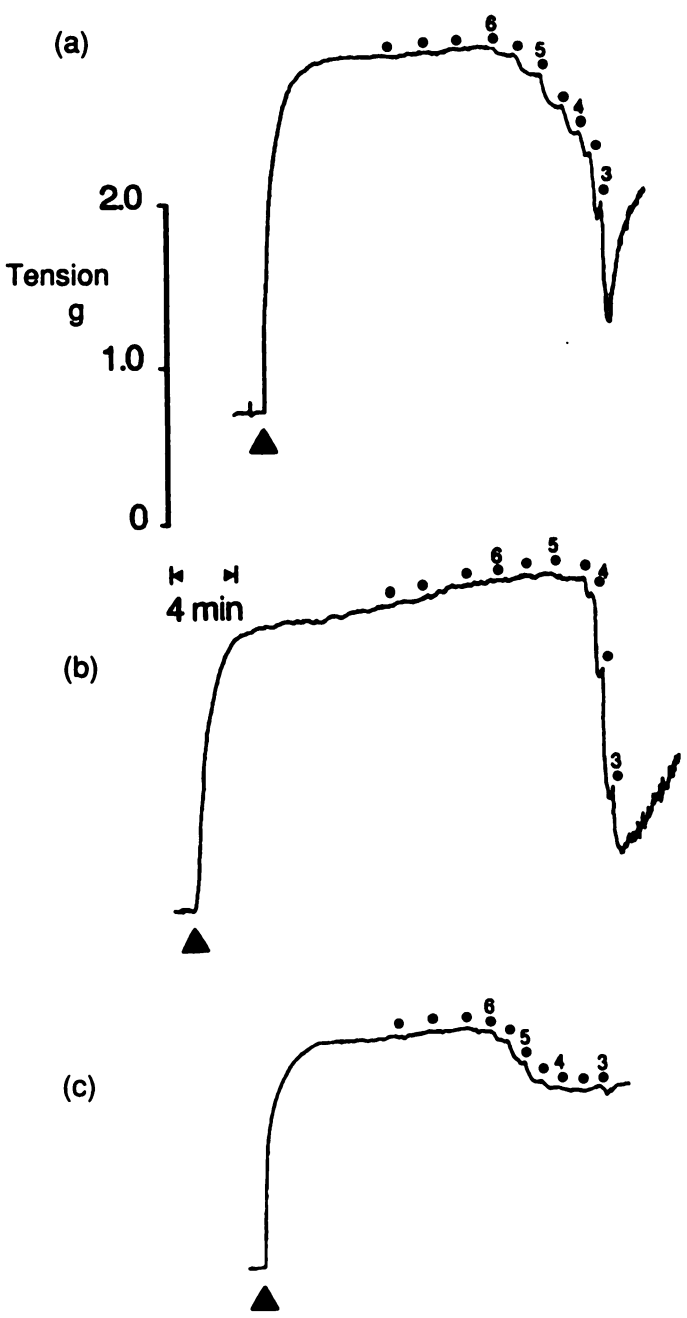

Fig. 1. Reproduction of original traces showing the relaxant responses of rabbit precontracted jugular vein to ADP-β-F. Event marker indicates the addition of U46619 (10^{-8} M) to contract the tissues. ●, indicate the cumulative administration of ADP-β-F with the associated numbers representing negative log molar drug concentration. a) Tissue with intact endothelium; b) tissue with intact endothelium in the presence of 8-SPT (3×10^{-4} M); c) tissue denuded of endothelium.

The rabbit jugular vein was chosen for this study because it exhibits well-defined relaxant responses to agonists at both P_{2Y} and P_1 purinoceptors and, unlike visceral smooth muscle, allows

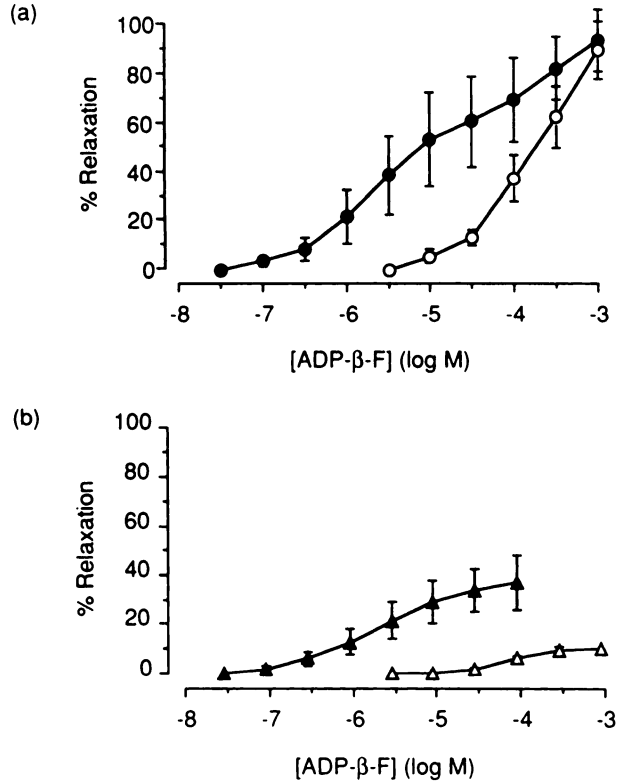

Fig. 2. Summary of mean data ± S.E. for the relaxant effects of ADP-β-F in the rabbit jugular vein. Relaxant responses are expressed as a percent of the U46619 contraction. a) Endothelium intact: (●) control; (○) in the presence of 8-SPT (3×10^{-4} M) (p[A_{50}] 3.98 ± 0.07, n = 5). b) Endothelium denuded: (▲) control (p[A_{50}] 5.58 ± 0.13, n = 6); (△) in the presence of 8-SPT (3×10^{-4} M).

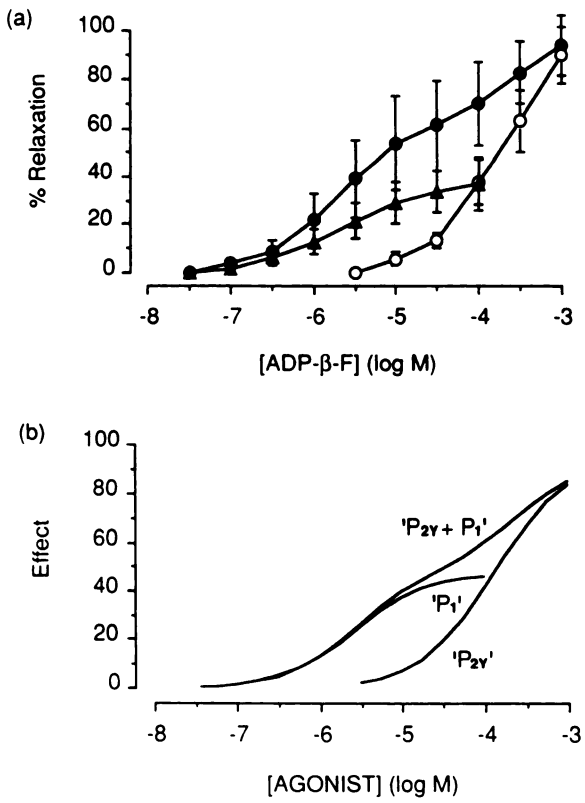

Fig. 3. Relaxant responses to ADP-β-F in rabbit jugular vein with intact endothelium either in the presence (○) or absence (●) of 8-SPT (3×10^{-4} M), or denuded of endothelium (▲). Comparison of the experimental data (a) with a computer simulation of the "one-agonist:two-receptor model" (Furchgott, 1981) (b). In panel b, the lines are labeled to indicate which receptor-mediated component each represents.

ready separation of P_1 from P_{2Y} components by simple removal of the endothelium. In addition, it appears to lack a significant population of constrictor P_{2X} purinoceptors, activation of which might have interfered with the analysis of relaxant responses. The potent P_{2X} agonist D-α,β-methylene ATP, which has a p[A_{50}] of 6.47 as a spasmogen of the rabbit ear artery (O'Connor *et al.*, 1990), does not contract denuded jugular veins at concentrations below 3×10^{-4} M (unpublished observations).

The selectivity of ADP-β-F is overtly suspect because it produces two kinetically and pharmacologically distinct relaxant responses in the jugular vein. Based on the observed sensitivity to 8-SPT, the established P_1 purinoceptor-selective antagonist, its primary effect is mediated at P_1 purinoceptors located on the smooth muscle. Endothelium-dependent relaxations presumably mediated by P_{2Y} purinoceptors occurred only at higher concentrations.

8-SPT has been reported to be a selective P_1 purinoceptor antagonist (Collis *et al.*, 1987) that lacks significant phosphodiesterase inhibitory properties (Gustafsson, 1984). This presumption appears reasonable based on the evidence presented here and unpublished observations with other ATP analogs, but cannot be tested in a definitive fashion because of the lack of a selective competitive receptor antagonist for this subtype. Reactive blue 2, which has been used for this purpose (Burnstock and Warland, 1987), demonstrates nonspecific properties in the jugular vein (unpublished observations), as were found in the rabbit portal vein (Reilly *et al.*, 1987).

Theoretical analysis showed that the results were qualitatively consistent with the one-agonist:two-receptor model of Furchgott (1981). Furchgott's model assumes that the stimuli produced by activation of the two receptor populations are additive and that the measured response is related saturably to the sum of the two stimuli. This is a pharmacological and not a molecular description, and its use here cannot and is not intended to provide insight into the transduction pathways linked to the two receptors. For example, it makes no allowance for synergistic or antagonistic interactions between the two pathways. The close agreement, therefore, between the model and the experimental data supports the contention that the multiphasic relaxant E/[A] curve produced by ADP-β-F in the intact jugular vein is simply the composite of activation of both smooth muscle P_1 purinoceptors and endothelial P_{2Y} purinoceptors. On this basis, the purely P_{2Y}-mediated component could be reliably quantified.

Hourani *et al.* (1988) failed to observe P_1 agonist properties of ADP-β-F in the *T. coli.* Our use of vascular rather than visceral smooth muscle may be advantageous in this respect because it allows selective abolition of the P_{2Y} component by removal of the endothelium. Hourani *et al.* also report a much higher P_{2Y} potency for ADP-β-F in the *Taenia* (p[A_{50}] 5.5) than our estimate from the jugular vein. One possible explanation, therefore, for this lack of agreement is a marked difference in the relative densities of P_1 and P_{2Y} purinoceptors between guinea pig *Taenia* and rabbit jugular vein. Hence, the jugular vein may favor expression of P_1-mediated responses and the *Taenia* may favor the expression of P_{2Y}-mediated responses.

ADP-β-S presented as a much more potent and selective P_{2Y} agonist than ADP-β-F, although a P_1-mediated component was

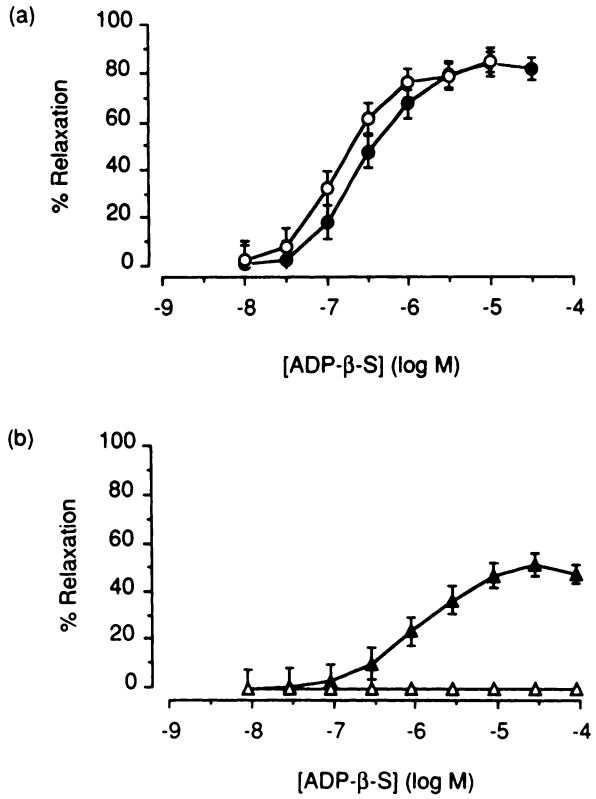

Fig. 4. Summary of mean data ± S.E. for the relaxant effects of ADP-β-S in the rabbit jugular vein. Relaxant responses are expressed as a percent of the U46619 contraction. a) Endothelium intact: (●) control, (○) in the presence of 8-SPT (3×10^{-4} M) (p[A_{50}] 6.86 ± 0.12, $n = 5$). b) Endothelium denuded: (▲) control (p[A_{50}] 5.73 ± 0.20, $n = 4$); (△) in the presence of 8-SPT (3×10^{-4} M).

evident in denuded tissues over a slightly higher concentration range. Based on p[A_{50}] values, the P_{2Y}/P_1 selectivity of ADP-β-S was 13, compared with 0.03 for ADP-β-F. In denuded tissues, 8-SPT caused a greater shift of the relaxation curve to ADP-β-S than the expected 100-fold shift found with NECA studies. The explanation for this is unclear, although we have observed weak non-P_{2X} spasmogenic effects of ADP-β-S in rabbit ear artery (unpublished observation), which would tend to oppose its dilator properties at high concentrations. [^{35}S] ADP-β-S has been reported to label a single high-affinity binding site in turkey erythrocyte membranes with properties consistent with that of a P_{2Y} purinoceptor (Cooper *et al.*, 1989).

The maximum relaxation produced by activation of P_1 purinoceptors by both ADP-β-F and ADP-β-S was much less than that produced by activation of P_{2Y} purinoceptors under the same conditions. In this system at least, ADP-β-F, although P_1-selective, can induce a greater relaxation, albeit at higher concentrations, *via* P_{2Y} purinoceptors.

In the *Taenia*, ADP-β-F is reported to be relatively resistant to degradation by ectonucleotidases, being broken down much less readily than ATP (Hourani *et al.*, 1988). ADP-β-S is similarly resistant (Welford *et al.*, 1987). Based on these studies, it is most likely that all the effects we have observed in the jugular vein are directly attributable to the parent compounds. We cannot, however, exclude the possibility that some degradation to adenosine contributed to the P_1 component of the responses. There is no information on how the capacity of the jugular vein to degrade ATP analogs compares with other tissues.

To summarize, in the rabbit jugular vein, relaxations produced by ADP-β-F were principally mediated through activation of P_1 purinoceptors, and the compound appeared to have only weak P_{2Y} agonist properties. It is, therefore, invalid to regard this agonist as a selective probe for P_{2Y} purinoceptors; however, ADP-β-S does show some degree of selectivity for the P_{2Y} purinoceptor and may be considered as a useful probe. If ADP-β-F is used in the classification of P_2 purinoceptors, appropriate precautions should be taken to eliminate its P_1 purinoceptor properties.

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