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Adenosine and compensatory renal growth

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The initiating factor(s) which triggers compensatory renal growth (CRG) or the nature of regulator(s) activated following the loss of kidney mass is still unknown. The existence of a negative feedback mechanism [1–3] and of stimulating factor(s) present in normal urine [4, 5] and plasma of unilaterally nephrectomized mammals [6–10] have been suggested. The involvement of the endocrine system, cyclic AMP, and cyclic GMP has also been considered [11–13].

A possible link between cell proliferation and adenosine (ADO) has been suggested [14–19]. Under normal conditions, ADO levels are barely detectable in most cells due to the combined action of ADase (adenosine aminohydrolase EC 3.5.4.4) which converts ADO to inosine in the metabolism of purine nucleotides and adenosine kinase (EC 2.7.1.20), which catalyzes the rephosphorylation of ADO into AMP. High concentrations of exogenous ADO are toxic to cultures of human lymphoblasts, fibroblasts [14–18] and leukemia cells [19]. ADO apparently inhibits growth in vitro by reducing pyrimidine nucleotides essential in RNA or DNA biosynthesis [14, 20]. Since a series of biochemical alterations which preceed DNA synthesis and cell division takes place in the contralateral kidney following unilateral nephrectomy, we have attempted to determine if ADase and ADO play some role in CRG. We find that ADO appears to have a biphasic effect. It inhibits thymidine incorporation into renal DNA but only at high concentrations in vitro; total inhibition of renal ADase in vivo, with the consequent elevation of intracellular ADO and far from arresting CRG, stimulates growth.

Methods

Materials. Adenosine and enzymes needed in the determination of ADase, ADO, ATP, ADP, and AMP were purchased from Boehringer Ingelheim Ltd., Ridgefield, Connecticut; thymidine [methyl-^{3}H] at a specific activity of 6.7 Ci/mmole was purchased from New England Nuclear Corporation, Boston, Massachusetts. Hank's medium (Grand Island Biological Company, Grand Island, New York) BSS 10X, without sodium bicarbonate and without phenol red was diluted to a normal concentration with double distilled water, and sterilized again before use. Coformycin was a gift from Drs. J. Douros and M. Suffness of the National Cancer Institute, Bethesda, Maryland. Reagent-grade chemicals were used throughout the experiments.

Nephrectomy. Male Sprague-Dawley rats (150 to 160 g; 40 to 45 days old) were used. Right nephrectomy was performed between 9 and 10 A.M. under a light ether anesthesia.

In vivo inhibition of renal ADase by coformycin (CF). The magnitude of the reduction of ADase activity and its duration were studied with different dosages of CF:

Low dosage (0.1 mg CF/kg body wt/24 hr). A solution of CF (0.1 mg/kg) diluted in 0.5 ml NaCl 0.9% was injected intraperitoneally into 16 rats around 9 A.M., and nephrectomy was performed 40 min afterwards. At the same time each following day, a group of four animals was sacrificed while the remaining animals received another dose of CF. The experiment ended on day 4.

High dosage (0.2 mg/kg body wt/12 hr). A solution of CF (0.2 mg/kg) diluted in 0.5 ml NaCl 0.9% was injected into 16 rats around 9 P.M. on a given day. A second injection was administered 12 hr later (9 A.M.), and unilateral nephrectomy was performed 40 min afterwards. The animals received CF injections every 12 hr until 12 hr prior to sacrifice time. A group of four animals was sacrificed 24, 48, 72, and 96 hr following the nephrectomy.

ADase and ADO levels were determined in both the removed and the regenerated kidneys. The tissue was homogenized in 0.1 M phosphate buffer, pH 7.0; the $\times 15\,000g$ supernatant was saved for assays. ADase activity was determined by the method of Hopkinson et al modified by Trotta and Balis [21]. ADO was determined by its conversion to inosine by ADase [22, 23]. The decrease in its absorbance at 265 nm was recorded spectrophotometrically.

Effect of coformycin on CRG. In vivo studies. Since only 12-hr injections of high dosage (0.2 mg/kg) appear to bring total inhibition of ADase for a long duration, the in vivo effects of CF on CRG were studied at this dosage; the injections and nephrectomies were carried out according to the schedule described earlier.

To dissociate the effect of starvation brought about by the CF treatment, rats were submitted to three different food regimes after they were unilaterally nephrectomized. A group of 16 control and 16 experimental animals was given free access to rat chow (Ralston Purina, St. Louis, Missouri) and water. A second group of rats (12 control and 12 experimental) was given access to water only. A third group of 6 control and 6 experimental rats was fed orally four times daily with powdered milk (SMA) dissolved in water; the amount given was equivalent to a

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total of 45 calories/100 g body wt per day. They also had free access to water. Three or four animals in each category were sacrificed at various times postnephrectomy (24, 48, 72, and 96 hr). The dry or wet weight and protein content of the remaining kidney was compared with those of the removed kidney taken as control. Unilaterally nephrectomized control rats received 0.9% NaCl in lieu of CF.

Effect of coformycin in CRG. In vitro studies. The incorporation of thymidine-^{3}H into DNA was studied with renal cortical slices obtained from rats having undergone a right nephrectomy 48 hr previously; this experiment was based on a previous study by Martel-Pelletier and Bergeron [3] which indicated that maximum synthesis of DNA was reached in CRG after 2 days. Cortical slices (300 to 400 μ) were cut with a Stadie-Riggs microtome. The operation temperature was maintained at 4°C. Each slice was divided into a control half and an experimental half. Each control or experimental flask contained three to four half-slices. Experimental slices were incubated at 37°C for 10 min in Hank's solution containing various concentrations of CF (0.5 to 10.0 μg/ml) in a total of 5 ml of medium. Thymidine-^{3}H (4 μCi/ml) was added and incubated for another 30 min. The control slices went through the same incubation conditions, but the medium did not contain CF. The procedure was stopped by placing the flasks on crushed ice. The slices were washed three times in 5% TCA and 95% alcohol, and DNA was extracted in 5% TCA (100°C for 10 min). The specific activity of DNA was expressed in cpm per 1000 O.D. units$_{600}$ read after a colorimetric reaction with diphenylamine.

Two other parallel experiments were performed: in one, renal slices were exposed to CF (10 μg/ml) and various concentrations of ADO (0.05 to 1 mM/ml); in another, they were exposed to ADO (0.25 to 2 mM/ml) without the presence of CF. Thymidine-^{3}H (4 μCi/ml) was added after a 10-min pre-incubation. The manipulation was carried out in the same manner as above.

Effect of CF on the levels of renal adenine nucleotides and cyclic nucleotides. The levels of ATP, ADP, AMP, and cAMP were examined under the following circumstances:

- Normal rats
- Unilaterally nephrectomized rats
- Normal rats receiving high dosage of CF
- Unilaterally nephrectomized rats receiving high dosage of CF.

The CF injections were carried out according to the schedule described earlier, however, an extra dose was given 40 min prior to sacrifice. The animals were anesthesized with an intraperitoneal injection of Somnotol (MTC Pharmaceuticals, Hamilton, Canada; 0.09 ml/100 g body wt) 24 hr following the nephrectomy or 24 hr following the second injection (9 A.M.) of CF. Kidneys were removed with minimum handling and were snap-frozen and crushed with flat-nose pliers precooled in liquid nitrogen. The frozen tissues were plunged into liquid nitrogen and subsequently weighed and extracted in 10% perchloric acid solution according to standard procedures.

ATP, ADP, and AMP were determined by enzymatic method. cAMP was estimated with a kit prepared by Amersham Corporation, Arlington Heights, Illinois.

Results

In vivo effect of CF on renal ADase and ADO. Our data shows that 40 min following the first intraperitoneal injection of 0.1 mg CF/kg body weight total inhibition of ADase occurred in the kidneys in 10 out of 16 animals. The ADO concentration was found to be 1.34 μmoles ± 0.19/g tissue (N = 10). In the other six rats, ADase activity was reduced and ADO was not detectable. The ADase examined in the remaining kidney recovered 65% of its initial activity 24 hr following a single injection, but the degree of recovery was reduced with continuing treatment at 24-hr intervals. Only 26 to 30% of the initial ADase activity was found on days 3 and 4.

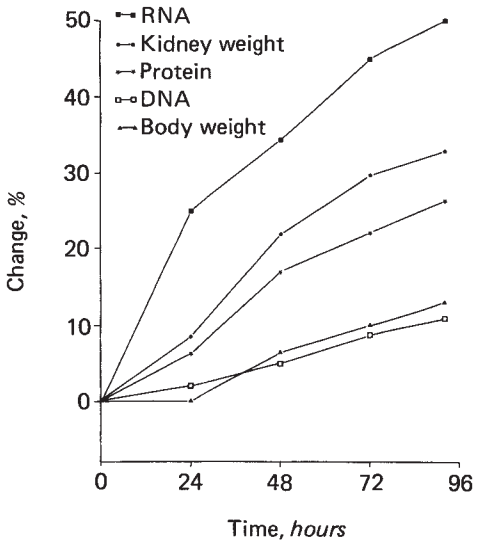

Fig. 1. *Effect of right unilateral nephrectomy on the principal parameters of normal rats fed ad libitum.* Each *point* is the mean of four animals.

When the dose was increased to 0.2 mg/kg/12 hr, the renal ADase activity measured 40 min after the second injection was found to be completely inhibited in 22 out of 27 animals. The ADO concentration was 0.95 μmole ± 0.16/g tissue (N = 22). This value is lower than the value of 1.34 μmoles presented earlier when ADO concentration was measured almost immediately upon the inhibition induced by the first CF injection. Some ADO thus appears to be either metabolized via alternative pathways different from inosine or transported out of the organ. With repeated CF injections at 12-hr intervals, only traces of renal ADase were found in the remaining kidney when it was examined 12 hr following a final injection. Although again no ADO was detected, the level of ADO measured in an extra group of rats with an extra dose of CF given 40 min prior to sacrifice — the aim of which was to eliminate those newly occurred traces of ADase — was again around 0.95 μmole as indicated earlier. Whenever total inhibition of ADase was maintained, ADO remained at the same level throughout. The in vivo effect of CF on CRG, therefore, was studied at the high dosage.

In vivo effect of coformycin on CRG. Unilateral nephrectomy induced CRG in the remaining kidney of normal rats fed a regular diet. The RNA, DNA, and protein content as well as the renal mass also increased [24, 25] (Fig. 1). CF-treated animals were listless and had no appetite. Their food intake was minimal: They took only 10 to 15% of the amount of food consumed by the controls. As seen in Figure 2**A**, **B**, and **C**, their

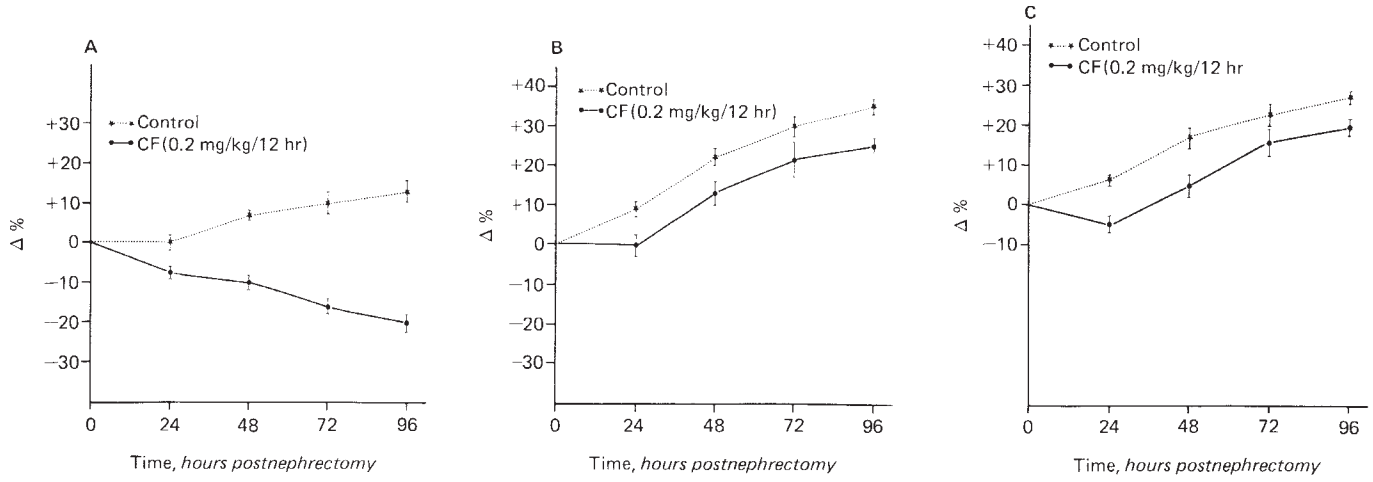

Fig. 2. *Changes in body weight* (**A**), *kidney wet weight* (**B**), *and kidney protein* (**C**) *content of uninephrectomized rats fed ad libitum.* The CF-treated rats, because of their diminished food intake brought about by CF, show a considerable loss in body weight, but CRG was not inhibited. Each *point* is the mean ± SEM (N = 4).

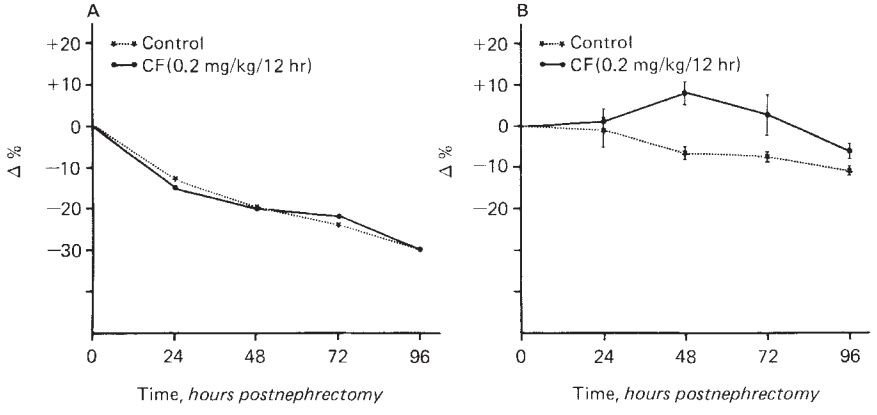

Fig. 3. *Changes in body weight* (**A**) *and kidney dry weight* (**B**) *of uninephrectomized fasting rats.* An identical loss in body weight was recorded in both control and CF-treated rats. The mass of the remaining kidney of control rats also dropped, but that of the CF-treated rats showed a gain within the first 72 hr post-nephrectomy. Each *point* is the mean ± SEM (N = 3).

body weight shows a dramatic drop which is almost at the same level as that observed in fasting rats (Fig. 3**A**); however, CRG, judged from the kidney wet weight and protein content, still took place, although to a lesser extent than in well-fed control rats. Due to the correlation between kidney weight, protein, RNA, and DNA content seen in Figures 1 and 2**B** and **C**, dry kidney weight was subsequently used as a parameter in the assessment of CRG. Since starvation is a well known factor which prevents CRG from occurring [26, 27], we compared the normal and CF-treated fasting rats. The same degree of body weight loss was seen in both groups (Fig. 3**A**). However, when the dry weights of the regenerating kidneys were determined, the CF-treated rats showed a different response; their kidney dry weights actually increased within the first 72 hr, despite starvation (Fig. 3**B**).

When the rats were given an isocaloric amount of food, the CF-treated rats also rated better than their control counterparts. An increment of 23 and 31% (N = 3) over the control kidney was noted at 72 and 96 hr postnephrectomy, respectively, in CF-treated rats, as compared to an increment of 16 and 20% (N = 3) in the control rats. All animals in both groups regained their original body weight.

In vitro incorporation of thymidine-^3H into DNA of renal cortex. The presence of CF in the incubating medium does not exert any inhibitory effect on the incorporation of thymidine-^{3}H into the DNA of the regenerating renal cortex slices; in fact, one observes a slightly higher incorporation (Table 1), which rules out a cytotoxic effect of CF. When one considers the short duration of the in vitro experiment, there is a possibility that this slight acceleration could be more significant in vivo. This could explain the higher rate of CRG observed in CF-treated rats as noted earlier.

When ADO was added to the medium, the situation changed dramatically as a marked inhibition was noted: 46% with 0.5 mM ADO/ml and 68% with 1.0 mM ADO/ml (Table 2).

To separate the potentializing effect of CF, the influence of external ADO alone was studied. An inhibition was observed, but many times the concentraton of ADO was needed to obtain the same degree of inhibition (Table 3).

Effect of CF on the levels of adenine nucleotides and cyclic nucleotides. The values of ATP (2.09 ± 0.21 μmoles/g tissue; N = 9), ADP (1.67 ± 0.15 μmoles/g tissue; N = 9), AMP (0.077 ± 0.025 μmoles/g tissue; N = 3), and cAMP (696 ± 38 pmoles/g tissue; N = 9) found in normal rat kidneys were not altered by

Table 1. In vitro effect of coformycin on the incorporation of thymidine-^{3}H into regenerating renal cortex slices[a]

Coformycin *μg/ml*	DNA specific activity *cpm/1000 D.O.*$_{600}$	%Δ
0	150.2 ± 12.2	
0.5	157.8 ± 14.6	+5.0 (NS)
0	127.7 ± 12.6	
1.0	128.6 ± 9.3	(NS)
0	153.8 ± 10.0	
4.0	164.5 ± 8.5	+6.9 (NS)
0	169.2 ± 7.4	
10.0	176.4 ± 7.9	+4.2 (NS)

[a] Values are the mean ± SEM (N = 4); NS, not significant.

Table 2. In vitro effect of coformycin (10 μg/ml) and external ADO on the incorporation of thymidine-^{3}H into regenerating renal cortex slices[a]

Adenosine *mM/ml*	DNA specific activity *cpm/1000 D.O.*$_{600}$	% Inhibition
0	155.2 ± 3.5	NS
0.05	151.8 ± 8.2	
0	178.3 ± 13.1	46.1
0.5	96.0 ± 5.0	($P < 0.01$)
0	136.6 ± 15.1	68.4
1.0	43.1 ± 6.5	($P < 0.005$)

[a] Values are the mean ± SEM (N = 4).

CF treatment or unilateral nephrectomy under our experimental conditions just described.

Discussion

ADO is produced, released, absorbed, and metabolized by most animal tissues. As shown in Figure 4, the two major metabolic pathways of ADO are the conversion to AMP by adenosine kinase and the transformation to inosine by ADase.

A delicate balance between the two enzymes seems to regulate ADO concentrations. Under normal conditions, ADO is maintained at a very low level ranging from 1 to 30 nmoles per g of wet weight of tissue, and from 0.03 to 2.6 mM in body fluids [28]. In general, ADO tends to remain at the cell site of production rather than escape into the general circulation, but it does not accumulate in the cell unless an ADase inhibitor is present [29].

ADase is distributed widely in all tissues of the body. It is bound tightly to CF upon the distribution of the latter into tissues; the recovery of the enzyme activity is related to the quantity of drug administered, its clearance into urine, and the ability of the tissue to generate new enzyme activity [22].

Renal ADase in all normal adult rats was found to be approximately 150 mU/mg protein as reported previously [23]. Its stability is remarkable: Identical values were found at different times (2 to 96 hr) after unilateral nephrectomy. Renal ADO is not detectable when ADase is not completely inhibited which is why we carried out our studies with a high dosage of CF. According to Chassin et al [22], the injection of 2′-deoxycoformycin produces a reduction of ADase activity to

Table 3. In vitro effect of external ADO in the absence of CF on the incorporation of thymidine-^{3}H into regenerating renal cortex slices

Adenosine *mM/ml*	DNA specific activity *cpm/1000 D.O.*$_{600}$	% Inhibition
0	120.3 ± 9.0[a]	5.4
0.25	113.8 ± 8.2	($P = 0.13$)
0	139.4 ± 7.0[a]	18.5
0.5	113.6 ± 7.7	($P < 0.005$)
0	134.2 ± 8.2[a]	31.8
1.0	91.4 ± 6.2	($P < 0.005$)
0	131[b]	
2.0	78	40.4

[a] Values are the mean ± SEM (N = 4).
[b] N = 1.

less than 10% in many rat tissues (kidney, liver, heart, muscle, and so forth) even at the low dosage of 0.1 mg/kg, but a substantial degree of recovery seems to take place within 8 to 12 hr in most tissues, with the exception of erythrocyte ADase which remains very low.

A wide range of physiological and a number of biochemical effects of ADO have been reported. The addition of exogenous ADO is associated with the inhibitions of cell growth, immune response, coronary vasodilation, delayed neurotransmission, lypolysis, and the inhibition or stimulation of hormone secretion and so forth. Some of these were shown to be related to the biochemical effects which include activation of the adenylate cyclase systems, inhibition of pyrimidine synthesis, and alteration of phosphoribosyl pyrophosphate metabolism.

The growth inhibition effect of ADO has been demonstrated in cultures of human lymphoblasts and fibroblasts [14–18]. Impairment of thymidine incorporation into DNA was shown to occur in mitogen-induced lymphocytes in the presence of ADO, but, as shown in this study this occurs only when a millimolar concentration was reached. The potentialization of these phenomena by different ADase inhibitors, such as EHNA (erythro-9(2-hydroxy3-nonyl) adenosine hydrochloride) or CF in combination with ADO was also found in previous in vitro studies [15, 16, 18, 19]. All indications point to the toxic role of high concentrations of ADO, most likely due to the inhibition of pyrimidine synthesis brought about by changes in adenine nucleotide levels [14, 20]. This could explain why there is increased ADase activity in kidney-developing cells [23] and adult intestinal villus cells which carry out exogenous ADO absorption [22].

The concentration of ADO appears to be an important factor in the outcome of its biological or biochemical effect. The biphasic effect of ADO on several adenylate cyclase systems has been well demonstrated: activation occurring at a low concentration through one receptor site and inhibition at a higher concentration through another receptor site [30, 31]. While the toxic effect of high ADO concentrations is well documented in cell cultures, much less has been reported on its stimulatory effect. Carson and Seegmiller [15] also observed a dual action of ADO on thymidine incorporation into human lymphocytes, analogous to the one described here in kidney cells.

Since the levels of purine nucleotides and the adenylate cyclase activity were not modified in the presence of an intracellular concentration of 0.95 μM of ADO, this seems to

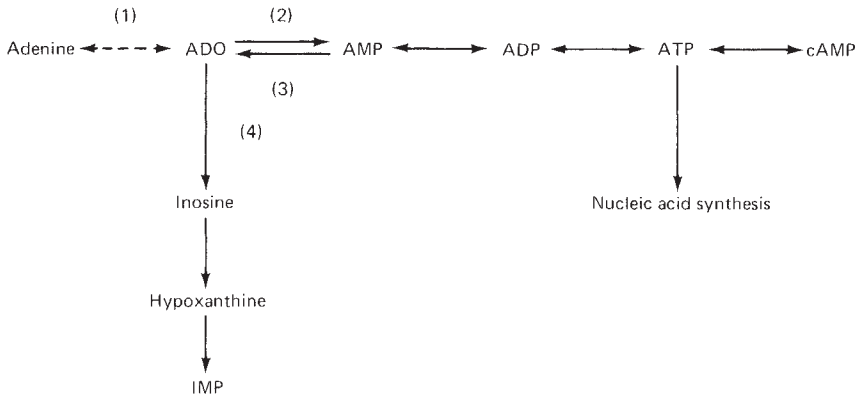

Fig. 4. *Metabolism of ADO* (1) *adenosine phosphorylase;* (2) *adenosine kinase;* (3) *5'-nucleotidase and nonspecific phosphatase;* (4) *ADase.*

indicate that the ADO effect is not due to its direct transformation into purine nucleotides or cAMP, nor to its modulating effect on their levels. ADO could thus play the role of a local messenger, as suggested by Arch and Newsholme [28], exerting a direct effect on the cytoplasmic components which are active in stimulating DNA synthesis [32–34]. This action could be similar to the one exerted by diadenosine tetraphosphate in the initiation of DNA replication [35, 36].

Our studies suggest that a low intracellular concentration of ADO can enhance CRG and suppress the CRG inhibition induced by starvation, but they do not demonstrate a key role for ADO in the initiation of CRG: Renal ADase activity was not modified nor was the intracellular ADO concentration increased following mononephrectomy [23]. However, this report brings indirect evidence in favor of a negative feedback mechanism essential for CRG to occur.

Summary. The role of ADO and ADase in CRG was studied in unilaterally nephrectomized rats by using a specific ADase inhibitor, CF. ADO appears to have a biphasic effect. In vitro, ADO can inhibit the incorporation of thymidine-^{3}H into DNA of regenerating renal cortex slices but only at high concentrations: Inhibition was 19% at 0.5 mM and 32% at 1.0 mM ADO/ml of incubation medium. The same concentrations of ADO combined with CF (10 μg/ml) yielded higher inhibitory effects: 46% at 0.5 mM and 68% at 1.0 mM. In contrast, when regenerating renal cortical slices were incubated with CF alone without external ADO, thymidine-^{3}H incorporation into DNA was higher in these than in controls. When a complete inhibition of ADase was achieved in vivo by injecting CF into rats, renal ADO increased from minimal amounts to 0.95 μmole/g kidney ± 0.16 (N = 22). CRG took place in coformycin-treated rats even when rats were fasting. The levels of ATP, ADP, AMP, and cAMP were not modified under the above conditions. We postulate that enhanced CRG after CF treatment in unilaterally mononephrectomized rats could be due to the intracellular action of ADO acting as an alternate messenger of cell proliferation.

Acknowledgments

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