Br. J. Pharmacol. (1994), **113**, 463–470

Effects of divalent cations on the potency of ATP and related agonists in the rat isolated vagus nerve: implications for P_2 purinoceptor classification

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1 By use of a 'grease-gap' technique, the depolarizing effects of adenosine 5'-triphosphate (ATP) and ATP analogues on the rat isolated vagus nerve were determined in normal and in Ca^{2+}/Mg^{2+}-free ($+1 \times 10^{-3}$ M ethylenediamine tetraacetic acid) physiological salt solution (PSS).

2 In normal PSS, ATP produced concentration-dependent depolarization responses but the concentration-effect curve to ATP was incomplete and a maximum effect was not achieved. The threshold concentration for depolarization was 1×10^{-5} M and at the highest concentration tested (1×10^{-3} M) the peak amplitude of the response to ATP only amounted to 71% of the depolarization produced by a near maximal response to 5-hydroxytryptamine (5-HT, $.1 \times 10^{-5}$ M).

3 In Ca^{2+}/Mg^{2+}-free PSS, ATP produced depolarization responses at much lower concentrations and of markedly larger amplitude. Under these conditions, the threshold concentration for depolarization was $1-3 \times 10^{-7}$ M and the maximal response to ATP amounted to 526% of the response to 5-HT (1×10^{-5} M) in normal PSS. The concentration-effect curve to ATP was sigmoid, with a defined maximum effect and a mean EC_{50} value of 1.2×10^{-6} M.

4 In contrast to the effects on responses to ATP, the absence of divalent cations in the PSS did not modify the effective concentrations of either α,β-methylene ATP or 5-HT. However, the maximum responses to both α,β-methylene ATP and 5-HT were significantly increased in Ca^{2+}/Mg^{2+}-free PSS.

5 The depolarizing effects of several analogues of ATP were determined in Ca^{2+}/Mg^{2+}-free PSS. ATP-γ-S and 2-methylthioATP were of similar potency to ATP (respective equi-effective molar ratios (EMRs) of 1.9 and 1.3, where ATP = 1) and similar maximum responses were obtained. α,β-Methylene ATP, β,γ-methylene ATP and β,γ-imido ATP were considerably less potent than ATP, analysis yielding mean EMRs of 48.9, 85.0 and 60.0, respectively. Maximum responses to these latter three agonists were not obtained at the highest concentrations tested ($1 \times 10^{-4}-3 \times 10^{-4}$ M). Benzoyl ATP, adenosine 5'-O-(2-thiodiphosphate) and adenosine diphosphate produced only small depolarizing responses at high concentrations ($> 1 \times 10^{-4}$ M). Adenosine monophosphate, adenosine and uridine 5'-triphosphate each had little or no depolarizing effect in Ca^{2+}/Mg^{2+}-free PSS.

6 These data demonstrate that in the absence of divalent cations the excitatory actions of some, but not all, purine nucleotides in the rat vagus nerve are markedly potentiated. In Ca^{2+}/Mg^{2+}-free PSS, the rank order of agonist potencies was ATP = 2-methylthioATP = ATP-γ-S >> α,β-methylene ATP = β,γ-imido ATP = β,γ-methylene ATP. These findings are in stark contrast to our previous observations in normal PSS where the rank order of agonist potencies for these nucleotides was α,β-methylene ATP > ATP-γ-S > β,γ-imido ATP = β,γ-methylene ATP > 2-methylthioATP > ATP.

7 We suggest that the two different rank orders of potency can be explained by differential metabolism involving Ca^{2+}/Mg^{2+}-dependent ectonucleotidases. If so, these data indicate that ATP and 2-methylthioATP are inherently more potent than α,β-methylene ATP as agonists at neuronal P_{2X} purinoceptors in the rat vagus nerve. The possible implications of these findings to the present system for subclassifying P_2 purinoceptors are profound.

Keywords: P_2 purinoceptors; rat vagus nerve; depolarization; ATP; 2-methylthioATP; α,β-methylene ATP

Introduction

The diverse actions of adenosine 5'-triphosphate (ATP) on many physiological systems are mediated via P_2 purinoceptors (see Burnstock, 1990, for review). Evidence from pharmacological studies indicates that P_2 purinoceptors are heterogeneous, and to date, at least five different receptor subtypes termed P_{2X}, P_{2Y}, P_{2T}, P_{2Z} and P_{2U} purinoceptors have been proposed (Burnstock & Kennedy, 1985; Gordon, 1986; O'Connor *et al.*, 1991). This concept of purinoceptor heterogeneity is corroborated by the recent cloning of two genes believed to encode the functional P_{2Y} and P_{2U} receptors (Lustig *et al.*, 1993; Webb *et al.*, 1993). In view of the paucity of available P_2 purinoceptor antagonists, the primary basis on which this classification has been founded is the differential relative agonist potencies of ATP and ATP analogues in different systems. For example, P_{2X} purinoceptor-mediated responses are characterized by the order of agonist potencies of α,β-methylene ATP > β,γ methylene ATP > ATP = 2 methylthioATP, whilst P_{2Y} purinoceptors are characterized by a rank order of potencies of 2 methylthioATP > ATP > α,β-methylene ATP = β,γ methylene ATP (Burnstock & Kennedy, 1985).

Recently, we described the depolarizing actions of ATP on the rat isolated vagus nerve and characterized the receptors mediating this response using a range of ATP analogues (Trezise *et al.*, 1993). Of the agents tested, α,β-methylene ATP was the most potent, whilst ATP and 2-methylthioATP were only weak agonists and on this basis we postulated the

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involvement of P_{2X} purinoceptors. In a follow up study, we attempted to elucidate the ionic mechanisms underlying this depolarization by examining the effect of various ion substitutions on responses produced by α,β-methylene ATP (Trezise *et al.*, 1994). Our data showed that these responses, like those mediated via 5-HT_3 receptor activation, depend on Na^+ influx and, thus, provide evidence that the purinoceptor in the vagus nerve activates a cation channel (Trezise *et al.*, 1994). Interestingly, removal of Ca^{2+} and Mg^{2+} from the physiological salt solution produced a large augmentation of α,β-methylene ATP-induced depolarization without markedly changing the EC_{50} value (Trezise *et al.*, 1994). In view of evidence indicating that in some systems removal of divalent cations increases the agonist potency of ATP, for example in rat mast cells (Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988), human fibroblasts (Fine *et al.*, 1989) and in bovine aortic endothelial cells (Motte *et al.*, 1993), we decided to examine whether depolarization responses of the rat vagus nerve produced by ATP as well as other analogues of ATP were differentially affected by removal of divalent cations.

A small part of this work was reported previously to the British Pharmacological Society (Trezise *et al.*, 1994).

Methods

Extracellular recordings of agonist-induced depolarizations were made from segments of rat isolated cervical vagus nerve as previously described (Trezise *et al.*, 1993). Briefly, male AHA Wistar rats (200–270 g) were stunned by a blow to the head, decapitated, and the two 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, at a rate of 1–2 ml min^{-1}, with a modified physiological salt solution (PSS; see below for composition), preheated to 27°C and gassed with 95% O_2/5% CO_2. Drugs were applied at known concentrations into the superfusate of the first compartment only.

Experimental protocols

Determination of agonist responses using a single concentration-effect curve protocol In some experiments a single agonist concentration-effect curve only was constructed. Thus, after an initial 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). Repeated exposures (2–3) to 5-HT (1×10^{-5} M) were performed until reproducible depolarization responses were obtained. After a 30 min wash period, a concentration-effect curve to either ATP, an ATP analogue or 5-HT was constructed non-cumulatively using serially increasing concentrations. Each concentration of agonist was applied for 2–3 min during which time a peak effect was reached. An interval of 45 min was left between each agonist application. In some experiments, after the initial responses to 5-HT, the PSS was changed to one containing no divalent cations (Ca^{2+}/Mg^{2+}-free PSS – see below). This procedure caused a depolarization response in its own right, which was sustained, but further depolarization responses could still be induced (see results). After a further 30 min re-equilibration period in Ca^{2+}/Mg^{2+}-free PSS, a single concentration-effect curve for a given agonist was constructed.

Determination of relative agonist potencies using a two concentration-effect curve protocol In Ca^{2+}/Mg^{2+}-free PSS it was possible to construct two consecutive concentration-effect curves to ATP in the same preparation. In these experiments, an initial 30 min equilibration period was allowed. After this time, the first concentration-effect curve to ATP (3×10^{-7}–1×10^{-4} M) was constructed non-cumulatively by applying serially increasing concentrations of drug for 3 min at 30 min intervals. Following a further 30 min re-equilibration period, a second concentration-effect curve to either ATP or test agonist was constructed as before. One preparation in four served as a time-matched control preparation to monitor any spontaneous changes in sensitivity to ATP during the course of the experimental period.

Analysis of results

The depolarization responses produced by agonists were measured as the peak change (μV) in the d.c. potential between the two compartments. In the single concentration-effect curve protocol experiments, responses to agonists were expressed as a percentage of the response to 5-HT (1×10^{-5} M). In the two concentration-effect curve protocol experiments, relative agonist potencies were determined by expressing all responses as a percentage of the maximum response to ATP obtained from the first concentration-effect curve. Equi-effective molar ratios (EMRs) were obtained by dividing the equi-effective concentration of the test agonist by that of ATP measured at the 50% response level. In some cases where the maximum response to the test agonist did not achieve 50% of the maximum response to ATP measured from the first concentration-effect curve, EMRs were obtained from measurements at the 30% response level. Time-related changes in sensitivity to ATP were corrected for by dividing the test agonist EMR by the equivalent ratio obtained from the control preparation in which consecutive concentration-effect curves to ATP were constructed.

All data are expressed as mean ± 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 normal physiological salt solution (PSS) was as follows (mM in deionised water): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, D-glucose 11.1, $CaCl_2.6H_2O$ 1.3. In some experiments $CaCl_2$ and $MgSO_4$ were omitted and ethylene diaminetetracetic acid (EDTA, 1×10^{-3} M) was added; this is referred to as Ca^{2+}/Mg^{2+}-free PSS. 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), 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (benzoyl ATP), adenosine 5′-O-(2-thiodiphosphate) trilithium salt (ADP-β-s), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), ethylenediaminetetraacetic acid disodium salt (EDTA, all Sigma), 2-methylthioATP tetra sodium salt (Research Biochemicals Incorporated). All drugs were dissolved and diluted to the required concentration in the appropriate PSS, and stored on ice.

Results

Comparison of the effects of ATP in normal PSS and in Ca^{2+}/Mg^{2+}-free PSS

In normal PSS, ATP (1×10^{-5}–1×10^{-3} M) produced concentration-related, depolarization responses of the rat vagus

nerve. The threshold concentration of ATP for depolarization was 1×10^{-5} M and at the highest concentration tested (1×10^{-3} M) the amplitude of the response to ATP only amounted to $70.6 \pm 7.8\%$ ($n = 6$) of the response to 5-HT (see Figures 1a, 2a and 5). A maximal response to ATP was not obtained at a concentration of 1×10^{-3} M and thus an EC_{50} value could not be determined. The effects of higher concentrations were not determined due to the possible complicating factor of significant chelation of divalent cations by ATP (Bartfai, 1979).

Changing the perfusate from normal PSS to Ca^{2+}/Mg^{2+}-free PSS produced a sustained depolarization response which was $300.0 \pm 40.1\%$ ($n = 4$) of the response to 5-HT (1×10^{-5} M). In this Ca^{2+}/Mg^{2+}-free PSS, there was a marked decrease in the threshold concentration of ATP required for depolarization ($1-3 \times 10^{-7}$ M; Figure 1b). The

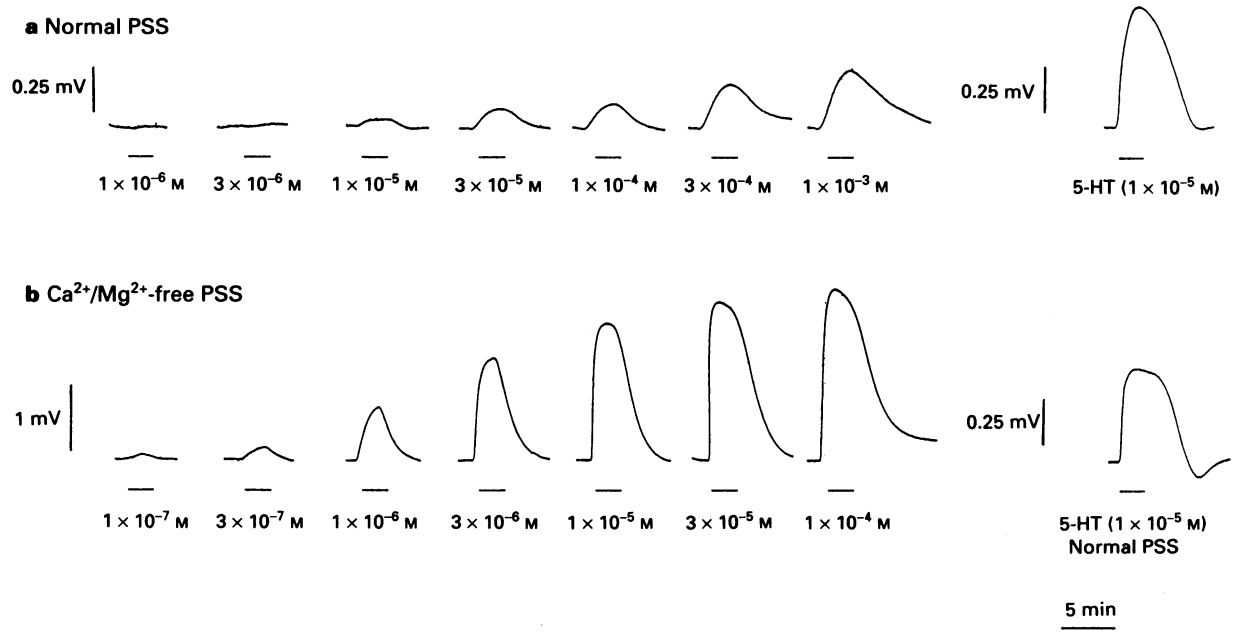

Figure 1 Discontinuous records of the effect of adenosine 5'-triphosphate (ATP) on extracellularly recorded membrane potentials in the rat isolated vagus nerve in (a) normal physiological salt solution (PSS) and (b) Ca^{2+}/Mg^{2+}-free PSS. An upward deflection indicates depolarization of the nerve 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. Note the marked differences in the potency and absolute amplitude of responses to ATP under the two experimental conditions. For comparison the near maximal depolarization response of each preparation to 5-hydroxytryptamine (5-HT, 1×10^{-5} M) in normal PSS is shown.

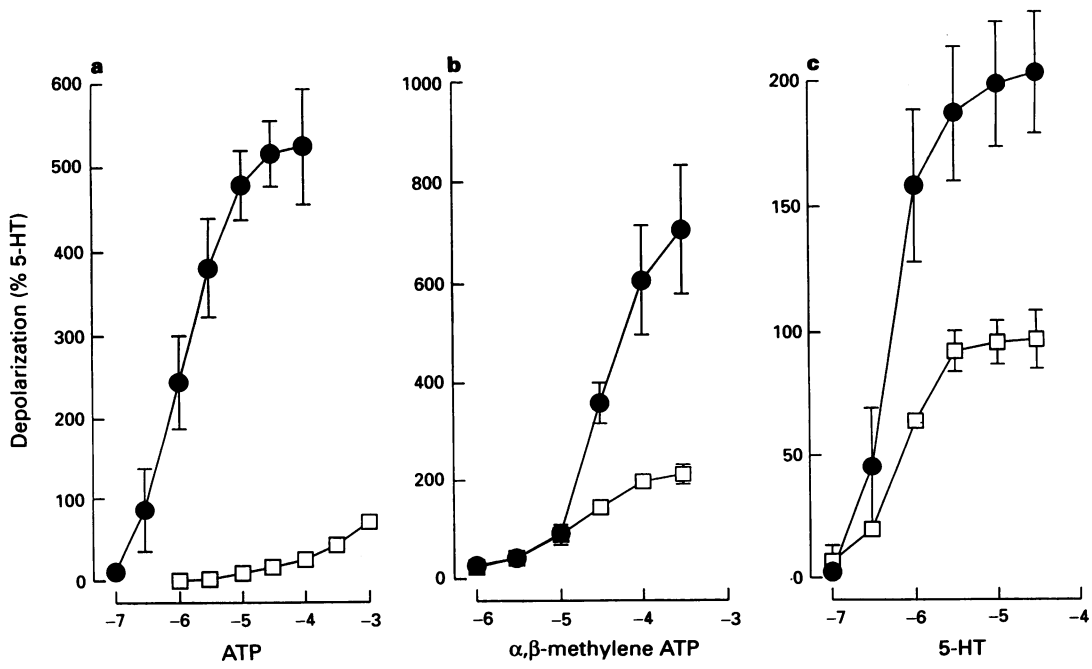

Figure 2 Comparison of depolarizing responses of the rat isolated vagus nerve produced by (a) adenosine 5'-triphosphate (ATP), (b) α,β-methylene ATP and (c) 5-hydroxytryptamine (5-HT) in either normal physiological salt solution (PSS, □) or in Ca^{2+}/Mg^{2+}-free PSS (●). Each point represents the mean with s.e.mean of n determinations ($n = 4-10$). The abscissa scales show 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) in normal PSS in the same preparation (see methods).

concentration-effect curve to ATP was complete, with a clearly defined maximum response at a concentration of 1×10^{-4} M and a mean EC_{50} value of 1.19×10^{-6} M (0.27–5.20), $n = 4$. The mean maximum amplitude of the response to ATP in Ca^{2+}/Mg^{2+}-free PSS was 525.6 ± 68.0% ($n = 4$) of the response to 5-HT (1×10^{-5} M), measured in normal PSS in the same preparation (Figure 2a). In normal PSS, when a sustained depolarization response (301.3 ± 23.4% of the 5-HT response) was induced by KCl (6×10^{-3} M added), the amplitude of the depolarization response to ATP (3×10^{-4} M) was not affected (control 53.7 ± 4.3%, test 46.9 ± 9.0%, $n = 4$).

Comparison of the effects of α,β-methylene ATP in normal PSS and in Ca^{2+}/Mg^{2+}-free PSS

In normal PSS, α,β-methylene ATP (10^{-6}–3×10^{-4} M) produced concentration-dependent depolarization responses with a mean EC_{50} value of 1.18×10^{-5} M (0.90–1.61) and a maximal response that amounted to 190.1 ± 15.0% ($n = 10$) of the initial response to 5-HT (1×10^{-5} M). The amplitude of depolarization responses to α,β-methylene ATP was significantly increased in Ca^{2+}/Mg^{2+}-free PSS; the response at the highest concentration tested was 727.1 ± 127.4% ($n = 5$) of the initial response to 5-HT (1×10^{-5} M), determined in normal PSS ($P < 0.05$; Figure 2b). Although, in the absence of a clearly defined maximum response, a true EC_{50} value could not be calculated, there was no apparent change in the concentrations of α,β-methylene ATP required to produce depolarization in Ca^{2+}/Mg^{2+}-free PSS compared to normal PSS. Similarly, the concentrations required for depolarization by both β,γ-methylene ATP and β,γ-imido ATP were not modified by removal of divalent cations, although an increase in the amplitude of responses was observed (data not shown).

Comparison of the effects of 5-HT in normal PSS and in Ca^{2+}/Mg^{2+}-free PSS

In normal PSS, 5-HT (1×10^{-7}–3×10^{-5} M) produced concentration-dependent depolarization responses with a mean EC_{50} value of 6.7×10^{-7} M (4.4–10.0) and a maximal response that was 96.7 ± 2.0% ($n = 5$) of the initial response to 5-HT (1×10^{-5} M). The amplitude of depolarization responses to 5-HT were significantly increased in Ca^{2+}/Mg^{2+}-free PSS; the mean maximum response was 204.0 ± 25.0% ($n = 5$) of the initial response to 5-HT (1×10^{-5} M), determined in normal PSS ($P < 0.05$; Figure 2c). However, there was no significant change in the sensitivity to 5-HT, such that in Ca^{2+}/Mg^{2+}-free PSS the mean EC_{50} value was 7.6×10^{-7} M (2.4–23.7).

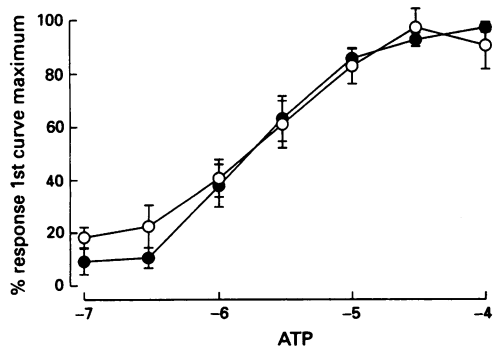

Figure 3 Reproducibility of concentration-effect curves to adenosine 5'-triphosphate (ATP) for depolarizing responses of the rat isolated vagus nerve in Ca^{2+}/Mg^{2+}-free physiological salt solution. First curve (●), second curve (○). Each point represents the mean with s.e.mean of n determinations ($n = 8$). The abscissa scale shows the log molar concentration of ATP and the ordinate scale the depolarizing response expressed as a percentage of the maximum response to ATP determined from the respective first concentation-effect curve.

Determination of relative potencies of purinoceptor agonists in Ca^{2+}/Mg^{2+}-free PSS

Reproducibility of concentration-effect curves to ATP In order to determine relative purinoceptor agonist potencies a two concentration-effect curve protocol was adopted (see Methods). With this protocol depolarization responses to ATP were found to be reproducible (Figure 3); the mean concentration-ratio between the first and second concentration-effect curves was 1.07 (0.45–2.53), $n = 8$. There was no statistically significant difference in the maximum responses obtained to ATP from the first and second concentration-effect curves.

Effects of ATP analogues 2-MethylthioATP and ATP-γ-S (both 1×10^{-7}–3×10^{-4} M) both produced concentration-related depolarization responses of the rat vagus nerve in Ca^{2+}/Mg^{2+}-free PSS (see Figure 4a, 4b and 5). The mean maximum response to each agent (second concentration-effect curve) was not statistically significantly different from the corresponding maximum response to ATP in the control preparation (ATP control 81.8 ± 9.3%, 2-methylthioATP 87.1 ± 8.8%, $n = 4$; ATP control 90.1 ± 10.3%, ATP-γ-S 95.6 ± 7.5%, $n = 5$). Both agonists were approximately equipotent with ATP (mean corrected EMRs of 1.3 (0.3–6.4) and 1.9 (0.8–4.4), for 2-methylthioATP and ATP-γ-S, respectively).

α,β-Methylene ATP, β,γ-methylene ATP, and β,γ-imido ATP (each 1×10^{-6}–3×10^{-4} M) were less potent agonists than ATP. The mean corrected EMRs were as follows: α,β-methylene ATP 48.8 (29.7–80.2), $n = 8$; β,γ-methylene ATP 85.0 (52.0–138.8), $n = 9$; β,γ-imido ATP 60.0 (37.6–96.0), $n = 6$. Under these experimental conditions, the response to α,β-methylene ATP at the highest concentration tested (3×10^{-4} M) was smaller than the maximum response to ATP (see Figure 4c). In contrast, in the single concentration-effect curve studies the response to α,β-methylene ATP was somewhat larger than the maximum response to ATP (Figures 2a and b). This variability could reflect the differences in the experimental protocols used, but is more likely to be explained by normal biological variation. Since a clearly defined maximum for α,β-methylene ATP was not obtained in either series of experiments one cannot make a quantitative comparison of the relative maxima *vis-a-vis* that of ATP.

Benzoyl ATP and ADP-β-S (both 1×10^{-6}–1×10^{-4} M) produced only small depolarizing responses at the highest concentrations tested and, thus, EMRs were not determined (see Figure 4d). The peak amplitude of the depolarization response to benzoyl ATP (1×10^{-4} M) and ADP-β-S (1×10^{-4} M) was 30.3 ± 8.7% ($n = 4$) and 28.9 ± 12.0% of the maximal response to ATP, respectively ($n = 5$).

ADP, AMP or adenosine (all 1×10^{-6}–1×10^{-4} M) had no marked effect on membrane potential in Ca^{2+}/Mg^{2+}-free PSS. The peak amplitude of the responses to each of these agents at the highest concentration tested (1×10^{-4} M) was 13.7 ± 8.0% ($n = 4$), 3.9 ± 2.7% ($n = 5$) and 0.5 ± 0.5% ($n = 4$) of the maximal response to ATP, respectively. UTP was without effect at concentrations up to 1×10^{-3} M ($n = 4$).

Discussion

The major finding of the present study was that removal of divalent cations had differential effects on the depolarizing actions of ATP, certain ATP analogues and of 5-HT in the rat isolated vagus nerve. A marked increase in potency (i.e. lowering of the threshold concentration and the EC_{50} value) of ATP, 2-methylthioATP and ATP-γ-S was observed when Mg^{2+} and Ca^{2+} ions were removed. In contrast, the potencies of α,β-methylene ATP, β,γ-methylene ATP and β,γ-imido ATP were not affected by divalent cations. In addition, for

all of the agonists, removal of divalent cations produced an increase in the maximum response (i.e. augmentation). The precise mechanisms underlying these processes of potentiation and augmentation have not been investigated directly in this study. However, the fact that some but not all, agonists were potentiated, whilst the effects of all agonists tested were augmented suggests that the mechanisms involved are distinct.

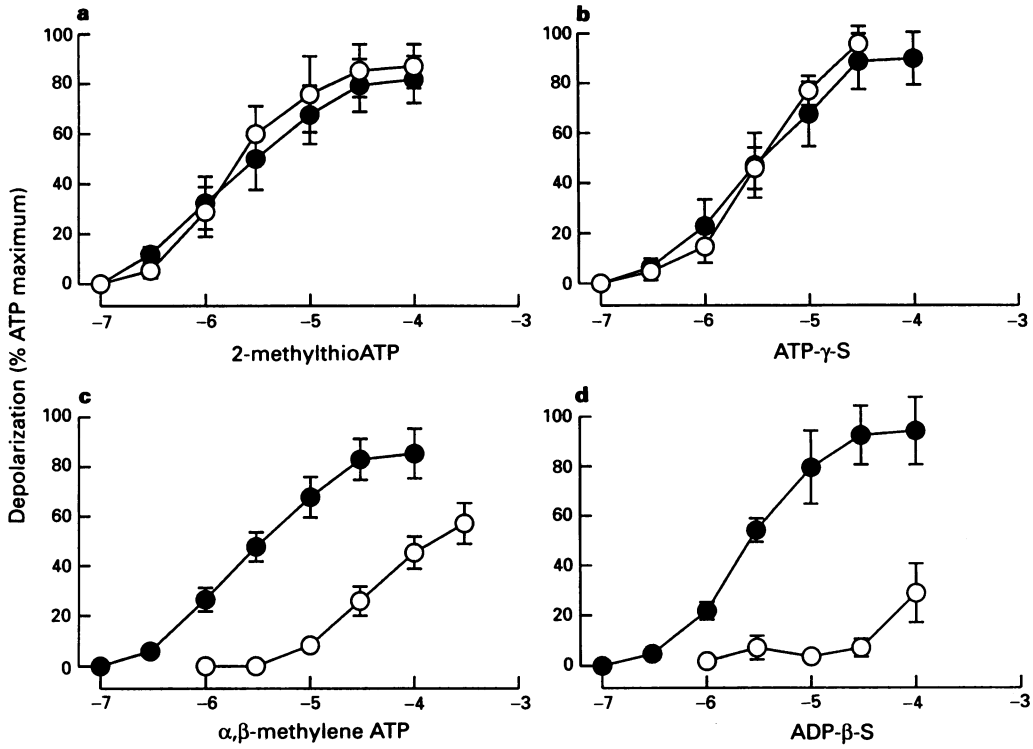

Figure 4 Comparison of the depolarizing effects of adenosine-5-triphosphate (ATP) with (a) 2-methylthio ATP, (b) ATP-γ-S, (c) α,β-methylene ATP and (d) ADP-β-S on the rat isolated vagus nerve in Ca^{2+}/Mg^{2+}-free physiological salt solution. In each panel the time-matched control responses to ATP are represented by (●) and the responses to test agonist by (○). Each point represents the mean with s.e.mean of n determinations ($n = 4-8$). The abscissa scales show the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the maximum response to ATP determined from the first concentration-effect curve.

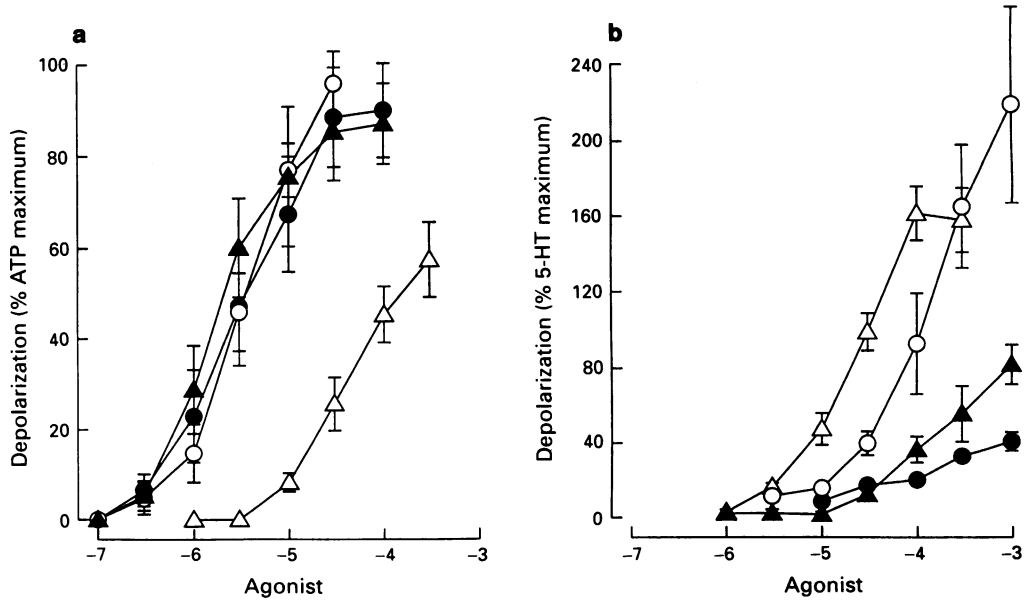

Figure 5 Comparison of the depolarizing effects of adenosine-5'-triphosphate (ATP) and ATP analogues on the rat isolated vagus nerve in (a) Ca^{2+}/Mg^{2+}-free PSS and (b) normal PSS (some data from Trezise *et al.*, 1993). In each panel the depolarization responses to ATP (●), ATP-γ-S (○), 2-methylthioATP (▲) and α,β-methylene ATP (△) are shown. Each point represents the mean with s.e.mean of n determinations ($n = 4-9$). The abscissa scales show the log molar concentration of drug and the ordinate scale the depolarizing response expressed either as a percentage of the maximum response to ATP determined from the first concentration-effect curve (a) or as a percentage of the depolarization response to 5-hydroxytryptamine (5-HT, 1×10^{-5} M) (b). Note the marked differences in the potencies of ATP, ATP-γ-S, and 2-methylthioATP, but not α,β-methylene ATP, in the absence and presence of divalent cations.

The augmentation of depolarization responses of the rat vagus nerve induced by ATP and the ATP analogues tested was not restricted to purines in that depolarization responses evoked by 5-HT were also augmented. It has previously been shown by use of a 'sucrose-gap' recording technique that depolarization responses of the rat internal carotid nerve, induced by 5-HT, are augmented by removal of divalent cations (Nash & Wallis, 1981). The mechanism underlying this phenomenon does not appear to be related to the change in membrane potential observed when divalent cations were removed since in this study we have shown that a similar depolarization response produced by increasing the extracellular K^+ concentration did not augment the response to ATP. Evidence from studies on single cells is consistent with these observations from whole nerve preparations that divalent cations reduce both ATP- and 5-HT-induced membrane currents. In neurones isolated from sensory and parasympathetic cardiac ganglia of the rat, the amplitude of depolarization responses induced by ATP are markedly reduced when the extracellular concentration of divalent cations is increased (Krishtal & Marchenko, 1984; Krishtal *et al.*, 1988a; Bean *et al.*, 1990; Fieber & Adams, 1991). Similarly, whole cell currents induced by 5-HT in rat dorsal root and rabbit nodose ganglion neurones are attenuated by Ca^{2+} and Mg^{2+} (Robertson & Bevan, 1991; Peters *et al.*, 1993). There are several possible explanations for these inhibitory effects of divalent cations including channel block or an effect on desensitization, but, at least in the case of ATP, an action at or close to the ion channel itself seems probable since the single channel conductance measured in membrane patches is also markedly increased on removal of divalent cations (Krishtal *et al.*, 1988a; Bean *et al.*, 1990).

In addition to the augmentation of responses to ATP, removal of divalent cations also markedly increased its potency, causing a large leftward shift of the concentration-effect curve and a decrease in the EC_{50} value. In contrast, the potencies of α,β-methylene ATP, β,γ-methylene ATP, β,γ-imidoATP and 5-HT in causing depolarization remained unchanged (see Figures 2 and 5). One possible explanation for the increase in potency of ATP could be that in the absence of divalent cations a greater proportion of ATP exists in an active ATP^{4-} form. In physiological salt solutions ATP^{4-} forms complexes primarily with Mg^{2+} and Ca^{2+}, and also to a much lesser extent with Na^+, K^+ and H^+. The proportion of these various complexes depends on the temperature, pH and ionic composition of the solution and the concentration of ATP (Bartfai, 1979; see Lustig *et al.*, 1992). By manipulating these factors it is possible to vary the concentration of complexed and uncomplexed ATP in a given solution. Using this approach, evidence has been obtained that certain purinoceptors preferentially recognise the ionised, tetrabasic form of ATP (ATP^{4-}; Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988; Fine *et al.*, 1989; Lustig *et al.*, 1992; Motte *et al.*, 1993). It would seem that ATP^{4-} also has agonist effects in the vagus nerve in that ATP produced marked responses in the absence of Ca^{2+} and Mg^{2+}. It is difficult, however, to assess precisely the contribution (if any) that the increase in the concentration of ATP^{4-} that occurs on removal of divalent cations may have made to the increase in potency of ATP. It can be estimated (Cockcroft & Gomperts, 1980) that the maximum increase in the ATP^{4-} concentration when divalent cations are omitted, compared to normal PSS, is 23 fold, for the range of ATP concentrations that we tested. However, since the nature of the concentration-effect curves for ATP were very different in normal PSS and Ca^{2+}/Mg^{2+}-free PSS (see Figure 2a) a quantitative estimate of the difference in potency could not be made. Nevertheless, there was a 50–100 fold difference in the threshold concentrations for the depolarization response to ATP under these different conditions suggesting that an increase in the concentration of ATP^{4-} when divalent cations are omitted cannot be the sole explanation for the increase in potency of ATP.

A more plausible explanation for the increase in potency of ATP is that in the whole nerve preparation removal of Ca^{2+} and Mg^{2+} prevents the metabolic breakdown of ATP by cell surface ectonucleotidase enzymes. It is well established that Ca^{2+}/Mg^{2+}-dependent isoforms of ectonucleotidase exist and that the preferred substrate for the enzyme in many cases is ATP complexed with Mg^{2+} (see Nagy, 1986 for review). The finding that responses to 2-methylthioATP, another nucleotide susceptible to metabolic breakdown (Welford *et al.*, 1986), were also markedly potentiated in Ca^{2+}/Mg^{2+}-free PSS is consistent with this hypothesis. Conversely, the methylene- and imido-analogues of ATP, α,β-methylene ATP, β,γ-methylene ATP and β,γ-imido ATP, were not potentiated in Ca^{2+}/Mg^{2+}-free PSS, which adds further support to this idea since these simple modifications to the ATP molecule have been shown to confer resistance to ectonucleotidases (Cusack & Hourani, 1984; Hourani *et al.*, 1985; Welford *et al.*, 1986; 1987). ATP-γ-S was also potentiated in Ca^{2+}/Mg^{2+}-free PSS. Although there is evidence to suggest that this nucleotide is resistant to metabolism in endothelial cells (Cusack *et al.*, 1983) and in certain visceral smooth muscles (Welford *et al.*, 1986; 1987), in frog skeletal muscle low concentrations of ATP-γ-S ($< 10\ \mu M$) are broken down as rapidly as ATP (Cascalheira & Sebastião, 1992). Taken together, these findings of differential modification of the potencies of ATP and ATP analogues suggest that removal of divalent cations from the PSS might increase the potency of ATP by preventing the influence of metabolizing enzymes.

In normal PSS, the rank order of agonist potencies for depolarizing the rat vagus nerve was α,β-methylene ATP > ATP-γ-S > β,γ-methylene ATP = β,γ-imido ATP > 2-methylthioATP ≥ ATP (Trezise *et al.*, 1993; see Figure 5). This profile is similar to that described for the P_{2X} purinoceptor subtype (Burnstock & Kennedy, 1985). In contrast, in Ca^{2+}/Mg^{2+}-free PSS, the rank order of agonist potencies was ATP = 2-methylthioATP = ATP-γ-S > α,β-methylene ATP ≥ β,γ-methylene ATP = β,γ-imido ATP, a profile more consistent with the involvement of a P_{2Y} purinoceptor (Burnstock & Kennedy, 1985). Thus, using this range of nucleotides in the rat vagus nerve, it is possible to demonstrate profiles of agonist potencies that resemble either the P_{2X} or the P_{2Y} purinoceptor subtype, simply by modifying the composition of the PSS. Since it seems highly unlikely that a fundamental change in the nature of the receptor structure could have occurred simply by removal of divalent cations this finding demands an alternative explanation (see below).

In the present study, 2-methylthioATP was a very potent agonist in the absence of divalent cations. This agent is believed to be a selective agonist for P_{2Y}, compared to P_{2X}, purinoceptors (Burnstock & Kennedy, 1985; Cusack, 1993). This might suggest the involvement of P_{2Y} purinoceptors, and not P_{2X} purinoceptors, in mediating depolarization of the rat vagus nerve. However, it would be difficult to argue that P_{2Y} purinoceptors are involved since ADP-β-S and ADP, other potent P_{2Y} purinoceptor agonists (Martin *et al.*, 1985; Berrie *et al.*, 1989; Boyer *et al.*, 1989), were only weak agonists in Ca^{2+}/Mg^{2+}-free PSS. The weak activity of UTP, ADP and benzoyl ATP, even in the absence of divalent cations, suggests that P_{2U}, P_{2T} or P_{2Z} purinoceptors are not involved (see Cusack, 1993). One could propose the involvement of a further purinoceptor subtype to explain the data in this study but given the profound effect of divalent cations on the potency of ATP this would seem imprudent. In attempting to arrive at an explanation, we have therefore assumed that all of the purine agonists are acting predominantly at a common receptor, believed to be of the P_{2X} type (see below).

Under the present purinoceptor classification system, P_{2X} purinoceptors are described as ligand-gated cation channels, whilst P_{2Y} purinoceptors utilise G-protein coupling for signal transduction (Kennedy, 1990; see Bean, 1992 and Dubyak & El-Moatassim, 1993 for reviews). The P_{2X} purinoceptor has yet to be characterized structurally but a P_{2Y} receptor gene, however, has recently been cloned (Webb *et al.*, 1993). The

P_{2Y} receptor protein that this gene encodes contains seven helical transmembrane domains, as would be expected for a G-protein-linked receptor. Although there is yet no structural proof that the P_{2X} purinoceptor forms part of a channel, there is good functional data, analogous to that obtained for the nicotinic and the 5-HT_3 receptor, to indicate that this is the case. Thus, in rat nodose ganglion (vagal) neurones there is convincing evidence that ATP opens cation channels (Krishtal & Marchenko, 1984; Krishtal *et al.*, 1988a; Bean, 1990). We have confirmed the dependence of the purinoceptor-mediated depolarization response of the equivalent whole nerve preparation on extracellular monovalent cations (Trezise *et al.*, 1994). The extremely short latency of onset (<50 ms) of the response to ATP in single cells (Krishtal *et al.*, 1988a; Bean, 1990; Fieber & Adams, 1991) precludes the involvement of any known G-protein-coupled receptor system and suggests that these cation channels are directly ligand-gated. Thus, on a transductional basis and by implication structural, the purinoceptor(s) on the vagus nerve would be classified as a P_{2X} purinoceptor.

Interestingly, in experiments on single cells, several workers have previously shown that ATP, and in some studies 2-methylthioATP, are more potent agonists than α,β-methylene ATP in causing depolarization via the opening of ligand-gated cation channels (e.g. rat vas deferens, Friel, 1988; rabbit ear artery, Benham & Tsien, 1987; guinea-pig bladder, Inoue & Brading, 1990; rat superior cervical ganglion neurones, Cloues *et al.*, 1993; rat parasympathetic neurones; Allen & Burnstock, 1990; Fieber & Adams, 1991; rat nucleus solitarii neurones, Ueno *et al.*, 1992). However, in the corresponding whole tissue preparations, ATP and 2-methylthioATP are less potent agonists than α,β-methylene ATP (e.g. guinea-pig bladder, Inoue & Brading, 1990; rabbit ear artery, O'Connor *et al.*, 1990; rat vagus nerve, Trezise *et al.*, 1993). Our findings of different rank orders of potencies for ATP, 2-methylthioATP and α,β-methylene ATP in the absence and presence of divalent cations may offer some explanation for this paradox. In studies on single cells in which drugs are applied very rapidly, the metabolism of ATP and of other hydrolysable agonists is likely to be negligible within the period that the response is measured (latency of onset 10–50 ms). In contrast, in whole cells where diffusion is limiting, metabolism may become the rate limiting event and the most metabolically stable compounds will appear the most potent. Therefore, it might be anticipated that measurement of agonist potencies in whole preparations in the absence of divalent cations will prevent agonist metabolism and provide a much more reliable measurement of purinoceptor agonist potency, and moreover, one that is consistent with studies on single cells. In our experiments in Ca^{2+}/Mg^{2+}-free PSS, the threshold concentration and potency (EC_{50} value 1.19×10^{-6} M) of ATP, and the rank order of potency of ATP analogues, were remarkably similar to values estimated from studies on single rat nodose ganglion neurones (Krishtal *et al.*, 1988a; Bean *et al.*, 1990). Confirmation of this hypothesis (or otherwise) awaits the availability of compounds which will selectively inhibit ectonucleotidase enzymes.

In conclusion, the results of the present study suggest that ATP and 2-methylthio ATP are much more potent agonists at the putative P_{2X} purinoceptor in the vagus nerve than was first thought. Given the susceptibility of these agents to metabolic breakdown, it may be difficult to classify these receptors operationally unless more metabolically stable and selective agonists can be identified. As such agonists are chemically not readily accessible, more emphasis will need to be given to the search for potent and selective antagonists. Nevertheless, the findings of this study are important in as much as they suggest that ATP and 2-methylthioATP are actually considerably more potent than α,β-methylene ATP at P_{2X} purinoceptors in the rat vagus nerve. If so, these findings will have profound implications for the classification of P_2 purinoceptors.

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(Received February 24, 1994
Revised May 12, 1994
Accepted June 20, 1994)