British Journal of Pharmacology (2001) 134, 639–647

www.nature.com/bjp

Changes in extracellular pH and myocardial ischaemia alter the cardiac effects of diadenosine tetraphosphate and pentaphosphate

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1 The structural conformation of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) has been reported to alter as pH is reduced. As such, it is possible that the cardiac effects of Ap_4A and Ap_5A vary during acidosis and myocardial ischaemia due to changes in ligand structure, receptor proteins or intracellular signalling.

2 We investigated whether the cardiac electrophysiological and coronary vasomotor effects of Ap_4A and Ap_5A are preserved under conditions of extracellular acidosis (pH 6.5) and alkalosis (pH 8.5) and whether Ap_4A has any electrophysiological or antiarrhythmic effects during ischaemia.

3 Transmembrane right ventricular action potentials, refractory periods and coronary perfusion pressure were recorded from isolated, Langendorff-perfused guinea-pig hearts under constant flow conditions. The effects of 1 nM and 1 μM Ap_4A and Ap_5A were studied at pH 7.4, 6.5 and 8.5. The effects of 1 μM Ap_4A were studied during global low-flow ischaemia and reperfusion.

4 At pH 7.4, Ap_4A and Ap_5A increased action potential duration (APD_{95}) and refractory period (RP) and reduced coronary perfusion pressure. The electrophysiological effects were absent at pH 6.5 while the reductions in perfusion pressure were attenuated. At pH 8.5, Ap_4A increased RP but the effects of Ap_4A and Ap_5A on perfusion pressure were attenuated. During ischaemia, Ap_4A had no antiarrhythmic or electrophysiological effects.

5 These data demonstrate the importance of extracellular pH in influencing the effects of Ap_4A and Ap_5A on the heart and indicate that any potentially cardioprotective effects of these compounds during normal perfusion at physiological pH are absent during ischaemia.

British Journal of Pharmacology (2001) **134,** 639–647

Keywords: Acidosis; action potential duration; alkalosis; coronary circulation; diadenosine polyphosphates; electrophysiology; extracellular pH; ischaemia; refractory period; vasomotion

Abbreviations: Ap_4A, diadenosine tetraphosphate; Ap_5A, diadenosine pentaphosphate; APD_{95}, action potential duration at 95% repolarization; Ap_nA, diadenosine polyphosphates; HEPES, N-2-hydroxyethylpiperazine-N′-3-propanesulphonic acid; K_{ATP}, ATP-dependent K^+ channel; MES, 2-(N-morpholino) ethanesulphonic acid, pH_o, extracellular pH; pK_a, dissociation constant; TAPS, *N*-Tris (hydroxymethyl) methyl-3-aminopropane sulphonic acid

Introduction

Attempts to reduce the mortality associated with ischaemic heart disease have considered the importance of platelet activation in acute coronary syndromes due to the ability of platelet-derived compounds to alter myocardial perfusion and the electrophysiological properties of the heart (Flores, 1996; Flores *et al*., 1999a). A clear understanding of the underlying mechanisms and the ways through which the heart may become vulnerable to arrhythmogenesis following platelet activation remains a primary objective.

Diadenosine polyphosphates (Ap_nA) are naturally occurring compounds that are present in the myocardium and platelet dense granules (Jovanovic *et al*., 1998; Luo *et al*., 1999; Flores *et al*., 1999b) from where they may be secreted. They function as neurotransmitters and extracellular signalling molecules, altering platelet reactivity, vasomotion and cardiac electrophysiology (Flores *et al*., 1999b; Stavrou *et al*., 2001a).

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We have recently described the coronary vasomotor and cardiac electrophysiological effects of Ap_nA at physiologically-relevant concentrations in the guinea-pig (Stavrou *et al*., 2001a). For diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) these include coronary vasodilatation and increases in ventricular action potential duration and refractory period, changes which were they to occur during myocardial ischaemia, would be seen as potentially protective. Mechanisms responsible for these effects involve P_1- (adenosine) and P_2-purinergic receptors (Stavrou *et al*., 2001a).

The majority of studies have described the cardiovascular effects of Ap_nA under normoxic conditions in adequately-perfused cardiac preparations and as such, little is known of their effects during ischaemia. Jovanovic *et al*. (1998) reported that ischaemia reduced myocardial levels of Ap_5A in the guinea-pig while Ahmet *et al*. (2000a,b,c,d) described potentially cardioprotective effects of Ap_4A associated with preconditioning and cardioplegia. Humphrey *et al*. (1987) examined the effects of Ap_5A on the recovery of myocardial function following ischaemia. We are unaware of any studies that have investigated potential antiarrhythmic actions of Ap_nA during ischaemia.

During myocardial ischaemia, platelet activation occurs and extracellular pH (pH_o) is reduced to values <pH 6 (Hirche *et al*., 1980). Electrophysiological abnormalities develop during acidosis (Källner & Franco-Cereceda, 1998) and the sensitivity of P2 purinergic receptors is altered when pH_o is changed (see for example Wildman *et al*., 1999a, b and Clarke *et al*., 2000). Since Ap_nA produce their cardiac effects *via* purinergic receptor-mediated mechanisms (Flores *et al*., 1999b; Stavrou *et al*., 2001a) knowledge of changes in receptor sensitivity together with the observation of conformational changes in Ap_nA induced by acidosis (Kolodny *et al*., 1979) are particularly relevant with regard to establishing whether the potentially cardioprotective effects of Ap_4A and Ap_5A observed during normal perfusion persist in acidotic or ischaemic myocardium. If binding to receptors is impaired due to conformational, changes in the molecule or if receptor sensitivity and intracellular signalling are altered, it is conceivable that Ap_4A or Ap_5A might lose potentially beneficial effects against ischaemia-induced ventricular arrhythmias, but this is not known. To investigate this, we studied the coronary vasomotor and cardiac electrophysiological effects of Ap_4A and Ap_5A under conditions of extracellular acidosis and alkalosis. We also investigated whether Ap_4A had any electrophysiological or antiarrhythmic effects during myocardial ischaemia.

Methods

Perfusion technique

Hearts were removed from male guinea-pigs (Dunkin-Hartley, 400–500 g) which had been humanely killed and were mounted for Langendorff perfusion (Goulielmos *et al*., 1995). They were perfused at a constant flow rate (7 ml min^{-1}) with buffered Tyrode solution or Krebs-Henseleit buffer at 32°C.

To study the effects of alterations in pH_o, buffered Tyrode solution was used. For experiments at pH 7.4, HEPES-buffered Tyrode solution was used with the following composition (in mM): NaCl 132, HEPES (N-2-hydroxyethyl-piperazine-N′-3-propanesulphonic acid, pK_a 7.55) 10, KCl 4, $MgCl_2$ 1, NaH_2PO_4 0.4, $CaCl_2$ 1.8, glucose 6.1, Na pyruvate 5. This solution was titrated to pH 7.4 using 1 M NaOH and gassed with 100% O_2. To study the effects of extracellular acidosis, MES (2-(N-morpholino) ethanesulphonic acid, pK_a 6.15) was substituted for HEPES, and the solution titrated to pH 6.5 as described by Gögelein *et al*. (1998). To study the effects of extracellular alkalosis, TAPS (*N*-Tris(hydroxy-methyl)methyl-3-aminopropane sulphonic acid, pK_a 8.4) was substituted for HEPES, and the solution titrated to pH 8.5.

In experiments in which the effects of myocardial ischaemia were investigated, hearts were perfused with Krebs-Henseleit buffer (Goulielmos *et al*., 1995) which contained (in mM): NaCl 118.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.6, glucose 8.0, Na pyruvate 2.0. The buffer was gassed with 95% O_2/5% CO_2 to obtain pH 7.4.

Electrophysiological recordings

Heart rate was maintained constant at 3.3 Hz (198 beats min^{-1}, cycle length 303 ms between beats, just above the spontaneous sinus rate) by right ventricular pacing at twice the diastolic pacing threshold using square wave pulses 5 ms in duration from a stimulator (ST-02, Experimetria Ltd., Budapest, Hungary). Action potentials, refractory periods, electrograms and perfusion pressure were recorded and analysed (Goulielmos *et al*., 1995) using a digital data acquisition and analysis system (Po-Ne-Mah®, Gould Instrument Systems, Inc., Valley View, OH, U.S.A.; 12-bit resolution, frequency response 5 kHz, sampling rates 1 kHz for perfusion pressure, electrograms and action potentials). Electrograms were recorded as a volume-conducted ECG equivalent (lead II) using three silver electrodes fixed within the organ bath in a triangular arrangement. Transmembrane action potentials were recorded using the floating microelectrode technique from apical right ventricular epicardial cells (Goulielmos *et al*., 1995). Glass microelectrodes (tip resistances of 5–15 MΩ) filled with 3 M KCl were mounted on flexible silver wire (diameter 125 μm) coated with Teflon® except for 3 mm at the tip which formed a Ag/AgCl junction. Microelectrodes were connected to a differential FET-input instrumentation amplifier with an input impedance of 1.5 TΩ (built by the Department of Medical Engineering, Imperial College School of Medicine based on circuits described by Chapman & Fry, 1978). Action potentials were recorded as the potential between the intracellular microelectrode and a reference electrode (a KCl salt bridge connected *via* an Ag/AgCl junction) placed in the organ bath, as described by Penny & Sheridan (1983). All recordings were made from the apical region of the heart and multiple impalements were required to provide continuous electrophysiological data, but all data presented are based on single, stable impalements. Using this technique, stable impalements can be achieved for at least 20 s allowing 60 action potentials to be recorded and analysed. Action potential duration was measured at 95% repolarization (APD_{95}). Refractory periods were determined using the extrastimulus technique. Pacing threshold was determined prior to each measurement and the extrastimulus was introduced once after every eight regular beats at shorter coupling intervals and in decrements of 5 ms until failure to capture occurred. The effective refractory period was taken as the longest interval at which failure to capture occurred (Penny & Sheridan, 1983). Effects on heart rate were investigated in unpaced preparations.

Protocol – effects of acidosis/alkalosis

Hearts were perfused with HEPES-Tyrode buffer (pH 7.4) for a control period of 20 min and measurements made. Perfusion was then switched to either TAPS-Tyrode buffer (pH 8.5) or MES-Tyrode buffer (pH 6.5) which was allowed to flow for 30 min and measurements repeated. Perfusion was then continued with buffer (either TAPS-Tyrode or MES-Tyrode) containing 1 nM Ap_nA (either Ap_4A or Ap_5A) which was allowed to flow for 30 min. The concentration of Ap_nA was then increased to 1 μM and measurements were repeated following 30 min exposure to this concentration. Measurements were repeated at 5 min intervals during exposure to the two concentrations and peak/nadir responses are reported.

In pilot experiments we confirmed that perfusion of hearts ($n=4$) with HEPES-Tyrode buffer (pH 7.4) had no effect on the variables recorded, compared with perfusion with Krebs-Henseleit buffer. We also confirmed that the effects of 1 nM

and 1 μM Ap_4A and Ap_5A in HEPES-Tyrode buffer were the same as those which we have reported in Krebs-Henseleit buffer at the same pH (Stavrou *et al*., 2001a).

To confirm that the coronary circulation retained residual vasodilator capacity under acidotic conditions, we examined the ability of 100 μM adenosine to alter coronary perfusion pressure during perfusion with buffer at pH 6.5 in six hearts.

We studied the effects of Ap_nA at 1 nM and 1 μM because these are concentrations which produce transient and sustained reductions in coronary perfusion pressure, respectively and increases in APD_{95} (1 nM) and refractory period (1 nM and 1 μM) in guinea-pig hearts (Stavrou *et al*., 2001a).

In pilot experiments we attempted to reduce pH_o to values <6.5 but this resulted in electrophysiological and mechanical disturbances. We therefore restricted our study to investigating the effects of changes in pH_o of the order of ± 1 pH unit. This allowed us to attempt to produce changes in the pH of the intracellular and extracellular compartments which would be comparable to those occurring during ischaemia and to investigate the effects of changes in the directional flux of protons. Perfusion with acidotic buffer would be expected to produce an intracellular and extracellular acidosis and an influx of protons. During perfusion with an alkalotic buffer, an intracellular and extracellular alkalosis occurs with an outward flux of protons. During ischaemia, the intracellular compartment becomes acidotic first and an outward flux of protons occurs which, after equilibration, is followed by an extracellular acidosis.

Protocol – effects of ischaemia and reperfusion

Hearts were perfused with Krebs-Henseleit buffer for a control period of 20 min and measurements made. To study the effects of Ap_4A, perfusion was then continued with buffer containing 1 μM Ap_4A which was allowed to flow for 30 min. Measurements were repeated at 5 min intervals and peak/nadir responses are reported. Control hearts received drug-free buffer for an equivalent period of time. Global low-flow myocardial ischaemia was then induced by reducing perfusion flow rate to 10% of control for 30 min (Penny & Sheridan, 1983; Goulielmos *et al*., 1995) and measurements were repeated at 5 min intervals. Reperfusion was initiated by restoring flow rates to control for 15 min and measurements were repeated at 5 min intervals. The onset and incidence of ventricular tachycardia and fibrillation during ischaemia and reperfusion were noted (Walker *et al*., 1988).

We studied the effects of 1 μM Ap_4A because this concentration produces a maximal, maintained reduction in coronary perfusion pressure and increases refractory period under normal conditions (Stavrou *et al*., 2001a) and because plasma concentrations of Ap_4A following platelet stimulation may reach this level (reviewed in Flores *et al*., 1999b).

The investigation conformed with United Kingdom legislation, the *Animals (Scientific Procedures) Act, 1986*.

Experimental groups

The effects of acidosis and alkalosis on responses to Ap_4A and Ap_5A were studied in six hearts per group. The effects of Ap_4A and Ap_5A at pH 7.4 were also studied in six hearts in each group. The effects of ischaemia were studied in 10 control hearts and 10 hearts which received Ap_4A.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean. Analysis of Variance followed by Bonferroni's Multiple Comparison Test (for changes in coronary perfusion pressure) and Dunnett's Multiple Comparison Test (for changes in APD_{95}, refractory period and heart rate in unpaced hearts) as post tests, were used to identify where statistically significant differences had occurred in hearts in which the effects of acidosis/alkalosis on responses to Ap_nA were investigated. The effects of acidosis and alkalosis *per se* were compared using Student's paired *t*-test. In hearts in which the effects of ischaemia were examined, the effects of Ap_4A were compared to drug-free control conditions within the same group of hearts using Student's paired *t*-test. To compare the effects of Ap_4A on APD_{95} and refractory period *vs* drug-free conditions during ischaemia, Bonferroni's Multiple Comparison Test was used. The effects of Ap_4A on the onset time of arrhythmias were compared using Student's *t*-test, while its effects on the incidence of arrhythmias and recovery were compared using Fisher's exact test. $P \leqslant 0.05$ defines the probability value indicating statistical significance and the test is clearly stated with the results.

Drugs

All chemicals used for the preparation of the Krebs-Henseleit and Tyrode buffers were of analytic grade (Merck Ltd., Lutterworth, U.K.). Ap_nA were dissolved in buffer (Tyrode or Krebs-Henseleit).

Results

Vasomotor and electrophysiological effects of Ap_nA

Figures 1 and 2 illustrate the vasomotor and electrophysiological effects of Ap_4A and Ap_5A at 1 nM and 1 μM in HEPES-Tyrode buffer at pH 7.4. These were comparable to those which we have described previously in Krebs-Henseleit buffer at the same pH (Stavrou *et al*., 2001a). Transient reductions in coronary perfusion pressure, which recovered, were seen with 1 nM Ap_nA, as illustrated for Ap_4A in Figure 1a. When the concentration was increased to 1 μM, larger and persistent, statistically significant reductions in perfusion pressure were observed. With both Ap_4A and Ap_5A, APD_{95} and refractory period were increased, as reported previously (Stavrou *et al*., 2001a). These effects are illustrated in Figure 2.

Effects of acidosis

Extracellular acidosis reduced heart rate (in unpaced hearts, from 168 ± 6 to 129 ± 5 beats min^{-1}, $P<0.0001$, paired *t*-test, $n=12$) and coronary perfusion pressure (from 37.5 ± 2.4 to 21.4 ± 2.6 mmHg, $P<0.0001$, paired *t*-test, $n=12$), but did not alter APD_{95} (183.4 ± 0.8 *vs* 186.9 ± 1.5 ms, $n=12$) or refractory period (150.4 ± 1.6 *vs* 155.0 ± 2.6 ms, $n=12$) in paced hearts.

The effects of Ap_4A and Ap_5A were not seen in acidotic conditions such that APD_{95} and refractory period were unchanged in the presence of these compounds, Table 1. The ability of Ap_4A and Ap_5A to alter coronary perfusion

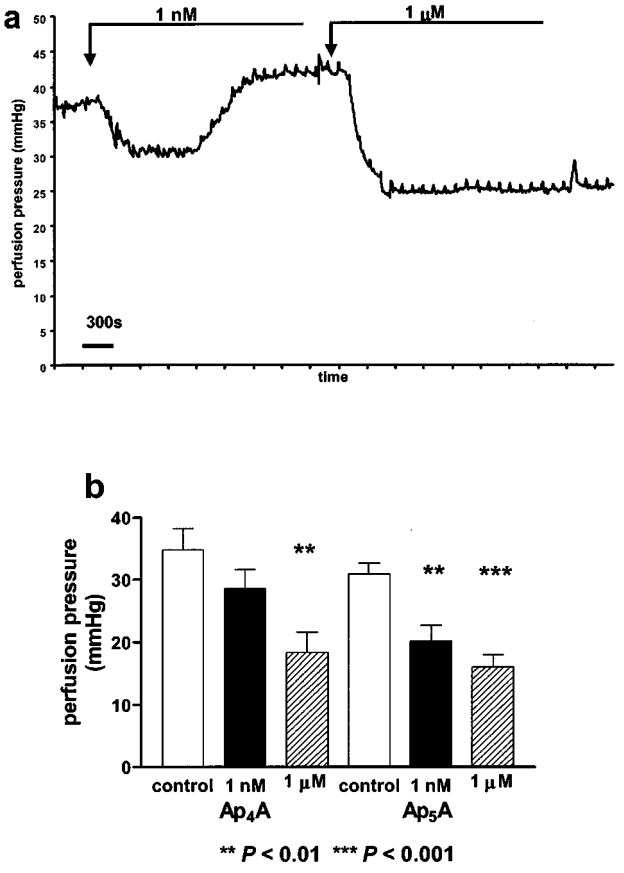

Figure 1 Effects of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) on coronary perfusion pressure at pH 7.4. (a) illustrates a typical recording (representative of five similar experiments which had similar results) from a heart receiving 1 nM Ap_4A followed by 1 μM. Exposure to 1 nM Ap_4A produced a transient reduction in perfusion pressure that recovered, while exposure to 1 μM Ap_4A produced a larger, maintained reduction in perfusion pressure. (b) illustrates the mean nadir responses of hearts to Ap_4A and Ap_5A at 1 nM and 1 μM. Data were obtained from six hearts for each compound and statistical significance determined by Bonferroni's Multiple Comparison test *vs* respective Ap_nA-free conditions (control).

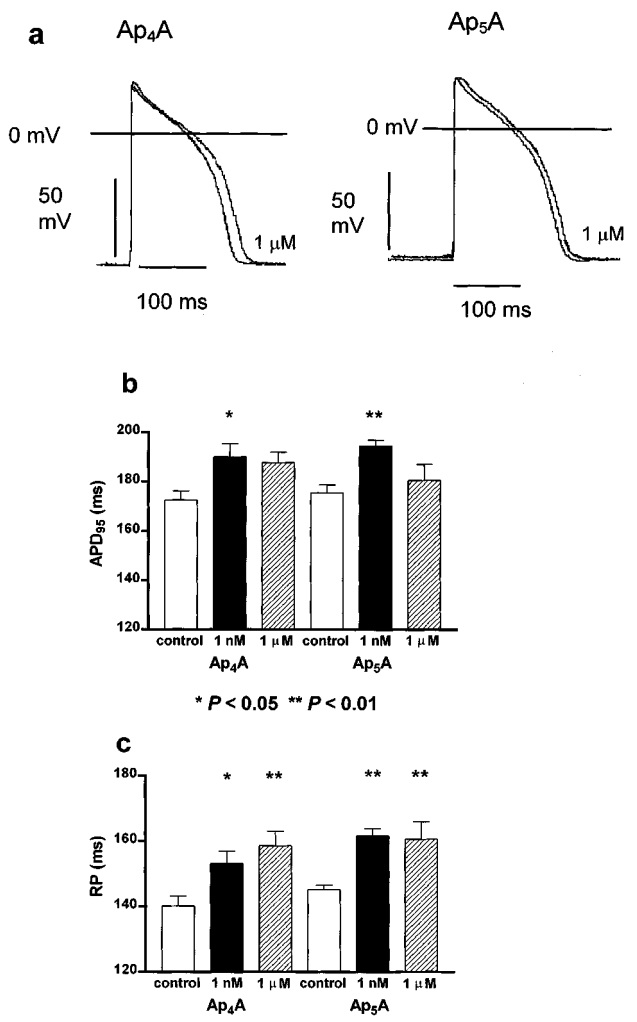

Figure 2 Effect of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) on ventricular action potential duration and refractoriness at pH 7.4. (a) illustrates representative action potential recordings from hearts before and after 1 μM Ap_4A and Ap_5A (representative of five similar experiments with both compounds that had similar results). (b) and (c) illustrate the effects of Ap_4A and Ap_5A at concentrations of 1 nM and 1 μM on action potential duration measured at 95% repolarization (APD_{95}) and refractory period (RP), respectively. Data were obtained from six hearts for each compound and statistical significance determined by Dunnett's Multiple Comparison test *vs* respective Ap_nA-free conditions (control).

pressure was attenuated, since perfusion pressure was unchanged in the presence of 1 nM Ap_4A and was only slightly (non-significantly, by Bonferroni's Multiple Comparison test) reduced in the presence of 1 μM Ap_4A, Table 1. Heart rate tended to decrease slightly during exposure to Ap_4A in unpaced hearts but was unchanged during exposure to Ap_5A, Table 1.

Although acidosis produced a reduction in coronary perfusion pressure, the coronary circulation retained its vasodilatory capacity since addition of 100 μM adenosine resulted in a further reduction in perfusion pressure (from 22.8 ± 1.3 to 18.8 ± 1.5 mmHg, $P = 0.04$, paired t-test, $n = 6$).

Effects of alkalosis

Alkalosis *per se* did not significantly affect the measured variables. Heart rate was unchanged in unpaced hearts (165.4 ± 4.8 *vs* 168.9 ± 8.2 beats min^{-1}, $n = 12$) while coronary perfusion pressure, APD_{95} and refractory period were unchanged (37.7 ± 3.6 *vs* 44.3 ± 4.8 mmHg, 176.5 ± 3.4 *vs* 176.3 ± 3.1 ms, 146.7 ± 2.3 *vs* 148.3 ± 3.1 ms, respectively, $n = 12$).

The ability of Ap_4A to increase refractory period was preserved (from 142.5 ± 2.1 to 160.0 ± 3.0 ms with 1 μM, $P < 0.01$, Bonferroni's Multiple Comparison Test, $n = 6$) (Figure 3). A similar, but non-significant trend was observed with Ap_5A and with APD_{95} (Figure 4). The ability of Ap_4A and Ap_5A to alter perfusion pressure was also attenuated (Figures 3 and 4). Although slight reductions in perfusion pressure were seen in the presence of Ap_4A, these did not reach statistical significance (from 38.1 ± 2.5 to 30.9 ± 3.2 mmHg with 1 nM Ap_4A and 33.3 ± 4.2 mmHg with 1 μM Ap_4A, $n = 6$; P = N.S., Bonferroni's Multiple Comparison test). Under alkalotic conditions, Ap_5A had little effect on perfusion pressure: (from 50.5 ± 8.9 to 49.4 ± 9.9 mmHg with 1 nM Ap_5A and

46.8±5.4 mmHg with 1 μM Ap_5A, $n=6$, $P=$N.S., Bonferroni's Multiple Comparison test). Heart rate was unchanged in unpaced hearts during exposure to 1 nM Ap_4A and Ap_5A and tended to decrease slightly during exposure to 1 μM Ap_4A and Ap_5A (from 166.8±14.1 to 153.7±26.1 beats min^{-1} with 1 μM Ap_4A, $n=6$, and from 170.9±9.6 to 164.8±9.5 beats min^{-1} with 1 μM Ap_5A, $n=6$, $P=$N.S. in both cases, Bonferroni's Multiple Comparison test), (Figures 3 and 4).

Effects of ischaemia

Normal perfusion of hearts with 1 μM Ap_4A produced the expected changes in APD_{95} (from 168.6±1.7 to 174.0±6.3 ms), refractory period (from 149.5±7.0 to 162.0±2.4 ms, $P<0.02$, paired t-test, $n=10$) and coronary perfusion pressure (from 41.3±2.1 to 29.9±1.6 mmHg, $P<0.0001$, paired t-test, $n=10$), (Figure 5). These changes were comparable to those that we

Table 1 Effects of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) under acidotic conditions (pH 6.5)

	APD_{95} (ms)	*Refractory period* (ms)	*Perfusion pressure* (mmHg)	*Heart rate* (beats min^{-1})
pH7.4	184.4±1.0	150.8±3.3	38.4±3.1	159.7±9.8
pH6.5	187.2±2.3	150.8±4.6	26.5±3.2*	125.5±9.0
+1 nM Ap_4A	184.2±0.6	153.6±5.0	27.0±3.1	112.7±11.1
+1 μM Ap_4A	184.0±0.5	157.4±2.3	20.7±2.6	105.8±12.4
pH 7.4	181.8±0.8	150.0±0.0	36.7±4.0	176.1±3.8
pH 6.5	186.5±1.9	159.2±1.5	15.4±3.0**	131.6±4.3**
+1 nM Ap_5A	189.1±1.5	162.6±3.8	13.6±2.9	124.4±7.5
+1 μM Ap_5A	187.0±2.5	164.7±1.2	11.6±2.1	129.9±9.5

Data were obtained from six hearts before and after admission of diadenosine tetraphosphate and another group of six hearts before and after admission of diadenosine pentaphosphate. APD_{95}, action potential duration measured at 95% repolarization. *$P<0.05$, **$P<0.001$ *vs* pH 7.4, paired t-test.

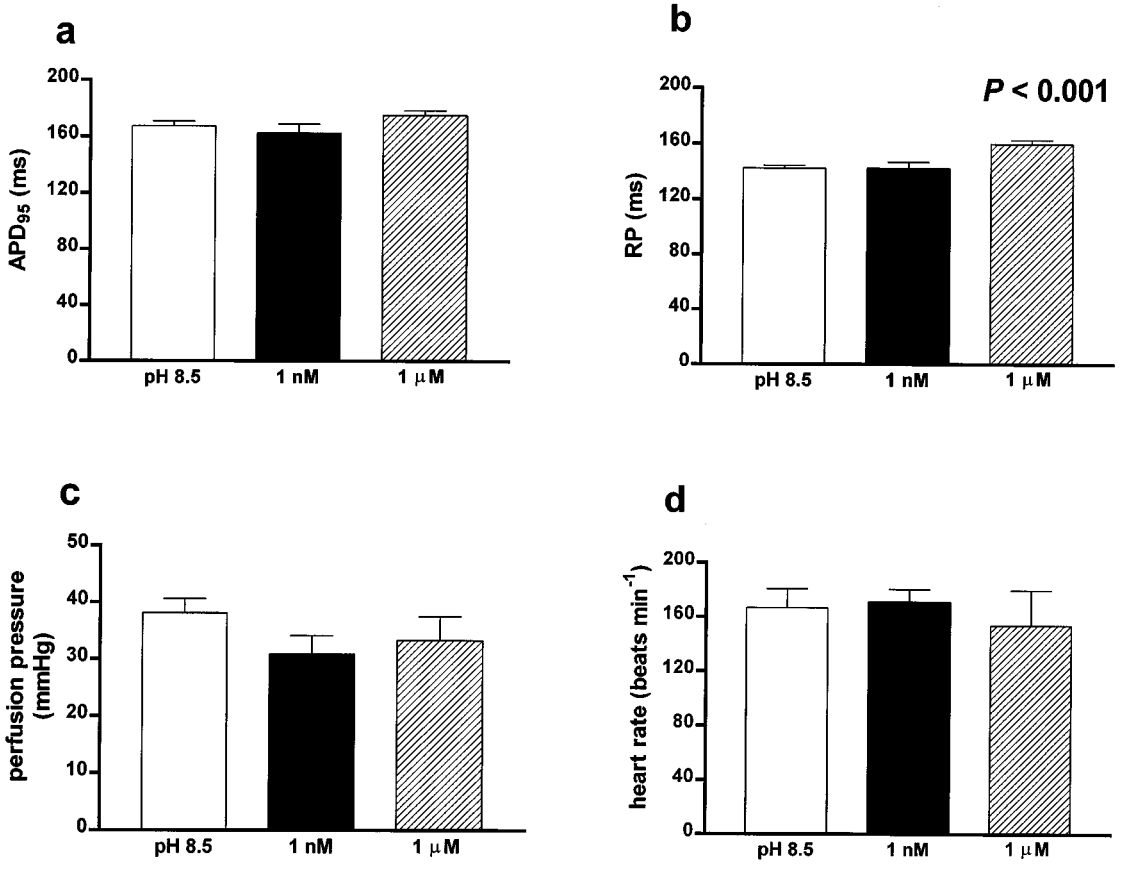

Figure 3 Effects of diadenosine tetraphosphate at 1 nM and 1 μM on action potential duration measured at 95% repolarization (APD_{95}, a), refractory period (RP, b), coronary perfusion pressure (c) and heart rate (in unpaced hearts, d) under alkalotic conditions (pH 8.5). Diadenosine tetraphosphate had no effect on APD_{95} or RP at 1 nM under alkalotic conditions, but at 1 μM RP was increased. APD_{95} tended to be increased by 1 μM diadenosine tetraphosphate, but this effect did not reach statistical significance (by Dunnett's Multiple Comparison test). Perfusion pressure and heart rate also tended to be reduced slightly by 1 μM diadenosine tetraphosphate but these changes did not reach statistical significance (by Bonferroni's and Dunnett's Multiple Comparison tests, respectively). Data were obtained from six hearts before (pH 8.5) and after admission of diadenosine tetraphosphate and the statistically significant difference indicated was made using Dunnett's test *vs* diadenosine tetraphosphate-free conditions (pH 8.5).

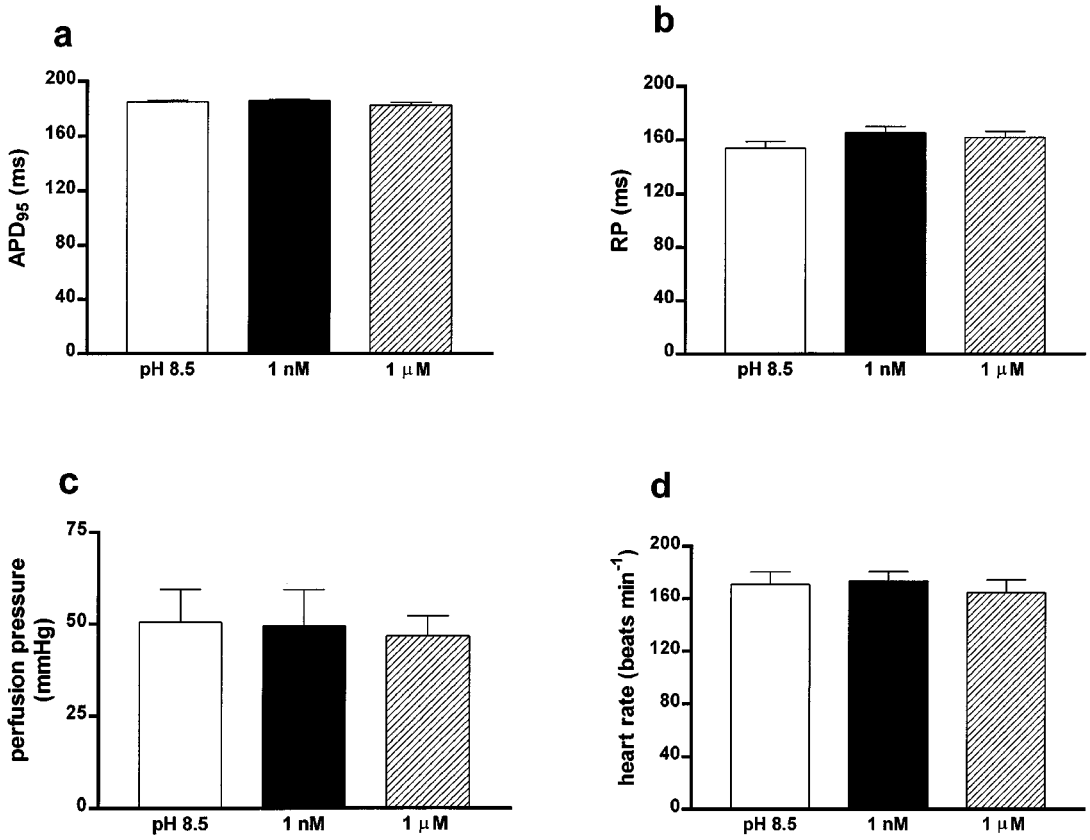

Figure 4 Effects of diadenosine pentaphosphate at 1 nM and 1 μM on action potential duration measured at 95% repolarization (APD_{95}, a), refractory period (RP, b), coronary perfusion pressure (c) and heart rate (in unpaced hearts, panel d) under alkalotic conditions (pH 8.5). Diadenosine pentaphosphate had no statistically significant effect on APD_{95} or RP under alkalotic conditions (by Dunnett's Multiple Comparison test), but RP tended to be increased. Perfusion pressure and heart rate tended to be reduced slightly by 1 μM diadenosine pentaphosphate but these changes did not reach statistical significance (by Bonferroni's and Dunnett's Multiple Comparison tests, respectively). Data were obtained from six hearts before (pH 8.5) and after admission of diadenosine pentaphosphate.

have reported previously (Stavrou *et al*., 2001a) and those illustrated in Figures 1 and 2. APD_{95} and refractory period were reduced during myocardial ischaemia to comparable extents in both groups of hearts (i.e. those receiving Ap_4A or drug-free controls) such that no statistically significant differences were observed between them, (Figure 5) (P = N.S. at all time points, Bonferroni's Multiple Comparison test, $n = 10$ in both groups). The onset times and incidences of ventricular arrhythmias during ischaemia and recovery during reperfusion were all similar between the groups, (Figure 5), although the presence of Ap_4A appeared to produce a trend for a greater incidence and earlier onset of arrhythmias with recovery in fewer hearts (P = N.S., Student's t-test and Fisher's exact test).

Discussion

This study has shown that pH_o influences the cardiac electrophysiological and coronary vasomotor effects of Ap_4A and Ap_5A and that Ap_4A has no antiarrhythmic effects during myocardial ischaemia. Novel observations are: (1) the lack of effect of Ap_4A and Ap_5A on cellular electrophysiology and coronary vasomotion under acidotic conditions; (2) the preservation of the electrophysiological effects of Ap_4A and the absence of the vasomotor effects of Ap_4A and Ap_5A under alkalotic conditions; and (3) the lack of antiarrhythmic effects of Ap_4A during ischaemia. These data indicate that any potentially cardioprotective effects of these compounds during normal perfusion are absent during ischaemia. This suggests that further work is required to confirm the presence of beneficial effects of non-hydrolysable Ap_nA analogues that have been synthesized for therapeutic use in conditions of platelet activation (Chan *et al*., 1997) under the conditions in which they might be required clinically.

We studied the effects of acidosis and alkalosis on the cardiac responses to Ap_4A and Ap_5A because of the members of the Ap_nA family, they have effects under normal conditions which if preserved, were most likely to have beneficial effects when pH_o is altered (Stavrou *et al*., 2001a). We investigated the effects of Ap_4A during ischaemia because the actions of this compound have been widely investigated in various models and because of the two compounds, the effects of Ap_4A would be more likely to be beneficial (Stavrou *et al*., 2001a).

Extracellular pH may be manipulated experimentally by various means. One method is to alter the composition of the buffer (e.g. Källner & Franco-Cereceda, 1998). Since Ca^{2+}

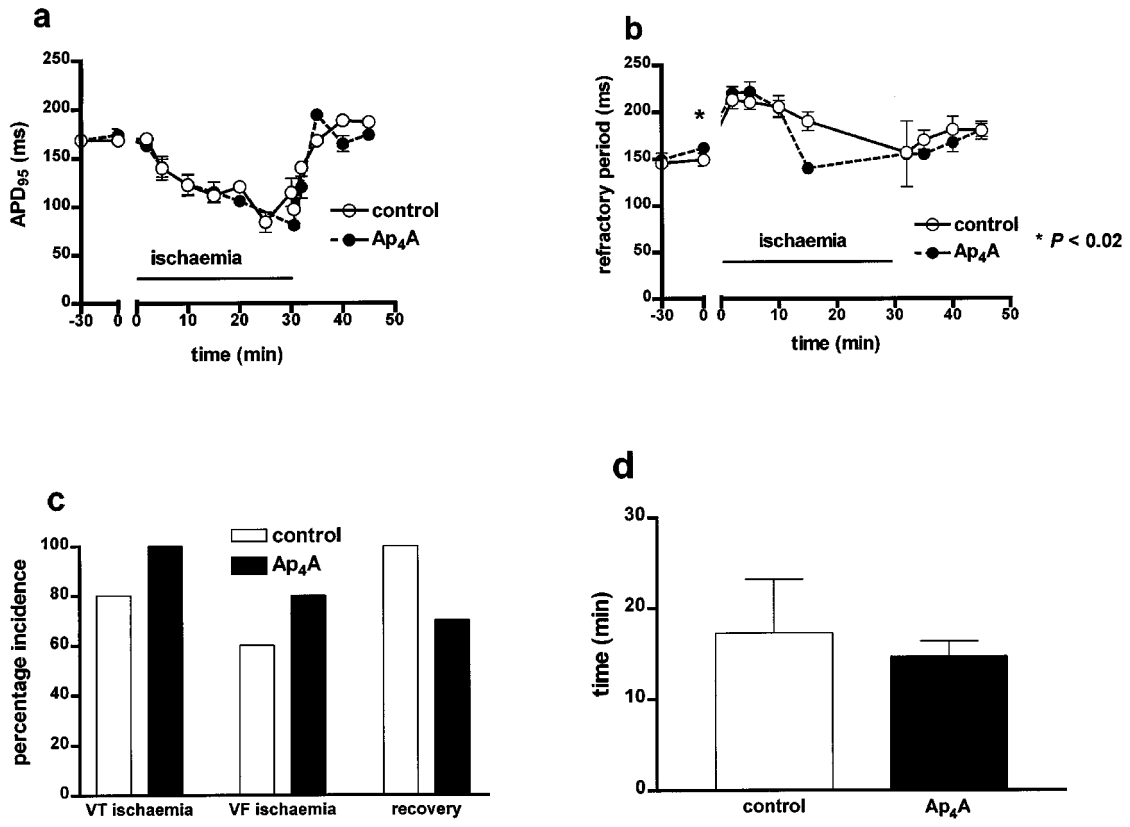

Figure 5 Effects of diadenosine tetraphosphate (Ap_4A) at 1 μM during myocardial ischaemia and reperfusion. (a) Illustrates the effects of Ap_4A on action potential duration measured at 95% repolarization (APD_{95}) and (b) illustrates the effects on refractory period. No statistically significant differences (by Bonferroni's Multiple Comparison test) were detected between the groups. (Refractory periods were not measured between 15 and 30 min of ischaemia due to the risk of triggering ventricular arrhythmias). (c) Illustrates the percentage incidence of ventricular tachycardia (VT) and fibrillation (VF) during ischaemia in control hearts and hearts receiving Ap_4A, together with the incidence of spontaneous recovery from these arrhythmias during reperfusion. No statistically significant differences were observed (by Fisher's exact test). (d) Illustrates the onset time of ventricular tachycardia during ischaemia in the two groups of hearts. No statistically significant differences (by Student's *t*-test) were observed. Thus, Ap_4A had no antiarrhythmic or electrophysiological effects during ischaemia and reperfusion. Interestingly, the presence of Ap_4A was associated with a trend for an earlier onset and greater incidence of arrhythmias during ischaemia together with recovery in fewer hearts. Data were obtained from 10 control (drug-free) hearts and 10 hearts which received 1 μM Ap_4A.

and HCO_3^- form ion pairs in solution (Fry & Poole-Wilson, 1981; Wu & Fry, 1998) this method suffers from the disadvantage that Ca^{2+} activity may be altered, with attendant, secondary effects on cardiac function. An alternative is to change the CO_2 content of the gas mixture (Fry & Poole-Wilson, 1981) or to use buffered Tyrode solutions (Gögelein *et al*., 1998). We chose this latter method and were careful to prepare buffers using agents with pK_a values as close as practically possible to the desired pH.

Perfusion of hearts under acidotic conditions produced the expected effects of reduction in heart rate and coronary perfusion pressure (Fry & Poole-Wilson, 1981; Källner & Franco-Cereceda, 1998; Ralevic, 2000). The absence of effects of Ap_4A and Ap_5A under acidotic conditions was paralleled by the lack of antiarrhythmic effects of Ap_4A during ischaemia. Interestingly, the electrophysiological effects of Ap_4A were preserved under alkalotic conditions, while those of Ap_5A were attenuated. The vasomotor effects of both compounds were attenuated under acidotic and alkalotic conditions.

Myocardial ischaemia produces an intracellular acidosis which occurs before the development of an extracellular acidosis. The intracellular Na^+ and Ca^{2+} overload associated with ischaemia arises due to an outward flux of protons (the direction of which is mimicked by extracellular alkalosis) which, after equilibration, leads to extracellular acidosis. Thus, ischaemia is likely to produce changes in intracellular signalling components and receptor proteins before the conformation of the Ap_nA is affected by the change in pH_o.

A recent study by Beauloye *et al*. (2001) reported that ischaemia inhibits insulin signalling in the heart by decreasing intracellular pH. Although changes in intracellular signalling associated with ischaemia were beyond the scope of our study, they may be relevant especially as Jovanovic *et al*. (1998) considered Ap_5A as an intracellular signalling molecule involved in the cardiac response to metabolic stress. They found that ischaemia induced a 10 fold decrease in myocardial concentration of Ap_5A which allowed a high probability of ATP-dependent K^+ (K_{ATP}) channel opening which supports earlier observations that high intracellular concentrations of Ap_nA inhibit K_{ATP} channel activity (Jovanovic *et al*., 1997). The loss of such an inhibitory effect during ischaemia would tend to enhance the ischaemia-induced reduction in APD_{95}, precluding any antiarrhythmic effects.

One mechanism that could account for our observations relates to evidence that changes in pH_o influence purinergic receptor activity (King *et al*., 1996; Wildman *et al*., 1999a, b; Clarke *et al*., 2000 and Zhong *et al*., 2000) or that P2-receptors can distinguish between fully ionized and other forms of agonist (Lustig *et al*., 1992). Another mechanism relates to observations by Kolodny *et al*. (1979) of conformational changes in Ap_4A and Ap_5A induced by extracellular acidosis. These authors reported that while Ap_nA adopt a stable, intramolecularly stacked conformation at physiological pH, Ap_4A and Ap_5A alone assume a unique 'folded' unstacked conformation in which the phosphate chains are shielded on both sides by the adenine rings when pH_o is reduced, stabilizing the molecule through electrostatic interactions between the negatively charged phosphate groups and the partially positively charged adenine rings.

In the guinea-pig heart, the electrophysiological effects of Ap_4A are mediated *via* P_1- (adenosine) and P2-purinergic receptors, while the vasomotor effects are mediated *via* P_2-receptors (Stavrou *et al*., 2001a). Impaired binding of Ap_4A to these receptors would thus be likely to result in the attenuation of vasomotor and electrophysiological effects. The relative preservation of the electrophysiological effects of Ap_4A under alkalotic conditions but the absence of vasomotor effects suggests that under these conditions, binding to P_2-receptors but not P_1- (adenosine) receptors is impaired or that intracellular signalling pathways activated by agonist binding to the receptors may be altered by these conditions.

It is widely known that alterations in pH_o can stimulate release of nitric oxide and prostacyclin from the vascular endothelium (Mitchell *et al*., 1991 and 1992; Demirel *et al*., 1993; Franco-Cereceda *et al*., 1994; Gurevicius *et al*., 1995; Hiley *et al*., 1995; Coessens *et al*., 1996). In the guinea-pig, the coronary vasomotor and cardiac electrophysiological effects of Ap_nA are mediated *via* release of prostanoids and both prostanoids and nitric oxide, respectively (Stavrou *et al*., 2001b) and it is possible that the lack of effect of Ap_nA when pH_o is altered could be due to release of these mediators induced by the change in pH_o. This is unlikely because the electrophysiological effects of Ap_4A were preserved under alkalotic conditions while the vasomotor effects were attenuated under acidotic conditions rather than ablated even though the coronary circulation retained vasodilator capacity. Potential limitations of our study relate to possible small changes in affinity of Ap_nA associated with changes in pH_o which might be overcome with higher agonist concentrations and effects associated with the use of buffers in which extracellular bicarbonate is absent, unlike the situation *in vivo* where the CO_2-HCO_3^- system is important. With regard to the latter point, Bountra & Vaughan-Jones (1989) have, however, commented on the similarity of the effects of changes in intracellular pH on contraction in cardiac muscle under experimental conditions using HEPES-buffered solutions and CO_2-HCO_3^--buffered solutions.

Ap_4A had no antiarrhythmic effect during ischaemia in this model. Interestingly, the presence of Ap_4A was associated with a trend for an earlier onset and greater incidence of arrhythmias during ischaemia and recovery during reperfusion in fewer hearts. We are unaware of any other studies that have examined electrophysiological effects of Ap_nA during ischaemia. Of the studies that have examined the effects of Ap_4A, beneficial effects in terms of improvements in post-ischaemic contractile function but not against reperfusion-induced arrhythmias were reported (Ahmet *et al*., 2000a, b, c, d). An earlier study by Humphrey *et al*. (1987) reported no beneficial effects of Ap_5A on post-ischaemic myocardial function.

In conclusion, this study has shown that extracellular acidosis alters the cardiac electrophysiological and coronary vasomotor effects of Ap_4A and Ap_5A and that Ap_4A has no antiarrhythmic or electrophysiological effects during myocardial ischaemia. This suggests that under conditions of platelet activation *in vivo*, Ap_nA do not have cardioprotective effects (discussed by Flores *et al*., 1999b).

Supported by British Heart Foundation project grant PG/99183 and a Physiological Society Vacation Studentship (ref. 92021) for Caroline Beck.

References

AHMET, I., SAWA, Y., NISHIMURA, M., ICHIKAWA, H. & MATSUDA, H. (2000a). Diadenosine tetraphosphate (AP_4A) mimics cardioprotective effect of ischemic preconditioning in the rat heart: contribution of K_{ATP} channel and PKC. *Basic Res. Cardiol.,* **95,** 235–242.

AHMET, I., SAWA, Y., NISHIMURA, M., KITAKAZE, M. & MATSUDA, H. (2000b). Cardioprotective effect of diadenosine tetraphosphate (AP_4A) preservation in hypothermic storage and its relation with mitochondrial ATP-sensitive potassium channels. *Transplantation,* **69,** 16–20.

AHMET, I., SAWA, Y., NISHIMURA, M. & MATSUDA, H. (2000c). Cardioprotective effect of diadenosine tetraphosphate (AP_4A) cardioplegia in isolated rat hearts. *Heart Vessels,* **15,** 30–34.

AHMET, I., SAWA, Y., NISHIMURA, M., YAMAGUCHI, T., KITAKAZE, M. & MATSUDA, H. (2000d). Myocardial protection using diadenosine tetraphosphate with pharmacological preconditioning. *Ann. Thorac. Surg.,* **70,** 901–905.

BEAULOYE, C., BERTRAND, L., KRAUSE, U., MARSIN, A.-S., DRESSELAERS, T., VANSTAPEL, F., VANOVERSCHELDE, J.-L. & HUE, L. (2001). No-flow ischemia inhibits insulin signaling in heart by decreasing intracellular pH. *Circ. Res.,* **88,** 513–519.

BOUNTRA, C. & VAUGHAN-JONES, R.D. (1989). Effect of intracellular and extracellular pH on contraction in isolated, mammalian cardiac muscle. *J. Physiol.,* **418,** 163–187.

CHAN, S.W., GALLO, S.J., KIM, B.K., GUO, M.J., BLACKBURN, G.M. & ZAMECNIK, P.C. (1997). P^1,P^4-dithio-P^2,P^3-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate: A novel antiplatelet agent. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 4034–4039.

CHAPMAN, R.A. & FRY, C.H. (1978). An analysis of the cable properties of frog ventricular myocardium. *J. Physiol.,* **283,** 263–282.

CLARKE, C.E., BENHAM, C.D., BRIDGES, A., GEORGE, A.R. & MEADOWS, H.J. (2000). Mutation of histidine 286 of the human P2X4 purinoceptor removes extracellular pH sensitivity. *J. Physiol.,* **523,** 697–703.

COESSENS, B.C., MILLER, V.M. & WOOD, M.B. (1996). Endothelin-A receptors mediate vascular smooth muscle response to moderate acidosis in the canine tibial nutrient artery. *J. Orthop. Res.,* **14,** 818–822.

DEMIREL, E., LASKEY, R.E., PURKERSON, S. & VAN BREEMEN, C. (1993). The passive calcium leak in cultured porcine aortic endothelial cells. *Biochem. Biophys. Res. Commun.,* **191,** 1197–1203.

FLORES, N.A. (1996). Platelet activation during myocardial ischaemia: a contributory arrhythmogenic mechanism. *Pharmacol. Ther.,* **72,** 83–108.

FLORES, N.A., BOTCHWAY, A.N.S., STAVROU, B.M. & SHERIDAN, D.J. (1999a). Cardiac electrophysiological effects of platelet-derived substances. *Exp. Physiol.,* **84,** 253–274.

FLORES, N.A., STAVROU, B.M. & SHERIDAN, D.J. (1999b). The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc. Res.,* **42,** 15–26.

FRANCO-CERECEDA, A., KÄLLNER, G. & LUNDBERG, J.M. (1994). Cyclo-oxygenase products released by low pH have capsaicin-like actions on sensory nerves in the isolated guinea pig heart. *Cardiovasc. Res.,* **28,** 365–369.

FRY, C.H. & POOLE-WILSON, P.A. (1981). Effects of acid-base changes on excitation-contraction coupling in guinea-pig and rabbit cardiac ventricular muscle. *J. Physiol.,* **313,** 141–160.

GÖGELEIN, H., HARTUNG, J., ENGLERT, H.C. & SCHÖLKENS, B.A. (1998). HMR 1883, a novel cardioselective inhibitor of the ATP-sensitive potassium channel. Part 1: effects on cardiomyocytes, coronary flow and pancreatic β-cells. *J. Pharmacol. Exp. Ther.,* **286,** 1453–1464.

GOULIELMOS, N.V., ENAYAT, Z.E., SHERIDAN, D.J., COHEN, H. & FLORES, N.A. (1995). Nitric oxide and prostacyclin modulate the alterations in cardiac action potential duration mediated by platelets during ischaemia. *Cardiovasc. Res.,* **30,** 788–798.

GUREVICIUS, J., SALEM, M.R., METWALLY, A.A., SILVER, J.M. & CRYSTAL, G.J. (1995). Contribution of nitric oxide to coronary vasodilation during hypercapnic acidosis. *Am. J. Physiol.,* **268,** H39–H47.

HILEY, C.R., BOTTRILL, F.E., WARNOCK, J. & RICHARDSON, P.J. (1995). Effects of pH on responses to adenosine, CGS 21680, carbachol and nitroprusside in the isolated perfused superior mesenteric arterial bed of the rat. *Br. J. Pharmacol.,* **116,** 2641–2646.

HIRCHE, H., FRANZ, C., BÖS, L., BISSIG, R., LANG, R. & SCHRAMM, M. (1980). Myocardial extracellular K^+ and H^+ increase and noradrenaline release as possible early causes of early arrhythmias following acute coronary artery occlusion in pigs. *J. Mol. Cell. Cardiol.,* **12,** 579–593.

HUMPHREY, S.M., HOLLISS, D.G. & CARTNER, L.A. (1987). Influence of inhibitors of ATP catabolism on myocardial recovery after ischemia. *J. Surg. Res.,* **43,** 187–195.

JOVANOVIC, A., ALEKSEEV, A.E. & TERZIC, A. (1997). Intracellular diadenosine polyphosphates. A novel family of inhibitory ligands of the ATP-sensitive K^+ channel. *Biochem. Pharmacol.,* **54,** 219–225.

JOVANOVIC, A., JOVANOVIC, S., MAYS, D.C., LIPSKY, J.J. & TERZIC, A. (1998). Diadenosine 5′,5″-P^1,P^5-pentaphosphate harbors the properties of a signaling molecule in the heart. *FEBS Lett.,* **423,** 314–318.

KÄLLNER, G. & FRANCO-CERECEDA, A. (1998). Ion channels involved in the release of calcitonin gene-related peptide by low pH, prostacylin and capsaicin in the isolated guinea-pig heart. *Eur. J. Pharmacol.,* **352,** 223–228.

KING, B.F., ZIGANSHINA, L.E., PINTOR, J. & BURNSTOCK, G. (1996). Full sensitivity of P_{2X2} purinoceptor to ATP revealed by changing extracellular pH. *Br. J. Pharmacol.,* **117,** 1371–1373.

KOLODNY, N.H., KISTENEFF, E., REDFIELD, C. & RAPAPORT, E. (1979). Ap_4A and Ap_5A prefer folded unstacked conformations at pH 4-5, in sharp contrast with Ap_2A and Ap_3A. *FEBS Lett.,* **107,** 121–124.

LUO, J., JANKOWSKI, J., KNOBLOCH, M., VAN DER GIET, M., GARDANIS, K., RUSS, T., VAHLENSIECK, U., NEUMANN, J., SCHMITZ, W., TEPEL, M., DENG, M.C., ZIDEK, W. & SCHLUTER, H. (1999). Identification and characterization of diadenosine 5′,5‴-P^1,P^2-diphosphate and diadenosine 5′,5‴-P^1,P^3-triphosphate in human myocardial tissue. *FASEB J.,* **13,** 695–705.

LUSTIG, K.D., SPORTIELLO, M.G., ERB, L. & WEISMAN, G.A. (1992). A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionized forms of ATP and UTP. *Biochem. J.,* **284,** 733–739.

MITCHELL, J.A., DE NUCCI, G., WARNER, T.D. & VANE, J.R. (1991). Alkaline buffers release EDRF from bovine cultured aortic endothelial cells. *Br. J. Pharmacol.,* **103,** 1295–1302.

MITCHELL, J.A., DE NUCCI, G., WARNER, T.D. & VANE, J.R. (1992). Different patterns of release of endothelium-derived relaxing factor and prostacylin. *Br. J. Pharmacol.,* **105,** 485–489.

PENNY, W.J. & SHERIDAN, D.J. (1983). Arrhythmias and cellular electrophysiological changes during myocardial 'ischaemia' and reperfusion. *Cardiovasc. Res.,* **17,** 363–372.

RALEVIC, V. (2000). Effect of a decrease in pH on responses mediated by P2 receptors in the rat mesenteric arterial bed. *Eur. J. Pharmacol.,* **406,** 99–107.

STAVROU, B.M., LAWRENCE, C., BLACKBURN, G.M., COHEN, H., SHERIDAN, D.J. & FLORES, N.A. (2001a). Coronary vasomotor and cardiac electrophysiologic effects of diadenosine polyphosphates and nonhydrolyzable analogs in the guinea pig. *J. Cardiovasc. Pharmacol.,* **37,** 571–584.

STAVROU, B.M., SHERIDAN, D.J. & FLORES, N.A. (2001b). Contribution of nitric oxide and prostanoids to the cardiac electrophysiological and coronary vasomotor effects of diadenosine polyphosphates. *J. Pharmacol. Exp. Ther.,* **298,** 531–538.

WALKER, M.J., CURTIS, M.J., HEARSE, D.J., CAMPBELL, R.W.F., JANSE, M.J., YELLON, D.M., COBBE, S.M., COKER, S.J., HARNESS, J.B., HARRON, D.W., HIGGINS, A.J., JULIAN, D.G., LAB, M.J., MANNING, A.S., NORTHOVER, B.J., PARRATT, J.R., RIEMERSMA, R.A., RIVA, E., RUSSELL, D.C., SHERIDAN, D.J., WINSLOW, E. & WOODWARD, B. (1988). The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. *Cardiovasc. Res.,* **22,** 447–455.

WILDMAN, S.S., KING, B.F. & BURNSTOCK, G. (1999a). Modulation of ATP-responses at recombinant rP2X4 receptors by extracellular pH and zinc. *Br. J. Pharmacol.,* **126,** 762–768.

WILDMAN, S.S., KING, B.F. & BURNSTOCK, G. (1999b). Modulatory activity of extracellular H^+ and Zn^{2+} on ATP-responses at rP2X1 and rP2X3 receptors. *Br. J. Pharmacol.,* **128,** 486–492.

WU, C. & FRY, C.H. (1998). The effects of extracellular and intracellular pH on intracellular Ca^{2+} regulation in guinea-pig detrusor smooth muscle. *J. Physiol.,* **508,** 131–143.

ZHONG, Y., DUNN, P.M. & BURNSTOCK, G. (2000). Pharmacological comparison of P2X receptors on rat coeliac, mouse coeliac and mouse pelvic ganglion neurons. *Neuropharmacology,* **39,** 172–180.

(Received February 19, 2001
Revised July 5, 2001
Accepted July 12, 2001)