Characterization of purinoceptors mediating depolarization of rat isolated vagus nerve

¹D.J. Trezise, I. Kennedy & P.P.A. Humphrey

Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 As part of a broader study to characterize neuronal purinoceptors, the effects of adenosine 5'-triphosphate (ATP) and a range of ATP analogues were investigated on the extracellularly recorded membrane potential of the rat isolated vagus nerve, using a 'grease-gap' technique.

2 ATP evoked depolarization of the rat vagus nerve. The concentration-effect curve to ATP was not monophasic: at the lower concentrations (1×10^{-5} – 1×10^{-3} M) the curve was shallow (< 50% of the near maximal response to 5-hydroxytryptamine (5-HT)) whilst at higher concentrations the relationship between concentration and amplitude of depolarization was steeper (> 135% of the response to 5-HT at the highest concentration tested, 1×10^{-2} M). On washout of the high drug concentrations large after-hyperpolarizations were often observed.

3 α,β -methylene ATP (1×10^{-6} – 3×10^{-4} M), β,γ -methylene ATP (1×10^{-6} – 1×10^{-3} M), and 5'-adenylylimidodiphosphate (β,γ -imido ATP; 1×10^{-6} – 1×10^{-3} M) were all more potent than ATP and produced large depolarizations of the rat vagus nerve at the highest concentrations tested (> 150% of the response to 5-HT). The overall rank order of potency was α,β -methylene ATP > β,γ -methylene ATP = β,γ -imido ATP > ATP.

4 In contrast, 2-methylthio ATP (1×10^{-6} – 1×10^{-3} M) produced relatively small depolarizations (< 100% of the response to 5-HT). As was the case with low concentrations of ATP, the concentration-effect curve to 2-methylthio ATP was very shallow.

5 Adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine and adenosine 5'-O-(2-thiodiphosphate) (ADP- β -s; all 1×10^{-6} – 1×10^{-3} M) evoked only small depolarizations of the vagus nerve, amounting to $47 \pm 2.5\%$, $40.8 \pm 7.8\%$, $33.7 \pm 3.3\%$ and $62.4 \pm 12.7\%$ of the response to 5-HT, respectively. Uridine 5'-triphosphate (UTP; 1×10^{-6} – 1×10^{-3} M) was inactive.

6 The P_2 purinoceptor antagonist, suramin (1×10^{-5} M– 1×10^{-4} M), antagonized responses to α,β -methylene ATP. The nature of this antagonism was not, however, consistent with simple competitive kinetics between agonist and antagonist. Depolarizations produced by β,γ -methylene ATP and β,γ -imido ATP were also attenuated by suramin (1×10^{-4} M), but in contrast, suramin had no effect on responses to ADP, 2-methylthio ATP, ADP- β -S or 5-HT.

7 In addition to its antagonist effects, suramin (10^{-4} M) markedly increased the maximum amplitude of the depolarization produced by ATP.

8 It is concluded that a heterogeneous receptor population mediates depolarization of the rat vagus nerve by purine nucleotides. Importantly, the large amplitude depolarizations to α,β -methylene ATP, β,γ -methylene ATP and β,γ -imido ATP are mediated via receptors that share many characteristics of the classical P_{2x} receptor. In contrast, the relatively small depolarizing effects of ADP, ADP- β -S and 2-methylthio ATP were suramin-resistant. Although it appears that other purinoceptors are present, these data suggest that the rat vagus nerve may serve as a useful preparation for studying the pharmacology of neuronal P_{2x} receptors.

Keywords: Rat vagus nerve; ATP; P_2 purinoceptors; suramin; depolarization

Introduction

It is widely accepted that adenosine 5'-triphosphate (ATP) acts as an excitatory co-transmitter of certain postganglionic parasympathetic, postganglionic sympathetic and sensory neurones (see Burnstock, 1990 for review). More recently direct evidence for a role for ATP in excitatory synaptic transmission has been provided (Evans *et al.*, 1992; Silinsky *et al.*, 1992). Neuromodulatory effects of purines in the central nervous system have also been described (Harms *et al.*, 1992; Sun *et al.*, 1992; Tschöpl *et al.*, 1992). As with other neurotransmitters, the neuronal effects of ATP are mediated through specific extracellular receptors. These receptors for ATP have been termed purinoceptors although they may belong to a larger family of nucleotide receptors (Burnstock & Kennedy, 1985; Cusack, 1993).

To date, relatively few studies have focused on the characterization of neuronal purinoceptors. Indeed, the framework

for the present purinoceptor classification system (Burnstock, 1978; Burnstock & Kennedy, 1985) is founded almost exclusively on studies in non-neuronal, often multicellular preparations. From these studies purinoceptors have been divided into P_1 receptors, at which adenosine and AMP are more active than ADP and ATP, and P_2 receptors for which the reverse rank order of agonist potency occurs. Both the P_1 and P_2 receptor groups have been further subdivided on the basis of relative potencies of adenosine and ATP analogues and on the antagonist profiles of drugs such as the methylxanthines and suramin (see Fedan & Lamport, 1990, and Cussack, 1993, for reviews). At least four different subtypes of P_2 purinoceptors have been proposed (Burnstock & Kennedy, 1985; Gordon, 1986). The P_{2x} and P_{2y} receptor subtypes were first described on the basis of differential relative potencies of ATP analogues in vascular and visceral smooth muscles such that α,β -methylene ATP is considered to be most potent at P_{2x} receptors and 2-methylthio ATP is most potent at P_{2y} receptors (Burnstock & Kennedy, 1985). P_{2t} and P_{2z}

¹ Author for correspondence.

receptors have been characterized in studies on platelets and mast cells, respectively (Gordon, 1986). Most recently a binding site resembling a P_2 purinoceptor in rat brain synaptosomes has been tentatively designated P_{2d} (Pintor *et al.*, 1993).

Electrophysiological experiments in single neurones and brain slices have provided some indication of the nature of purinoceptors mediating membrane depolarization (see Bean, 1992, and Illes & Nörenberg, 1993, for reviews). For example, structure-function relationships for purine nucleotide-evoked depolarization of rat cultured nodose ganglion neurones have been described (Krishtal *et al.*, 1988b) although no attempt was made to classify the receptor type(s) mediating this effect. In bullfrog dorsal root ganglion cells and rat and guinea-pig cardiac ganglia, membrane depolarization evoked by ATP is mediated via a P_2 purinoceptor which resembles the P_{2y} receptor (Allen & Burnstock, 1990; Tokimasa & Akasu, 1990; Fieber & Adams, 1991). In contrast, the purinoceptor that mediates excitation of neurones in the rat locus coeruleus paradoxically shares characteristics of both the P_{2x} and the P_{2y} receptor (Harms *et al.*, 1992; Tschöpl *et al.*, 1992; but see Illes & Nörenberg, 1993). However, the lack of simple quantitative neuronal assay systems for the measurement of relative agonist potencies and of antagonist affinities has undoubtedly hindered the characterization of neuronal purinoceptors.

We describe for the first time the depolarizing effect of ATP and ATP analogues on the extracellularly recorded membrane potential of the rat vagus nerve, measured by a 'grease-gap' technique. This preparation provides a technically convenient assay for the characterization of neuronal purinoceptors.

Methods

Extracellular recordings of agonist-induced depolarizations were made from segments of rat isolated cervical vagus nerve according to the method of Ireland & Tyers (1987). Briefly,

male AHA Wistar rats (200–270 g) were stunned by a blow to the head, decapitated, and the cervical vagus nerves rapidly excised. Segments of nerve, approximately 15–20 mm long, were desheathed under a dissecting microscope, and transferred to heated (27°C) two-compartment Perspex baths such that approximately 50% of the nerve lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. The d.c. potential between the two compartments was measured with silver-silver chloride electrodes connected to the preparation through agar-saline/filter paper bridges. Signals were amplified, filtered (0.5 Hz) and displayed on a chart recorder (Lectromed Multitrace 8). Each compartment of the bath was perfused continuously with Krebs solution, preheated to 27°C and gassed with 95% O_2 /5% CO_2 , at a rate of 1–2 ml min⁻¹. Drugs were applied at known concentrations into the perfusate of the first compartment only.

Experimental protocols

After a 30 min equilibration period the viability of each preparation was assessed by exposure to a near maximal concentration of 5-HT (1×10^{-5} M) for 2 min. Preparations that depolarized by less than 250 μ V were rejected (<15% of preparations). Repeated exposures to 5-HT (1×10^{-5} M) for 2 min at 30 min intervals were performed until reproducible depolarizations were obtained. After a further 30 min re-equilibration period, concentration-effect curves for agonist-induced depolarization were constructed non-cumulatively using serially increasing concentrations. Each concentration of agonist was applied for 2–2.5 min during which time a peak effect was reached. An interval of 45 min was left between agonist applications since in preliminary experiments depolarizing responses to purinoceptor drugs showed tachyphylaxis if shorter intervals were employed. Use of this protocol precluded construction of more than one concentration-effect curve in any one preparation. Relative agonist potencies and the effects of antagonists were thus determined by comparing with respective time- and vehicle-matched con-

Figure 1 Discontinuous records of the effect of (a) adenosine 5'-triphosphate (ATP) and (b) α,β -methylene ATP on extracellularly recorded membrane potentials in the rat isolated vagus nerve. An upward deflection indicates depolarization of the nerve trunk, calibrated in mV. Each concentration of drug was perfused for the time period indicated by the solid horizontal bar; 45 min was left between agonist applications. For comparison the near maximal depolarization to 5-hydroxytryptamine (5-HT, 1×10^{-5} M) in the same preparation is also shown.

trol curves from other preparations. Antagonist contact times of 45 min were employed.

Analysis of results

The depolarization evoked by purinoceptor agonists was measured as the peak change (μV) in the d.c. potential between the two compartments, and expressed as a percentage of the response to 5-HT ($1 \times 10^{-5} \text{ M}$). Due to the limited availability of some agents it was not always possible to achieve the maximum of the agonist concentration-effect curve. Thus, to measure agonist potency, the concentration of agonist required to produce the equivalent of 100% of the response to 5-HT ($1 \times 10^{-5} \text{ M}$) was estimated from individual concentration-effect curves. This is referred to as EC_{100} . A response of this magnitude lay on the linear part of the concentration-effect curve for the purinoceptor agonists that evoked marked depolarizations. Antagonist effects were quantified, where possible, by measuring lateral displacements of the concentration-effect curves at a level of 50% of the maximum measured control response to give concentration-ratios between antagonist-treated and control, vehicle-treated preparations. In cases where the antagonist depressed the agonist response such that parallel displacements of the concentration-effect curve were not observed and lateral displacements could not be calculated, the results are expressed as percent inhibition of the agonist response at the highest concentration tested.

Data are expressed as arithmetic mean $\pm$ s.e.mean or geometric mean with 95% confidence limits where appropriate. Differences between groups were assessed by Student's unpaired t test and considered significant when $P < 0.05$.

Drugs and solutions

The composition of the Krebs solution was as follows (mM in de-ionised water): NaCl 118, NaHCO_3 25, KCl 4.7, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6, KH_2PO_4 1.2, D-glucose 11.1, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 1.3. The following drugs were used: 5-hydroxytryptamine creatinine sulphate (5-HT), adenosine 5'-triphosphate disodium salt (ATP), α,β -methylene ATP lithium salt, uridine 5'-triphosphate sodium salt (UTP), β,γ -methylene ATP, 5'-adenylylimidodiphosphate lithium salt (β,γ -imido ATP), adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5'-O-(2-thiodiphosphate) trilithium salt (ADP- β -s, all Sigma), 2-methylthio ATP tetra sodium salt (Research Biochemicals Incorporated), suramin (Bayer). All drugs were dissolved and diluted to the required concentration in Krebs solution, and stored on ice.

Results

Effect of ATP

Adenosine 5'-triphosphate evoked concentration-related depolarizations of rat isolated vagus nerve, amounting to $138.4 \pm 22.0\%$ ($n = 4$) of the amplitude of the response to 5-HT at the highest concentration tested ($1 \times 10^{-2} \text{ M}$; see Figure 1a and Figure 2). At the higher concentrations some preparations displayed marked after-hyperpolarizations on washout of the drug. The concentration-effect curve to ATP was not monophasic; at concentrations up to $1 \times 10^{-3} \text{ M}$ the relationship between concentration and depolarizing effect was very shallow whilst higher concentrations (1×10^{-3} – $1 \times 10^{-2} \text{ M}$) produced a much steeper concentration-effect curve.

Effect of analogues of ATP

A range of ATP analogues were tested for activity on the rat vagus nerve (Figures 2 and 3). Of these, α,β -methylene ATP

Figure 2 Comparison of the depolarizing effects of α,β -methylene ATP ($\bullet$), β,γ -imido ATP (∇), β,γ -methylene ATP ($\blacktriangle$), 2-methylthio ATP ($\blacksquare$) and ATP ($\circ$) on rat isolated vagus nerve. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4-8$). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-hydroxytryptamine (5-HT, $1 \times 10^{-5} \text{ M}$).

Figure 3 Depolarizing effects of ADP (Δ), AMP ($\square$), adenosine ($\blacktriangle$) and ADP- β -S ($\blacktriangledown$) on rat isolated vagus nerve. For comparison the effect of α,β -methylene ATP ($\bullet$) is also shown. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4-8$). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-hydroxytryptamine (5-HT, $1 \times 10^{-5} \text{ M}$).

was the most potent with a threshold concentration for depolarization of $1 \times 10^{-6} \text{ M}$ and an EC_{100} value of $1.75 \times 10^{-5} \text{ M}$ (95% confidence intervals $0.97-3.18 \times 10^{-5} \text{ M}$, $n = 8$). The maximal effect of α,β -methylene ATP was $165.2 \pm 15.0\%$ of the response to 5-HT. Typically, depolarizations to α,β -methylene ATP reached the peak response more rapidly than responses to ATP and faded more rapidly in the continued presence of the drug (see Figure 1). Both β,γ -imido ATP and β,γ -methylene ATP were approximately 15 fold less potent than α,β -methylene ATP with respective EC_{100} values of $2.70 \times 10^{-4} \text{ M}$ ($1.49-4.92 \times 10^{-4} \text{ M}$, $n = 4$) and $3.48 \times 10^{-4} \text{ M}$ ($2.30-5.27 \times 10^{-4} \text{ M}$, $n = 4$) and evoked similar maximal depolarizations at the highest concentration tested ($1 \times 10^{-3} \text{ M}$; $184.3 \pm 20.0\%$ and $171.2 \pm 17.5\%$, respectively). In general, depolarizations to β,γ -imido ATP and β,γ -methylene ATP also achieved the peak effect more rapidly than responses to ATP, but the marked fade observed with α,β -methylene ATP was less apparent. Marked after-hyperpolarizations were observed on washout of high concentrations of these methylene and imido analogues of ATP. The concentration-effect curve to 2-methylthio-ATP was shallow, and at the highest concentration tested ($1 \times 10^{-3} \text{ M}$) the amplitude of the depolarizing effect of this agent was less than 100% of the response to

5-HT. Consequently, an EC_{100} value could not be determined. ADP, AMP and adenosine (all 1×10^{-6} M– 1×10^{-3} M) evoked relatively small depolarizations of the vagus nerve, the peak effects at 1×10^{-3} M being $47.0 \pm 2.5\%$ ($n = 4$), 40.8 ± 7.8 ($n = 4$) and $33.7 \pm 3.3\%$ ($n = 8$) of the response to 5-HT, respectively (see Figure 3). Similarly, depolarizations to ADP- β -S (1×10^{-6} – 3×10^{-4} M) were small in comparison to the 5-HT response (62.4 ± 12.7 , $n = 7$). Uridine 5'-triphosphate was inactive at concentrations up to 1×10^{-3} M.

Effect of suramin on depolarization to ATP analogues

Suramin (1×10^{-5} – 1×10^{-4} M) had no effect on the extracellularly-recorded membrane potential, but produced rightward displacement of the concentration-effect curve to α,β -methylene ATP (see Figure 4a). At concentrations of 1×10^{-5} M and 3×10^{-5} M the agonist concentration-ratios in the presence of suramin did not appear to be concentration-related (8.8 ± 3.1 , $n = 5$ and 3.8 ± 0.6 , $n = 4$, respectively). The analysis of these data was complicated by an increase in the maximum response to α,β -methylene ATP in the presence of suramin. At the highest concentration of suramin tested (1×10^{-4} M) the displacement appeared parallel and the mean concentration-ratio was 15.5 ± 5.6 ($n = 5$). This corresponds to a pK_B value of 5.04 ± 0.15 . Suramin (1×10^{-4} M) also antagonized responses to β,γ -imido ATP (see Figure 5a) and β,γ -methylene ATP; the percentage inhibition of depolarization at the highest concentration of agonist tested (1×10^{-3} M) was $67.1 \pm 3.1\%$ ($n = 4$) and $57.2 \pm 10.7\%$ ($n = 5$), respectively.

In contrast, depolarizations to 2-methylthio ATP (Figure 5b), ADP and ADP- β -S were resistant to antagonism by suramin (concentration-ratios of 0.93 ± 0.27 , 2.34 ± 0.68 and 1.61 ± 0.57 , all $n = 4$, respectively).

Effects of suramin on depolarizations to ATP

The maximum amplitude of the depolarization evoked by ATP was markedly enhanced by suramin (1×10^{-4} M; see Figure 5c).

Specificity of suramin

Concentration-effect curves to 5-HT (1×10^{-7} – 3×10^{-5} M) were constructed in the presence of suramin or vehicle fol-

Figure 4 Effect of suramin on depolarizations of rat isolated vagus nerve evoked by (a) α,β -methylene ATP and (b) 5-hydroxytryptamine (5-HT). Symbols indicate responses to agonist in the presence of vehicle (○) or in the presence of 1×10^{-5} M (▲), 3×10^{-5} M (■) or 1×10^{-4} M suramin (●). Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4-5$). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-HT (1×10^{-5} M). Note the rightward displacement of the α,β -methylene ATP concentration-effect curve by suramin, and the increase in the maximum response to the agonist.

Figure 5 Effect of suramin on depolarizations of rat vagus nerve to (a) β,γ -imido ATP, (b) 2-methylthio ATP and (c) adenosine 5'-triphosphate (ATP). The responses to each agonist in the presence of vehicle or suramin (1×10^{-4} M) are represented by (○) and by (●), respectively. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4-5$). In each panel the abscissa scale shows the log molar concentration of agonist and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-HT (1×10^{-5} M). Note that suramin antagonizes responses to β,γ -imido ATP but not 2-methylthio ATP. Depolarizations to ATP were augmented in the presence of suramin.

lowing the same protocol described for the ATP analogues. Maximal responses to 5-HT were achieved such that EC_{50} values could be calculated. 5-HT evoked concentration-related depolarizations of rat vagus nerve with a mean EC_{50} value of 4.79×10^{-7} M (3.04 – 7.51×10^{-7} M, $n = 4$). Suramin (1×10^{-5} , 3×10^{-5} , 1×10^{-4} M) had no significant effect on either the potency or maximum amplitude of depolarization produced by 5-HT (see Figure 4b).

Discussion

In the present study, using an extracellular recording system, we observed that ATP and other purine nucleotides depolarized the rat isolated vagus nerve. Although several other workers have shown that ATP excites neurones grown in culture from parasympathetic cardiac ganglia (Allen & Burnstock, 1990; Fieber & Adams, 1991) and nodose ganglia (Krishtal *et al.*, 1988a,b) this is, as far as we know, the first demonstration of a depolarizing effect of this agent on whole vagal nerve trunks.

The depolarizing action of ATP was marked but high concentrations were necessary. This, perhaps, reflects the rapid degradation of the nucleotide by ecto-nucleotidases to ADP, AMP and adenosine (see Welford *et al.*, 1987 and Hourani & Chown, 1989). In other neuronal preparations, adenosine evokes hyperpolarizing responses which offset the excitatory effects of ATP (e.g. Tschöpl *et al.*, 1992; Illes & Nörenberg, 1993). This would not, however, appear to be the case in the rat vagus nerve since adenosine itself evoked small depolarizations ($< 50\%$ of the response to 5-HT) rather than hyperpolarizations. Furthermore, in preliminary experiments responses to ATP were not modified by the adenosine antagonist 8-parasulphophenyltheophylline (3×10^{-5} M; data not shown). Another possibility is that ATP simultaneously activates an inhibitory purinoceptor on the vagus nerve. However, even though the marked depolarization evoked by high concentrations of ATP was often followed by a large after-hyperpolarization on washout of the drug it seems improbable that this reflects activation of an inhibitory receptor since there was no indication of an initial hyperpolarizing effect of any of the other purines tested. It seems more plausible that the after-hyperpolarization is due to activation of membrane Na^+/K^+ ATPases since similar, ouabain-sensitive after-hyperpolarizations have been reported with

other depolarizing agents (e.g. acetylcholine, Brown *et al.*, 1972). However, the ouabain-sensitivity of the after-hyperpolarization evoked by ATP has not been examined and so this possibility cannot be excluded.

The concentration-effect curve to ATP was clearly not monophasic; at low concentrations (1×10^{-5} – 1×10^{-3} M) a very shallow relationship between concentration and the amplitude of depolarization was observed, whilst at higher concentrations ($>1 \times 10^{-3}$ M) this relationship was much steeper. Importantly, the large amplitude depolarizations evoked by ATP could not be attributed to excitatory effects of either ADP, AMP or adenosine, since responses to these purines were relatively small. These data suggest that a P_2 , rather than a P_1 purinoceptor mediates the large amplitude depolarizations to ATP (Burnstock & Kennedy, 1985).

The relative potencies and amplitudes of depolarization of other ATP analogues support this proposal. Of the purines tested only three other agonists (α,β -methylene ATP, β,γ -imido ATP, and β,γ -methylene ATP) evoked depolarizations whose peak amplitude was comparable with the effect of high concentrations of ATP. Although true maximal responses for β,γ -imido ATP and β,γ -methylene ATP could not be determined (due to limited availability of these compounds) the amplitude of the depolarizations to these agents at the highest concentration tested were similar to the maximal effect of α,β -methylene ATP. α,β -methylene ATP was the most potent being some 15–20 fold more potent than both β,γ -imido ATP and β,γ -methylene ATP, and 330 fold more potent than ATP itself. Similar rank orders of agonist potency for actions at P_{2x} receptors in the guinea-pig bladder (Cusack & Hourani, 1984) and the rabbit ear artery (O'Connor *et al.*, 1990) have been described. Indeed, this high potency of α,β -methylene ATP is generally considered indicative of an action at this receptor subtype. Moreover, 2-methylthio ATP and ADP- β -S, purines that show certain selectivity for the P_{2y} purinoceptor subtype (Burnstock & Kennedy, 1985; Cusack 1993) were relatively weak. Although it is prudent to be cautious when characterizing receptor subtypes on the basis of relative agonist potencies, taken together, these observations are consistent with the view that a P_{2x} purinoceptor mediates the large amplitude depolarizations of the rat vagus nerve to purine nucleotides.

In other preparations activation of P_{2x} purinoceptors by α,β -methylene ATP, and to a lesser degree ATP, evokes pronounced tachyphylaxis (Kasakov & Burnstock, 1983; see Fedan & Lampion, 1990). This phenomenon is not considered to occur with P_{2y} purinoceptors (Burnstock & Kennedy, 1985). Although tachyphylaxis was not specifically investigated in the present study, it was clear that the depolarization produced by high concentrations of α,β -methylene ATP faded rapidly in the continued presence of the drug. In addition, consecutive responses to this agent became progressively smaller if less than 45 min was permitted between drug applications. Interestingly, the fade of depolarizations produced by ATP and the β,γ -methylene and β,γ -imido analogues of ATP was less marked. It remains to be seen whether these superficial observations on fade of response correlate in any way with tachyphylaxis and receptor desensitization. Furthermore, more work is needed to determine whether desensitization is a characteristic of P_{2x} receptors in general or, alternatively, whether it is a specific property of the agonist α,β -methylene ATP itself.

The finding that the P_2 purinoceptor antagonist, suramin (Dunn & Blakeley, 1988; Leff *et al.*, 1990) inhibited depolarizations to α,β -methylene ATP, β,γ -imido ATP and β,γ -methylene ATP provides further evidence for the involvement of a P_2 receptor. Under our experimental conditions suramin did not, however, behave simply as a classical competitive antagonist, in that the magnitude of the displacement of the agonist concentration-effect curve was not directly related to the antagonist concentration. Interestingly, in rabbit ear artery suramin was shown to equilibrate only very slowly with the P_{2x} receptor and the nature of the antagonism

was closely related to the incubation time (Leff *et al.*, 1990). Since in the present study the agonist concentration-effect curve took almost 5 h to construct it is possible that the complex nature of the antagonism is in some way related to the differing incubation times that the effects of each concentration of agonist were investigated at. The augmentation of responses to high concentrations of α,β -methylene ATP by suramin (1×10^{-5} M and 3×10^{-5} M, but not 1×10^{-4} M) is a further complicating factor. Indeed, somewhat surprisingly, suramin augmented rather than antagonized the responses to high concentrations of ATP. It seems unlikely that this reflects a non-specific membrane effect of suramin in that depolarizations to 5-HT were unaffected. A similar augmentation by suramin of contractions of the guinea-pig urinary bladder to α,β -methylene ATP, but not carbachol, has been reported (Hoyle *et al.*, 1990). It is plausible that low concentrations of suramin preferentially antagonize an inhibitory receptor activated by high concentrations of the purinoceptor agonist. Alternatively, suramin may bind to a subunit on the purinoceptor to facilitate allosterically agonist effects. Indeed, suramin has already been shown to modulate nicotinic receptor function in skeletal muscle by what seems to be an allosteric mechanism (Henning *et al.*, 1992). In the case of ATP, it is possible that suramin retards degradation via interaction with an ATPase, although this seems unlikely given the observation that very high concentrations of suramin (10 mM) are required to inhibit ecto ATPase in the guinea-pig urinary bladder (Hourani & Chown, 1989). No definitive evidence to support any of these hypotheses was obtained in the present study and therefore the mechanism(s) underlying this phenomenon remains unclear. Despite these complications, it is clear that suramin antagonizes the large amplitude depolarizations of the rat vagus nerve to the methylene and imido analogues of ATP thus supporting the proposal that the responses that they produce are mediated via P_2 purinoceptors.

In contrast, the small depolarizing responses to 2-methylthio ATP, ADP- β -S and ADP were resistant to suramin. These data imply that one or more purinoceptors, by definition distinct from the known classical suramin-sensitive P_2 purinoceptors, also mediate depolarization of the rat vagus nerve. Although the suramin-sensitivity of the P_{2u} receptor has not been reported it is unlikely that these responses are mediated via this receptor type since UTP was inactive as an agonist (Cusack, 1993). It is conceivable that these drugs activate a receptor that corresponds to the diadenosine polyphosphate binding site in rat brain synaptosomes, tentatively designated P_{2d} by Pintor and coworkers (1993). Another possibility is that these agents or their metabolites interact with adenosine (P_1) purinoceptors to evoke membrane depolarization; this supposition is at present the subject of investigation in our laboratory. Importantly, the suramin-resistance of these responses indicates that these agents are not partial agonists at the receptor activated by α,β -methylene ATP.

In summary, we have provided evidence that the rat cervical vagus nerve contains a heterogeneous purinoceptor population that mediates membrane depolarization. From the relative order of potencies of a series of ATP analogues, the predominant effect is mediated by a receptor which appears to be a P_{2x} receptor. Interestingly, from patch- and voltage-clamp studies on neurones cultured from parasympathetic intracardiac ganglia it appears that the excitatory effects of purines are mediated via a receptor that shares more similarities with the P_{2y} than the P_{2x} purinoceptor (Allen & Burnstock, 1990; Fieber & Adams, 1991). It remains to be established whether there is a genuine difference in the purine receptor population on the intracardiac neurones and on the axonal membranes of the cervical vagal nerve trunk or whether these contrasting findings can be attributed to the different methods used to study these receptors. For example, working with single cells may minimize some of the problems of purine nucleotide metabolism

associated with whole tissue experiments such that a more accurate index of the potency of the less stable purines (e.g. ATP and 2-methylthio ATP) is obtained. However, it is difficult to perform quantitative pharmacology using these methods and it is uncertain whether these neurones grown in culture express the same receptor types as the parent cells. The limitations of the available drug tools are another major problem, and the advent of more selective P_2 purinoceptor

agonists and antagonists will undoubtedly aid in addressing these issues. The findings from this study indicate that the rat vagus nerve preparation may prove to be a useful preparation in which to study the activity of such novel agents on neuronal P_{2x} receptors.

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