Diadenosine polyphosphate-stimulated gluconeogenesis in isolated rat proximal tubules

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Diadenosine polyphosphates released into the extracellular environment influence a variety of metabolic and other cellular activities in a wide range of target tissues. Here we have studied the impact of these novel nucleotides on gluconeogenesis in isolated rat proximal tubules. Gluconeogenesis was stimulated following exposure of isolated proximal tubules to a range of adenine-containing nucleotides including ADP, ATP, Ap₃A, Ap₄A, Ap₅A and Ap₆A. The concentration-dependence of ATP-, Ap₃A- and Ap₄A-mediated stimulation of gluconeogenesis was similar and was consistent with a role for these agents in the

physiological control of renal metabolism. Nucleotide-stimulated gluconeogenesis was diminished in the presence of agents that interfere with phospholipase C activation or intracellular Ca²⁺ metabolism, indicative of a role for polyphosphoinositide-mediated Ca²⁺ mobilization in the mechanism of action of ATP, Ap₃A and Ap₄A. The characteristics of binding of [2-³H]Ap₄A to renal plasma-membrane preparations suggest that Ap₄A mediates its effects on proximal tubule gluconeogenesis via interaction with P_{2y}-like purinoceptor(s) also recognized by extracellular ATP.

INTRODUCTION

Extracellular adenine nucleotides, through their interaction with specific purinoceptors, elicit a diverse range of cellular and metabolic responses in many different cell types. The impact of these effectors on the cardiovascular, nervous and immune systems has now been extensively studied [1,2]. Adenine nucleotides are released into the extracellular environment after discharge of a variety of storage granules. A significant proportion of the nucleotide content of dense granules of platelets [3], secretory granules of chromaffin cells [4] and neuronal synaptic granules [5] is in the form of diadenosine polyphosphates (Ap_nA; where *n* = 3, 4, 5, 6). Although Ap₄A and Ap₃A are the best characterized of the diadenosine polyphosphates, all of these unusual compounds appear to exert a wide range of effects on a variety of tissues [6,7]. In contrast with ATP, which is rapidly degraded in the extracellular environment, the diadenosine polyphosphates are characterized *in vivo* by relatively long half-lives [3]. As a consequence, these compounds may be particularly suited to a role in long-range signalling, exerting effects on target cells relatively distant from their site of release into the extracellular space.

A number of reports on the impact of the diadenosine polyphosphates on various aspects of renal cell physiology and biochemistry have appeared. Alterations in renal perfusion rate have been demonstrated [8], which may reflect the ability of nucleotides, including the diadenosine polyphosphates, to alter Cl[−] conductance in rat glomerular mesangial cells [9]. In addition, Ap₄A has been reported to cause activation of phospholipase C [10] and specific stimulation of Ca²⁺ influx into cultured mesangial cells [11]. Diadenosine polyphosphates also modulate Na⁺/H⁺ exchange in isolated rat cortical collecting-duct cells [12,13]. More recently, it has been demonstrated that a variety of diadenosine polyphosphates stimulate proliferation of cultured

mesangial cells and augment mesangial cell growth induced by other mitogenic agents [14,15].

It has been clear for some time that renal gluconeogenesis is sensitive to extracellular adenine nucleotides [16]. These effectors, acting via P_{2y}-like purinoceptors, bring about Ca²⁺-mediated [17] stimulation of gluconeogenesis in proximal tubular cells [18]. The purpose of the present investigation was therefore to assess the ability of a range of diadenosine polyphosphates to promote alterations in gluconeogenesis in isolated rat proximal tubules and to characterize the purinoceptor(s) and signalling system(s) involved. Furthermore the relationship between any such effects and those mediated by ATP and other mononucleotides was investigated in order to provide a better understanding of the relationship between the signalling processes mediated by these different compounds in proximal tubular cells.

MATERIALS AND METHODS

Chemicals

Biochemicals and reagents were obtained from the sources described previously [19] with the following exceptions. Adenosine 5'-[β-thio]diphosphate (ADPβ[S]) and nifedipine were from Sigma. Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt (PPADS) was obtained from Tocris Cookson Ltd., Bristol, U.K. U-73122 and U-73343 were obtained from LC Laboratories, Nottingham, U.K. All other chemicals were of the purest grade available from standard suppliers.

Animals

Male Wistar rats (University of Liverpool breeding colony) weighing 180–220 g were used throughout. Animals were fed *ad*

Abbreviations used: Ap₂A, diadenosine 5',5'''-P¹,P²-diphosphate; Ap₃A, diadenosine 5',5'''-P¹,P³-triphosphate; Ap₄A, diadenosine 5',5'''-P¹,P⁴-tetraphosphate; Ap₅A, diadenosine 5',5'''-P¹,P⁵-pentaphosphate; Ap₆A, diadenosine 5',5'''-P¹,P⁶-hexaphosphate; Ap₄, adenosine 5'-tetraphosphate; ADPβ[S], adenosine 5'-[β-thio]diphosphate; pp[CH₂]pA, adenosine 5'-[α,β-methylene]triphosphate; p[CH₂]ppA, adenosine 5'-[β,γ-methylene]triphosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt; U-73122, 1-(6-[[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione); U-73343, 1-(6-[[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrolidine-2,5-dione).

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libitum [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Preparation and incubation of isolated rat proximal tubules

Proximal kidney tubules were prepared essentially as described previously [20]. Briefly, immediately after rats had been killed by cervical dislocation, each kidney was perfused *in situ* with 5 ml of ice-cold Krebs–Henseleit buffer [21] supplemented with collagenase (1 mg/ml). The kidneys were then minced coarsely and suspended in 20 ml of Krebs–Henseleit buffer containing collagenase (1 mg/100 mg wet weight of tissue). The resulting suspension was incubated, with shaking (100 cycles/min) at 37 °C for 15–20 min. At the end of the digestion period the suspension was filtered through a plastic tea strainer; subsequently, 20 ml of Krebs–Henseleit buffer supplemented with 2.5 mM CaCl_2 was added to the digest. The resulting suspension was subjected to centrifugation (50 *g*; 1 min) and resuspension, repeated three times. The final resuspension buffer was supplemented with 2% (w/v) BSA. The recovered tissue consisted mainly of strands of between five and ten cells with intact membranes as judged by light microscopy. The metabolic integrity of preparations was assessed by assay of ATP content [22] and gluconeogenic capacity.

Preparation of kidney membrane extracts

Crude membrane extracts were prepared essentially as described by Hilderman et al. [23]. Briefly, immediately after rats had been killed by cervical dislocation, each kidney was removed and, after elimination of medullary tissue, the cortex was resuspended in 50 mM Tris/HCl, pH 7.7, and homogenized with a Ystral tissue disperser. The resulting homogenate was centrifuged for 10 min at 40 000 *g* at 4 °C. The pellet was resuspended in 20 vol. of 50 mM Tris/HCl, pH 7.7, and incubated at 37 °C for 10 min before re-centrifugation for 10 min at 40 000 *g* at 4 °C. The resulting pellet was resuspended in 50 mM Hepes, pH 7.5. These membrane preparations retained their Ap_4A -binding activity after storage at –70 °C for periods of up to 3 months.

For a limited number of experiments, preparations enriched in brush-border membranes were prepared using an aqueous two-phase partition technique essentially as described by Hammond et al. [24].

Binding assays

Ap_4A binding was assayed essentially as described by Hilderman et al. [23]. Briefly, each binding assay (final volume 620 μl) contained 67 mM Tris/HCl, pH 7.7, 100 μM MgCl_2 and 0.033 μM $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ (specific radioactivity 9000–10 000 c.p.m./pmol). Binding was initiated by addition of membrane fractions (10 μg), and incubations, in triplicate, were continued for up to 10 min at 25 °C. Initial experiments indicated that less than 5% of the $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ was degraded over the time course of the binding experiments. In addition, the extent and reversibility of binding, measured after a 10 min incubation, was not significantly different from binding characteristics measured after a 2 min incubation. Incubations were terminated by collection of membranes on Whatman GF/C glass microfibre filter discs, which were subsequently washed with 67 mM Tris/HCl, pH 7.7, containing 100 μM MgCl_2 . The discs were counted for radioactivity in the presence of 4 ml of Optiphase Safe scintillation fluid. Blanks contained all components except membrane preparation. Non-specific binding was estimated from the extent of binding of the radiolabelled ligand in the presence of 1 mM ATP. This was routinely less than 2% total binding.

HPLC analysis

The extent of breakdown of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ in portions of incubation medium was analysed by HPLC. After an appropriate incubation period, 200 μl of incubation medium was mixed with an equal volume of 0.4 M trichloroacetic acid and 400 μl of 0.6 M triethylamine in Arcton (1,1,2-trichlorotrifluoroethane). After vigorous shaking, the mixture was centrifuged at 12 000 *g* for 2 min at room temperature. Portions of the resulting upper aqueous layers were subjected to ultrafiltration (0.2 μm Millipore filter) before HPLC analysis. Nucleotide breakdown products were separated by Resource-Q ion-exchange chromatography. Samples (100 μl) prepared as described above were injected on to the column and then eluted using a linear gradient (21–560 mM) of NH_4HCO_3 , pH 9.6. Fractions (400 μl) were collected and 100 μl portions were counted for radioactivity in 3 ml of scintillation fluid.

Other assays

$[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ was synthesized from $[2\text{-}^3\text{H}]\text{ATP}$ as described previously [25]. Protein concentrations were determined using the method of Bradford [26]. Glucose production was assayed by the method of Trinder [27]. Gluconeogenic rates are based on the initial linear rate (over approx. 20 min) of glucose production observed after addition of nucleotide effectors and are expressed either as a percentage of the basal glucose-production rate or as nmol of glucose produced/h per mg dry weight of tubules.

RESULTS AND DISCUSSION

Adenine nucleotide-mediated stimulation of gluconeogenesis in isolated proximal tubules

Table 1 shows the effect of a range of adenine-containing effectors on the gluconeogenic activity of isolated rat proximal tubules incubated in the presence of either 10 mM lactate or 10 mM glutamine (gluconeogenic precursors). The substrate-supported glucose-production rates, which were maintained over a 1 h incubation period, were similar to those observed previously by ourselves [20] and other workers [28], thereby confirming the metabolic viability of the tubule preparations. Exogenous adenosine promoted an approx. 2-fold stimulation of gluconeogenesis from either substrate, and this presumably reflects A_2 purinoceptor-mediated activation of adenylate cyclase, with consequent

Table 1 Effect of different adenine-containing compounds on gluconeogenic activity of isolated rat proximal tubules

Rat proximal tubules were isolated and incubated in the presence of the indicated gluconeogenic substrate and effector as described in the Materials and methods section. Results are expressed as means $\pm$ S.E.M. for at least three independent isolated tubule preparations. The significance of differences between means was assessed by a paired *t* test. **P* < 0.05, compared with no effector in the presence of the same gluconeogenic substrate. Other differences were not significant. The basal gluconeogenic rate in the presence of 10 mM lactate only was 101 ± 8 nmol/h per mg dry weight and in the presence of 10 mM glutamine only was 89 ± 9 nmol/h per mg dry weight (mean $\pm$ S.E.M. for 10 independent tubule preparations).

Effector	Gluconeogenesis (% of basal flux)	
	Lactate	Glutamine
Adenosine (100 μM)	$181 \pm 10^*$	$199 \pm 17^*$
ADP (100 μM)	$133 \pm 6^*$	$157 \pm 8^*$
ATP (100 μM)	$137 \pm 5^*$	$156 \pm 2^*$
Ap_3A (100 μM)	$171 \pm 3^*$	$154 \pm 15^*$
Ap_4A (100 μM)	$163 \pm 8^*$	$176 \pm 14^*$

Table 2 Effect of nifedipine and U-73122 on gluconeogenic activity of isolated rat proximal tubules

Rat proximal tubules were isolated and incubated in the presence 10 mM lactic acid as gluconeogenic substrate and the indicated effector(s) as described in the Materials and methods section. Results are expressed as means $\pm$ S.E.M. for at least three independent isolated tubule preparations. The significance of differences between means was assessed by a paired *t* test. **P* < 0.05 compared with the value obtained in the absence of nifedipine or U-73122 as appropriate. Other differences were not significant. DMSO (1%, v/v), the carrier used for U-73122, had no significant effect on gluconeogenic rates.

Nucleotide effector	Gluconeogenesis (% of basal flux)		
	None	50 μ M Nifedipine	10 μ M U-73122
None	100	81 $\pm$ 5*	101 $\pm$ 1
ATP (100 μ M)	149 $\pm$ 9	80 $\pm$ 7*	120 $\pm$ 2*
Ap ₃ A (100 μ M)	169 $\pm$ 2	97 $\pm$ 14*	121 $\pm$ 2*
Ap ₄ A (100 μ M)	151 $\pm$ 8	100 $\pm$ 15*	132 $\pm$ 1*

production of cAMP in tubular cells [29,30]. The addition of 100 μ M dibutyryl-cAMP to isolated tubules also provoked increases in gluconeogenic activity similar to those observed with exogenous adenosine (results not shown). Exogenous ADP and ATP both stimulated substrate-supported gluconeogenesis, although to a lesser extent than adenosine. The 30–50% stimulation seen with these agonists was similar to that observed by other workers [16,18], who concluded that these effects were mediated via interaction of the nucleotides with tubular cell P_{2y}-like purinoceptor populations [18] and involved inositol triphosphate-dependent mobilization of intracellular Ca²⁺ stores [17]. In the current series of experiments, the P_{2y}-selective agonist, 2-methylthio-ATP, was also capable of stimulating gluconeogenic flux in isolated proximal tubules (132 $\pm$ 5 as a percentage of 10 mM lactate-stimulated gluconeogenesis: mean $\pm$ S.E.M. for three different proximal tubule preparations).

Table 1 shows that Ap₃A and Ap₄A, the predominant naturally occurring diadenosine polyphosphates, also enhanced substrate-supported gluconeogenesis. The enhancements were similar in magnitude to those observed in the presence of exogenous ADP or ATP. Table 2 shows the effect of nifedipine, a Ca²⁺-channel blocker [31], and U-73122, an inhibitor of phospholipase C activity [32], on gluconeogenesis in isolated rat proximal tubules. U-73122 had no appreciable effect on basal gluconeogenic flux; however, ATP-, Ap₃A- and Ap₄A-mediated stimulation of lactate-mediated gluconeogenesis was diminished in the presence of U-73122. In contrast, U-73343, an inactive analogue of U-73122, had no appreciable effect on gluconeogenic flux (results not shown). Nifedipine caused a significant decrease in basal nucleotide-independent gluconeogenic activity, which was not prevented by the presence of ATP, Ap₃A or Ap₄A. In addition, when incubations were carried out in the absence of Ca²⁺ (either nominal absence of Ca²⁺ or absence of Ca²⁺ in combination with 0.5 mM EGTA), the resulting diminution in the rate of basal gluconeogenic activity was not altered by the addition of ATP, Ap₃A or Ap₄A (results not shown).

Taken together these results indicate a possible role for phospholipase C activation and redistribution of Ca²⁺ in the nucleotide-dependent stimulation of gluconeogenesis. These findings are consistent with the observations of other workers on the impact of diadenosine polyphosphates on renal phospholipid metabolism [10] and Ca²⁺ homeostasis [11].

Figure 1 shows the concentration-dependence for ATP- and diadenosine polyphosphate-stimulated gluconeogenesis in iso-

Figure 1 Relationship between nucleotide concentration and stimulation of gluconeogenic activity of isolated rat proximal tubules

Rat proximal tubules were isolated and incubated in the presence of 10 mM glutamine as gluconeogenic substrate together with the indicated concentration of (a) ATP (■) or Ap₄A (●), (b) Ap₂A (■) or Ap₃A (●), or (c) Ap₃A (■) or Ap₆A (●). Results are expressed as means $\pm$ S.E.M. for at least three independent isolated tubule preparations.

lated proximal tubules. Ap₄A and ATP (at concentrations $\geq$ 10 μ M) caused significant stimulation of gluconeogenesis from 10 mM glutamine (Figure 1a) (or 10 mM lactate: results not shown). Other workers have reported a similar dose-response profile for ATP-dependent stimulation of gluconeogenesis in isolated rat proximal tubules [18]. Concentrations of Ap₄A in this range are physiologically significant. It has been suggested that at sites of nucleotide release (e.g. synaptic clefts, wounded blood vessel walls, etc.) concentrations of Ap₄A may reach 10–100 μ M [3]. In view of its relatively long half-life *in vivo* (see the Introduction), it is conceivable that Ap₄A may play a potentially important signalling role in the control of gluconeogenesis and other metabolic and cellular processes in renal tissues.

Figures 1(b) and 1(c) show the concentration-dependence of gluconeogenesis for lower and higher homologues of Ap₄A respectively. Ap₃A was characterized by a dose-response curve similar to that for Ap₄A. In contrast, Ap₂A, a synthetic homologue, was less effective. Ap₃A and, to a greater extent, Ap₆A were also able to stimulate gluconeogenic activity in isolated proximal tubules. Extracellular concentrations and turnover of Ap₃A appear to be similar to those reported for Ap₄A (see above). Unfortunately, little detailed information is available on

Figure 2 Displacement of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ from renal membrane preparations by other nucleotides

The displacement of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ from renal membrane preparations by other nucleotides was determined using the binding assay described in the Materials and methods section. In (a) displacement was by other diadenosine polyphosphates: $\blacksquare$, unlabelled Ap_4A ; $\blacktriangle$, Ap_2A ; $\circ$, Ap_3A ; $\bullet$, Ap_5A ; $\square$, Ap_6A . In (b) displacement was by adenine mononucleotides: $\blacksquare$, unlabelled Ap_4A ; $\blacktriangledown$, Ap_4 ; $\blacktriangle$, ATP; $\circ$, ADP; $\square$, AMP; $\bullet$, adenosine. In (c) displacement was by $\text{P}_{2\text{y}}$ -purineric effectors: $\blacksquare$, unlabelled Ap_4A ; $\bullet$, 2-methylthio-ATP; $\square$, ADP/ β [S]. In (d) displacement was by $\text{P}_{2\text{x}}$ -purineric effectors: $\blacksquare$, unlabelled Ap_4A ; $\bullet$, pp[CH₂]pA; $\square$, p[CH₂]ppA; $\circ$, PPADS. In (e) displacement was by UTP: $\blacksquare$, unlabelled Ap_4A ; $\square$, ATP; $\bullet$, UTP. Results are expressed as means $\pm$ S.E.M. for at least five independent membrane preparations.

the extracellular concentrations of Ap_5A and Ap_6A that may be achieved *in vivo*. Nonetheless, the present data indicate that each of the naturally occurring diadenosine polyphosphates, in common with ATP, has the potential to act as a signalling molecule involved in the control of renal gluconeogenesis.

Binding of Ap_4A to renal cortex membrane preparations

The binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ to membranes prepared from rat renal cortex was linear with membrane protein concentration (between 2 and 50 μg of membrane protein; results not shown). Binding was rapid and saturable, reaching a maximum of 19.7 ± 1.69 pmol/mg of membrane protein (mean $\pm$ S.E.M. for

four independent membrane preparations) within 2–5 min. Binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ was reversible throughout the time course studied, with the bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ effectively displaced by the addition of an excess of unlabelled ligand. These properties are consistent with the ability of Ap_4A and the other diadenosine polyphosphates to promote rapid changes in gluconeogenic flux and other metabolic processes in tubular cells (see above). In addition, these features are similar to those observed for the binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ to hepatic plasma-membrane preparations that we have previously reported [19].

The ability of a wide range of both natural and synthetic nucleotides to displace $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ from membrane binding sites was investigated. Figure 2(a) illustrates the effect of other

diadenosine polyphosphates on the binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ to membranes. The synthetic homologue Ap_2A did not displace bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$. In contrast, Ap_3A , Ap_5A and Ap_6A were all almost as effective as unlabelled Ap_4A as displacers of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$. These results are similar to those reported by ourselves [19] and other workers [23] on the binding of Ap_4A to membranes prepared from other tissues. The ability of adenine mononucleotides to displace bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ is illustrated in Figure 2(b). Whereas P_2 -purinoceptor-preferring ligands (ATP and ADP) and Ap_4 , a synthetic homologue, were effective displacers, P_1 -purinoceptor-preferring ligands (AMP and adenosine) were without significant displacing activity. This behaviour is reminiscent of the displacing ability of these effectors with respect to $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ bound to isolated rat liver cell plasma membranes [19] and suggests that Ap_4A may mediate its effects on tubular cell gluconeogenesis through interaction with a P_2 -like purinoceptor population also recognized by ATP [18].

We investigated the ability of a range of P_2 -specific agonists and antagonists to displace bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$. Figure 2(c) indicates that 2-methylthio-ATP and ADP β [S], which have frequently been used as specific agonists of the P_{2y} -purinoceptor subtype [33], were effective displacers. In the present experiments 2-methylthio-ATP proved to be a significantly more effective displacer than ADP β [S]. In contrast, pp[CH₂]₂pA and p[CH₂]₂ppA, which show a greater affinity for the P_{2x} -purinoceptor subtype [34], were less effective (Figure 2d). These displacing activities are consistent with Ap_4A binding a purinoceptor of the P_{2y} subtype. This differs from the situation in neural tissues and certain murine tissues [23,35], where a unique diadenosine polyphosphate-specific (P_{2d}) purinoceptor subtype has been described [36].

PPADS has previously been characterized as a potent P_{2x} antagonist [37], which has been reported to block P_{2x} -mediated Ap_4A -induced Ca^{2+} movements in guinea-pig urinary bladder [38]. More recently this compound has been used to discriminate between P_{2y} purinoceptor subtypes in endothelial cells [39]. From Figure 2(d) it appears that PPADS was a completely ineffective displacer of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ from renal membranes. This observation is consistent with the reported P_{2x} selectivity of PPADS. However, it does imply that the P_{2y} -like purinoceptor subtype(s) recognized by Ap_4A may be distinct from the PPADS-sensitive P_{2y} -purinoceptor subtype(s) identified in bovine aortic endothelial cells [39].

All G-protein-coupled P_2 -purinoceptors have recently been reclassified as members of the P_{2y} subtype. One of these (P_{2y3}), formerly known as the P_{2u} -purinoceptor, is responsible for mediating the signalling properties of extracellular UTP [40]. Interestingly, it has been reported that a cloned human P_{2y2} receptor, showing little affinity for 2-methylthio-ATP, readily binds Ap_4A [41]. UTP, like ATP, causes rapid inositol triphosphate formation in rat renal mesangial cells [42]; however, in contrast with the liver [43], there is little information available on the role of extracellular UTP as a modulator of renal metabolism. From the present studies it is clear that UTP is a significantly less effective displacer of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ than either ATP or unlabelled Ap_4A (Figure 2e). Therefore it appears that a P_{2y1} (2-methylthio-ATP-preferring) rather than a P_{2y2} (UTP-preferring) purinoceptor is primarily involved in mediating renal responses to extracellular Ap_4A .

Binding of Ap_4A to isolated proximal tubules

The experiments described above concern the characteristics of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ binding to a crude membrane preparation. We also carried out a limited analysis of the binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ to

Figure 3 Displacement of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ from isolated proximal tubules

The displacement of bound $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$, after 2 min at 37 °C, from isolated proximal tubules by other nucleotides was determined using the binding assay described in the Materials and methods section. ■, Unlabelled Ap_4A ; ○, ATP. Results are expressed as means $\pm$ S.E.M. for at least three independent isolated proximal tubule preparations.

isolated proximal tubule preparations (Figure 3). These experiments showed that ATP and unlabelled Ap_4A were similarly effective displacers of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ with half-maximal binding evident at displacer concentrations in the 5–10 μM range. These data are quantitatively similar to the nucleotide-sensitivity displayed by proximal tubule gluconeogenic flux (see above) and are consistent with a role for the observed binding phenomena in the physiological control of proximal tubule gluconeogenesis.

HPLC analysis revealed that Ap_4A was somewhat less stable in the presence of intact tubules than in the presence of the crude membrane preparation. In the presence of membrane preparations, approx. 10% of Ap_4A was degraded, mainly to AMP, over a 20 min incubation period. In contrast, in the presence of isolated tubules a similar degree of breakdown was observed after 5 min. Nonetheless, Ap_4A was still significantly more stable than ATP, which was effectively completely degraded to adenosine within 5 min in the presence of tubules (results not shown). These findings are of interest in the light of our preliminary observations on the binding and metabolism of Ap_4A by brush-border-membrane-enriched fractions, which indicated the presence of a significant Ap_4A -degrading activity (> 90% degradation of Ap_4A within 2 min). These latter observations presumably reflect the presence of abundant ectonucleotidase activities associated with brush-border membranes and suggest a role for the proximal tubule in the termination of extracellular diadenosine polyphosphate-mediated signalling processes.

Conclusions

The data described here indicate the potential role played by extracellular diadenosine polyphosphates in the control of renal metabolic and cellular activity. The naturally occurring diadenosine polyphosphates promoted increases in gluconeogenic flux in isolated proximal tubules similar in magnitude to those observed in the presence of other hormones and purinergic effectors. Therefore during adenine nucleotide release into the extracellular environment, e.g. release from platelets after haemorrhage, diadenosine polyphosphates such as Ap_4A may be significant mediators of the resulting gluconeogenic response. Given the relative stabilities of ATP and Ap_4A within the

extracellular environment, it might be expected that, after liberation from a site of injury, for example, Ap_4A may be a more potent signalling molecule than ATP in relatively distant target tissues (e.g. kidney). This notwithstanding, it does appear that, in addition to responding to increased concentrations of Ap_4A with receptor-mediated processes, the kidney also contributes to the termination of Ap_4A -mediated signalling activity through brush-border Ap_4A -degrading activities. In the absence of evidence supporting the existence of specific diadenosine nucleotide hydrolases in the brush-border membranes, the enzyme most likely to be responsible for degrading extracellular Ap_4A is alkaline phosphodiesterase I/nucleotide pyrophosphatase. This enzyme is significantly more abundant in kidney brush-border membranes than in liver [44,45], in agreement with our data on the relative stabilities of Ap_4A in the presence of liver and kidney membrane fractions [19].

Ap_4A appears to stimulate polyphosphoinositide-mediated alterations in renal tubular cell Ca^{2+} homeostasis in a fashion analogous to ATP and, to a more limited extent, Ap_4A action on other tissues. The characteristics of binding of $[2\text{-}^3\text{H}]\text{Ap}_4\text{A}$ to renal membrane preparations are consistent with recognition of a P_{2Y_1} -like purinoceptor indistinguishable from that responsible for ATP-mediated signalling in renal tissues.

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