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Effects of ATP-$MgCl_2$ and adenosine-$MgCl_2$ administration on intracellular ATP levels in the kidney

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The effects of exogenous administration of 1 mM [8-^{14}C]ATP-$MgCl_2$ and adenosine-$MgCl_2$ on intracellular accumulation of adenine nucleotides were examined in isolated, perfused rat kidneys. The kidneys were made filtering or non-filtering by increasing the colloid oncotic pressure of the perfusate solution in order to assess the relative contributions of the glomerular and peritubular routes in the uptake of the nucleotides. The results indicate that: (1) although labeled ATP is undetectable in the perfusate after 20 min, there is a significant accumulation of labeled ATP in the tissue and (2) although labeled adenosine-$MgCl_2$ administration also leads to labeled intracellular ATP, the total intracellular ATP is much less than with ATP-$MgCl_2$ administration.

Introduction

Studies from a number of laboratories, including ours, have shown that the administration of ATP-$MgCl_2$ is beneficial to the survival of animals after hemorrhagic shock, severe burns, sepsis-peritonitis, post-ischemic hepatic failure, bowel ischemia, and endotoxic shock [1–5]. In addition, recent studies utilizing the isolated perfused rat kidney preparation have shown that ATP-$MgCl_2$ accelerates the recovery of renal function after warm ischemia [6,7] or nephrotoxic injury from cis-platinum [8], gentamicin [9], or cyclosporine [10]. However, the mechanism by which ATP-$MgCl_2$ augments recovery of the injured cells still remains unclear.

Previous studies utilizing isolated kidney slices from normal animals have indicated that by incubating such slices in a medium containing [8-^{14}C]ATP, ATP was detected intracellular [11]. Moreover, the studies of Maxild [12] have indicated that exogenous ATP decreased O_2 consumption and affected *P*-aminohippurate transport in the cortical slices. The above studies therefore provide evidence for cellular uptake of ATP. However, since ATP is metabolized rapidly at the cell surface [13] the relative concentration of ATP versus adenosine in increasing intracellular ATP levels remains to be determined. The aim of the present study was therefore to determine the fate of exogenously administered labeled ATP-$MgCl_2$ in an in vitro perfused kidney preparation and to compare the increase in intracellular ATP with ATP-$MgCl_2$ versus adenosine-$MgCl_2$. Both filtering and non-filtering preparations were used to determine the relative contributions of glomerular filtration and peritubular uptake to the renal extraction of this complex.

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Materials and Methods

Animals. Male Holtzman rats (250–350 g in weight) were used as kidney donors for the isolated perfused rat kidney preparation. The rats were fasted overnight but allowed water ad libitum. They were anesthetized with sodium pentobarbitol (50 mg/kg, intraperitoneally) prior to experimentation.

Isolated perfused rat kidney preparation. The procedure for perfusing rat kidneys was the same as that described in detail previously [10,14]. Briefly, the abdominal cavity was exposed and the right ureter was cannulated with heparinized polyethylene 10 tubing. Following heparin injection (500 units/kg body weight) into the lower inferior vena cava, the right renal artery was cannulated with an 18 gauge needle via the superior mesenteric artery. Perfusion was immediately initiated from a gravity system so as not to interrupt flow to the kidney and to wash the renal vessels of blood. The kidney was freed from the animal and perfused in a closed circuit perfusion system at 37°C. Perfusate flow was continuously monitored by means of a Brooks flowmeter in series with the arterial cannula.

Perfusate composition. The basic perfusate consisted of a Krebs-Henseleit HCO_3^- buffer containing 1 mg/ml glucose, 0.5 mg/ml creatinine, a mixture of amino acids, trace amounts of [^{3}H]inulin (New England Nuclear, spec. act. 216 mCi/g) as the extracellular marker and 1 mM [8-^{14}C]ATP (New England Nuclear, spec. act. 2 mCi/mmol) combined with equimolar $MgCl_2$. In additional experiments 1 mM [8-^{14}C]adenosine (New England Nuclear, spec. act. 2 mCi/mmol) with equimolar $MgCl_2$ was used instead of the labeled ATP.

In the filtering kidney preparation, 7.5 g/dl bovine serum albumin (Fraction V) (Miles Laboratory, Indiana) was also added as colloid to provide a calculated oncotic pressure of 36 mmHg. These kidneys were perfused at a net mean renal arterial pressure of 90 mmHg. Function of this preparation has been previously shown to be constant over a 2 h period [15]. In the present experiments, glomerular filtration rate calculated from the inulin clearance during the last 10 min of perfusion show values of 0.46 ± 0.07 ml/min for ATP-$MgCl_2$ treated kidneys and 0.48 ± 0.09 ml/min for adenosine-$MgCl_2$ treated kidneys. For the non-filtering kidney preparation, 10.0 g/dl bovine serum albumin was added (calculated oncotic pressure, 58 mmHg) and the kidneys were perfused at a net mean renal arterial pressure of 65 mmHg. Earlier studies have shown that despite this lowered perfusion pressure, renal perfusate flow and transport of organic anions across the peritubular membrane remains intact [15]. Glomerular filtration rate could not be calculated in the present study because of the lack of urine formation. However, there was no significant change in inulin radioactivity in the perfusate of non-filtering kidneys implying negligible filtration.

The kidneys were perfused for 100 min and perfusate and urine samples were collected at selected intervals. The samples were immediately mixed with an equal volume of ice-cold, 10% trichloroacetic acid/0.1 mM HCl mixture and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was removed and assayed for ATP using the luciferase method. At the end of the perfusion period, small pieces of kidney cortex were frozen between aluminum blocks that had been cooled in liquid N_2. The frozen samples were then powdered with pestle and mortar (cooled on dry ice), homogenized and analyzed enzymatically for adenine nucleotides as described in detail previously [16].

Samples of end-perfusate and kidney tissue were also obtained and subjected to electrophoretic separation of the labeled nucleotides [11]. Approximately 90–95% of the applied radioactivity was recovered.

^{14}C and ^{3}H radioactivity in perfusate and urine were counted by liquid scintillation using jellified Aquasol (New England Nuclear). Settings for double isotope counting were used and counts were corrected for the crossover of ^{3}H into ^{14}C. Quenching was estimated by the external ratio technique. Statistical analyses were performed by paired and unpaired Student's *t*-test where appropriate. Values are expressed as means ± S.E.

Results

Effect of ATP-$MgCl_2$

Fig. 1 shows the levels of enzymatically measured ATP in the perfusate of filtering and non-

filtering kidneys perfused with 1 mM labeled ATP-$MgCl_2$. There was no significant difference in the perfusate ATP curves. In both kidney preparations, there was a steady decline in ATP levels with only barely detectable levels of ATP in the perfusate after 25 min of closed-circuit perfusion. In the filtering kidneys, only 3% of the total ATP added was detectable in the urine and the majority of this was excreted during the first 5 min of perfusion.

Table I shows the radioactivity present as adenine and hypoxanthine nucleotides in end perfusate and tissue following electrophoretic separation in the filtering and non-filtering preparation, expressed as a percent of the total dose. There was essentially no qualitative difference in the electrophoretic separation pattern for tissue or perfusate for either the filtering or non-filtering preparation. The principle breakdown product in the perfusate was inosine with lesser amounts of adenosine. In the tissue, inosine and adenosine were detected in addition to ATP and the other adenine nucleotides.

By calculating the tissue to perfusate (T/P) ratio of the nucleotide and subtracting the tissue to perfusate ratio of the extracellular marker, inulin, one gets a semi-quantitative assessment of the intracellular content or space of the nucleotide. For filtering kidneys, the T/P ratio of inulin

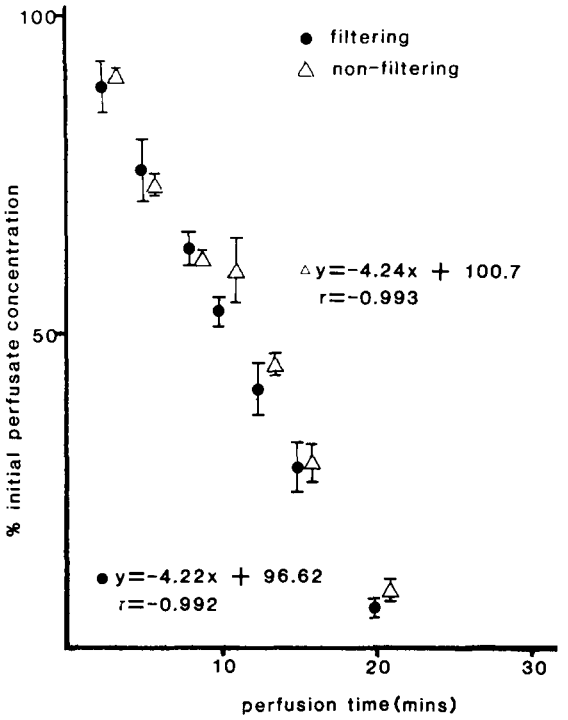

Fig. 1. Perfusate ATP levels. Levels of enzymatically measured ATP in perfusate of filtering (●) and non-filtering (△) isolated, perfused kidneys are shown above. The ordinate is the ATP level expressed as a percent of the initial perfusate ATP concentration. The abscissa is the perfusion time. The values are means ± S.E. of four kidneys in each group. There was no significant difference between the two curves.

TABLE I

DISTRIBUTION OF ADENINE NUCLEOTIDE IN TISSUE AND PERFUSATE IN FILTERING AND NON-FILTERING KIDNEYS WITH ATP-$MgCl_2$

The distribution of labeled adenine nucleotides in tissue and perfusate after 100 min of perfusion with [8-^{14}C]ATP-$MgCl_2$ in filtering and non-filtering isolated perfused kidneys is shown below. The radioactivity present as adenine and hypoxanthine nucleotides in tissue and perfusate was counted following electrophoretic separation and expressed as a percent of the total added radioactivity. Each column represents the mean ± S.E. of four kidneys. The values for tissue to perfusate ratio (T/P) of adeninine nucleotides are calculated from the data presented.

Nucleotide	Filtering kidneys			Non-filtering kidneys		
	perfusate	tissue	T/P ratio	perfusate	tissue	T/P ratio
ATP	1.5 ± 0.4	12.9 ± 1.1	31.1 ± 17.0	5.5 ± 0.5	20.3 ± 3.6	6.1 ± 0.5 *
ADP	1.3 ± 0.3	11.6 ± 0.4	22.0 ± 9.6	2.7 ± 0.5	13.0 ± 0.8	8.9 ± 1.0 *
IMP	2.2 ± 0.3	7.5 ± 1.1	8.3 ± 2.3	6.7 ± 2.8	5.0 ± 1.8	1.6 ± 0.3 *
AMP	2.7 ± 0.8	7.0 ± 1.1	5.4 ± 0.8	2.1 ± 1.0	7.2 ± 1.6	7.6 ± 3.7
Inosine	75.1 ± 1.6	32.4 ± 3.1	1.1 ± 0.2	62.1 ± 8.2	21.4 ± 2.6	0.6 ± 0.2
Adenosine	17.5 ± 3.0	28.7 ± 2.2	2.0 ± 0.4	20.9 ± 5.7	33.0 ± 3.2	3.0 ± 0.5

* $P < 0.05$ comparing the filtering to non-filtering group.

was 1.7 ± 0.2 and for non-filtering kidneys 0.8 ± 0.1. Table I also shows the T/P ratios of the nucleotides in the filtering and non-filtering kidneys. The predominant intracellular labeled nucleotide of filtering kidneys is ATP, followed by ADP, then IMP, with inosine and adenosine being relatively lower. The non-filtering kidneys, on the other hand, had an intracellular ATP, ADP, and IMP space significantly smaller than in filtering kidneys.

Table II shows the enzymatically determined tissue values of ATP, ADP and AMP in the filtering and non-filtering preparations. Filtering kidneys to which 1 mM ATP-$MgCl_2$ was added had significantly higher tissue ATP than control kidneys. However, there was no increase in tissue ATP levels of non-filtering kidneys compared to control filtering kidneys.

TABLE II

RENAL ADENINE NUCLEOTIDE LEVELS IN FILTERING AND NON-FILTERING KIDNEYS

Small pieces from in vitro filtering and non-filtering kidneys that were either perfused with the standard perfusate alone (control), or with the addition of 1 mM ATP-$MgCl_2$ or adenosine-$MgCl_2$ were frozen between aluminum tongs cooled in liquid nitrogen. Samples were homogenized and analyzed for adenine nucleotides as described in Methods. Values are means $\pm$ S.E. in μmol/g.

	Control filtering	1 mM ATP-$MgCl_2$		1 mM adenosine-$MgCl_2$ filtering
		filtering	non-filtering	
ATP	0.84 ± 0.06	1.20 ± 0.02 *,**	0.78 ± 0.07	1.03 ± 0.05 *
ADP	0.66 ± 0.01	0.59 ± 0.02 *,**	0.74 ± 0.06	0.68 ± 0.03
AMP	0.24 ± 0.02	0.21 ± 0.04	0.28 ± 0.06	0.27 ± 0.01

* $P < 0.05$ compared to control filtering kidneys.
** $P < 0.05$ compared to adenosine-$MgCl_2$ kidneys.

Effect of adenosine-$MgCl_2$

The distribution of radioactivity in adenine and hypoxanthine nucleotides in end perfusate sample and of kidneys perfused with 1 mM ATP-$MgCl_2$ to those kidneys perfused with 1 mM adenosine-$MgCl_2$ is shown in Table III. The electrophoretic pattern is qualitatively similar between the two groups although statistically the adenosine space is larger in the filtering kidneys perfused with 1 mM adenosine-$MgCl_2$. The principal nucleotide in the perfusate of both groups is inosine and adenosine, while in tissue, all nucleotides were found in similar concentrations.

Table III also compared the tissue to perfusate ratio between the ATP-$MgCl_2$ treated and the adenosine-$MgCl_2$ treated filtering kidneys. In this instance, there is also no significant difference in the intracellular accumulation of nucleotides in the two groups. The intracellular ATP space is relatively the largest nucleotide tissue space in both groups.

TABLE III

DISTRIBUTION OF ADENINE NUCLEOTIDES WITH ATP-$MgCl_2$ AND ADENOSINE-$MgCl_2$

The distribution of labeled adenine nucleotides in tissue and perfusate after 100 min of perfusion with [8-^{14}C]ATP-$MgCl_2$ or [8-^{14}C]adenosine-$MgCl_2$ in filtering isolated perfused kidneys is shown above. The radioactivity present as adenine and hypoxanthine nucleotides in tissue and perfusate was counted following electrophoretic separation and expressed as a percent of the total added radioactivity. Each column represents the mean $\pm$ S.E. of four kidneys. The values for tissue to perfusate ratios are calculated from the data presented.

Nucleotide	ATP-$MgCl_2$			Adenosine-$MgCl_2$		
	perfusate	tissue	T/P ratio	perfusate	tissue	T/P ratio
ATP	1.5 ± 0.4	12.9 ± 1.1	31.1 ± 17.0	1.0 ± 0.1	23.9 ± 0.6	39.7 ± 6.4
ADP	1.3 ± 0.3	11.6 ± 0.4	22.0 ± 9.6	0.8 ± 0.1	11.8 ± 2.2	25.5 ± 1.4
IMP	2.2 ± 0.3	7.5 ± 1.1	8.3 ± 2.3	0.8 ± 0.1	3.1 ± 0.2	7.3 ± 1.8
AMP	2.7 ± 0.8	7.0 ± 1.1	5.4 ± 0.8	0.3 ± 0.1	5.9 ± 1.5	0.5 ± 0.1
Inosine	75.1 ± 1.6	32.4 ± 3.1	1.1 ± 0.2	62.1 ± 9.3	24.2 ± 1.6	0.7 ± 0.2
Adenosine	17.5 ± 3.0	28.7 ± 2.2	2.0 ± 0.4	35.5 ± 9.4 *	31.2 ± 1.6	1.6 ± 0.1

* $P < 0.05$ compared to ATP-$MgCl_2$ group.

Table II shows that the absolute measured tissue ATP levels of adenosine-$MgCl_2$ treated kidney were higher than control kidneys but statistically lower than the ATP-$MgCl_2$ treated group.

Discussion

Exogenously administered ATP-$MgCl_2$ has been shown to increase the lowered intracellular ATP levels and to correct the altered membrane permeability following shock and ischemia [1,17, 18]. In addition, ATP-$MgCl_2$ has been demonstrated to be beneficial to the function of kidneys exposed to toxic agents [8–10,19]. Whether externally applied ATP-$MgCl_2$ performs these functions by entering or interacting with cell membranes is still unknown. Furthermore, it is still unclear whether the favorable effects are due to ATP itself or the degradation products. Accordingly, one of the aims of this study was to examine the fate of labeled ATP-$MgCl_2$ in a normal in vitro kidney preparation. 1 mM ATP (i.e., 1 μmol/ml) was used as the starting perfusate concentration since the intracellular ATP content of a normal kidney is between 1 and 2 μmol/g wet weight.

The results indicate that the exogenously administered ATP disappears rapidly in the recirculating system (Fig. 1). Quite unexpectedly, the rate of disappearance of ATP from the perfusate of non-filtering kidneys ($t_{1/2}$ 11.4 min) was not significantly different from that of filtering kidneys ($t_{1/2}$ 11.0 min). Since ATP is freely permeable through the glomerulus, one would expect a greater extraction of ATP by the filtering kidneys compared to the non-filtering preparation, where the glomerular route has been made non-functional. Since this was not the case, these findings can be explained only if either the glomerular apparatus is not permeable to ATP or if ATP is being degraded during recirculation through the kidney or through the perfusion system. With regards to the first possibility, it could be argued that because of the negative changes on the ATP molecule, that this compound cannot cross the negatively charged glomerular barrier. In the present studies, however, an equimolar amount of $MgCl_2$ was added with ATP which theoretically should decrease the negative charges on ATP to a single negative charge. Since PO_4 with a negative charge is freely permeable through the cell membrane, it could be argued that ATP-$MgCl_2$ complex should also be able to cross the plasma membrane. Studies have in fact shown in ATP can cross the intact plasma membrane of a number of tissues, including the kidney [11,12]. With regard to the second possibility, the degradation of ATP during mechanical circulation through the perfusion system is unlikely since pilot studies have shown that perfusate ATP activity is not significantly altered by recirculation through the apparatus without the kidney in place. There is, however, evidence that ATPase activity is present in the endothelial lining surface [13] and this may be responsible for the similar degradation rates of ATP in both filtering and non-filtering conditions. In addition, in filtering kidneys only less than 3% of the total added ATP is found in the urine. This may be due to several factors such as: (1) the urinary excretion of ATP may be low either because the filtered load of ATP is low due to a low circulating perfusate level of ATP or, less likely, because of an altered permeability of the glomerulus to ATP; (2) filtered ATP may be extensively absorbed by the tubular cells and (3) ATP in the tubular lumen may be broken down by brush-border enzymes. The results of this study do not permit the elucidation of these factors. Nonetheless, the results illustrated in Fig. 1 indicate that the disappearance of ATP from the perfusate is similar in both filtering and non-filtering preparations, and that the endothelia ATPase enzymes are responsible for the degradation of ATP in both models.

The electrophoretic pattern of the adenine nucleotides in tissue and perfusate at the end of experimentation is similar in both filtering and non-filtering preparations (Table I), indicating that these systems have identical pathways for ATP catabolism or uptake. Despite this qualitative similarity, however, glomerular filtration probably represents the major route for the intracellular accumulation of ATP since both the tissue to perfusate ratio of ATP (Table I) and the absolute tissue concentrations of ATP (Table II) are significantly lower in the non-filtering kidneys.

The mechanism for the increased intracellular accumulation of ATP in the isolated kidney pre-

paration may be a result either of uptake of intact ATP via luminal or peritubular transport mechanisms or of subsequent intracellular regeneration of ATP from the more non-polar metabolites, which enter the cell after ATP degradation in the ciruclation. The latter argument is partially supported by the experiments in which adenosine-$MgCl_2$ was added to the filtering kidney preparation in lieu of ATP-$MgCl_2$. These adenosine studies indicate that the electrophoretic pattern of tissue adenine nucleotides is similar for both adenosine or ATP administration, and that the tissue to perfusate concentration of ATP is also statistically similar.

Despite this similarity in intracellular distribution of adenine and hypoxanthine nucleotides in ATP-$MgCl_2$ and adenosine-$MgCl_2$ treated kidneys, the absolute ATP tissue concentration was significantly higher in the ATP-$MgCl_2$ group compared to the adenosine-$MgCl_2$ or untreated groups. This finding and the observation that ATP-$MgCl_2$ and not adenosine-$MgCl_2$ administration improved survival after hemorrhagic shock and hepatic ischemia [1,11,16,20], and renal function following renal ischemia [6,7], indicate that the beneficial effect and the intracellular accumulation of ATP-$MgCl_2$ cannot be explained solely on the basis of the metabolites of ATP. The finding that ATP and not adenosine is a potent inhibitor of the enzyme 5′-nucleotidase [21] may provide a partial explanation in this regard. Moreover, since intracellular ATP levels increased with adenosine-$MgCl_2$ but did not improve the function of the kidneys following ischemia [6], it is possible that the ATP synthesized from adenosine is located in a different compartment of the cell and may not be available for functional aspects. This suggestion is in line with our more recent findings which also showed that administration of adenosine-$MgCl_2$ did not enhance the functional recovery of isolated kidneys following ischemia (unpublished observations).

In conclusion, the present studies indicate that under normal conditions, the addition of ATP-$MgCl_2$ to the perfusate results in increased intracellular tissue ATP levels. Whether this increase is due to direct uptake of ATP or due to regeneration from ATP metabolites which are taken up by the cell still remains unresolved. It is clear, however, that glomerular filtration with subsequent absorption by tubular cells is the major route for the accumulation of ATP in the kidney and that intracellular ATP is significantly higher when ATP-$MgCl_2$ instead of adenosine-$MgCl_2$ is administered.

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