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Adenosine stimulates 5′-nucleotidase activity in rat mesangial cells via A_2 receptors

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Because A_2 adenosine receptor activation stimulates adenylate cyclase and cyclic AMP induces 5′-nucleotidase expression in rat mesangial cells, we examined the effect of adenosine and its analogs on 5′-nucleotidase activity in these cells. A_2 adenosine receptors were characterized using [^{3}H]5′-*N*-ethylcarboxamidoadenosine (NECA) as a tracer. There was a single group of receptor sites with a K_D value of 0.53 μM and a number of sites of 1,317 fmol/mg. [^{3}H]NECA binding was inhibited preferentially by A_2 adenosine analogs and antagonists. Similarly, the order of potency for cAMP stimulation was in favour of A_2 adenosine analogs. Rat mesangial cells expressed surface 5′-nucleotidase activity. Exposure of cells for 48 h to adenosine analogs showed that at low concentrations A_2 analogs stimulated 5′-nucleotidase activity. These results indicate that adenosine upregulates activity of 5′-nucleotidase, the enzyme responsible for its local formation, via A_2 receptor stimulation and increase in cAMP production.

5′-Nucleotidase, Rat; Mesangial cell; Adenosine, Cyclic AMP

1. INTRODUCTION

Ecto-5′-nucleotidase is a membrane ecto-enzyme present at the surface of cultured rat mesangial cells which hydrolyzes extracellular adenine nucleotides into adenosine [1]. This nucleoside is a powerful local mediator responsible for multiple effects in the glomerulus including decrease in glomerular filtration rate via mesangial cell contraction [2] and constriction of the afferent arteriole [3] as well as inhibition of renin release [4]. In keeping with these findings, adenosine has been considered to be the potential mediator of the tubuloglomerular feed-back response. Following an increase of sodium chloride concentration in the distal nephron, adenosine would be locally generated in excess and thereby responsible for the fall in glomerular filtration rate [5]. 5′-Nucleotidase appears to play a key-role in adenosine synthesis because inhibition of its activity by a specific inhibitor, α,β-methylene adenosine 5′-diphosphate, results in inhibition of adenosine production in the isolated perfused kidney [6] and renal tubular cells in culture [7]. Therefore, regulation of 5′-nucleotidase activity may strongly influence adenosine availability at its receptor sites. We have already shown that 5′-nucleotidase expression in cultured rat mesangial cells was stimulated by cAMP [8] and interleukin-1 [9], the latter effect depending in part on cAMP via prostaglandin E_2 stimulation. We also showed that 2-chloroadenosine (2-cADO), a stable analog of adenosine, increased basal 5′-nucleotidase activity in a dose-dependent manner [10]. Taken together, these results raise the question of whether adenosine upregulates the enzyme responsible for its production via a cAMP-mediated mechanism. In order to address this issue, we sought to characterize A_2 adenosine receptors on rat mesangial cells and to define their role in cAMP production and stimulation of 5′-nucleotidase activity.

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2. MATERIALS AND METHODS

2.1 *Materials*

The following materials were purchased from the corresponding suppliers: cell culture medium and 0.05% trypsin–0.02% EDTA from Flow Laboratories (Irvine, Ayrshire, UK); penicillin G and streptomycin sulphate from Gibco (Grand Island, NY); cell culture Petri dishes and plates from Nunc (Roskilde, Denmark) or Costar (Cambridge, MA); collagenase and adenosine analogs including 5′-*N*-ethylcarboxamidoadenosine (NECA), *R*-N^6-phenylisopropyladenosine (R-PIA), N^6-cyclohexyladenosine (CHA) and 2-cADO from Sigma (St. Louis, MO), nonxanthine phosphodiesterase inhibitor, RO 20-1724, from Biomedical Research (Plymouth, PA); [^{3}H]NECA (27 Ci/mmol) and [^{125}I]cAMP (2,000 Ci/mmol) from the Radiochemical Center (Amersham, UK); [^{3}H]thymidine (25 Ci/mmol) from Dositek (Orsay, France). The adenosine receptor antagonists 8-cyclopenthyl-1,3-dipropylxanthine (PD 116,948) and [*N*-(2-dimethylaminoethyl)-*N*-methyl-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purine-8-yl)] benzenesulfonamide (PD 155,199) which exhibit anti-A1 and anti-A2 properties, respectively, were a gift from Parke Davis, Warner Lambert (Ann Arbor, MI). All other chemicals were of reagent grade and were used without further purification.

2.2. *Mesangial cell culture*

Primary cultures of rat mesangial cells were obtained from collagenase-treated glomeruli as previously described [11]. Kidneys were removed under pentobarbital anesthesia from 100–120 g male Sprague–Dawley rats and glomeruli were isolated by sieving techniques and centrifugation. Collagenase-treated glomeruli were seeded in plastic flasks of 25 cm^2 in the presence of 5 ml RPMI-1640 medium buffered

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with 20 mM HEPES and supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin sulfate and 2 mM glutamine. Mesangial cells were subcultured on day 21. After treatment with 0.05% trypsin 0.02%, EDTA, primary cultures were passed through a 50 μm-sieve and transferred to Petri dishes. At confluency, mesangial cells were detached and seeded in 12-well plates at a density of 50,000 cells per ml. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air. The culture medium was changed every 2 days. Confluent mesangial cells in the second subculture were studied in all experiments. Cells exhibited typical morphological and biochemical features of mesangial cells [11,12].

2.3. *Binding studies*

Binding studies were performed on cells in 12-well plates. The medium was aspirated and the cells were washed 3 times with phosphate-buffered saline (PBS), pH 7.4, supplemented with 1 mM $MgCl_2$. Then cells (90 μg protein per well) were incubated in quadruplicate with 30–240 nM [^{3}H]NECA and the appropriate unlabeled reagents in a final volume of 500 μl of the same medium at 4°C for 60 min unless otherwise stated. The reaction was terminated by aspirating the medium and washing 3 times with 2 ml of ice-cold PBS. Cells were digested in 1 M NaOH and samples were taken for ^{3}H radioactivity and protein determinations. ^{3}H radioactivity was measured by liquid scintillation spectroscopy (Rackbeta 211, LKB, Bromma, Sweden) in 8 ml of scintillation solution (Lumac, Olen, Belgium) after pH of the sample had been adjusted to 7. Nonspecific binding was measured by incubating cells with [^{3}H]NECA in the presence of 1 mM unlabeled NECA. Specific binding was calculated by subtracting nonspecific binding from total binding. Results were expressed as fmol [^{3}H]NECA bound per mg of protein per 60 min. The data obtained in saturation binding studies at equilibrium were transformed according to Scatchard in order to calculate the K_D value and the number of sites. The K_D value was also calculated from the association (k_{+1}) and dissociation (k_{-1}) constants determined in kinetic studies. The dissociation curve was obtained by incubating cells with 90 nM [^{3}H]NECA for 60 min and then adding 1 mM unlabeled NECA for various periods of time (1–10 min).

2.4. *cAMP assay*

cAMP levels were also measured in cells grown in 12-well plates The culture medium was removed. The cells were washed three times with serum-free medium and then pretreated for one hour with 0.1 mM RO 20-1724 in serum-free medium. This agent being a nonxanthine phosphodiesterase inhibitor is not an adenosine receptor antagonist. The medium was aspirated and the cells were exposed to various concentrations of adenosine analogs in 1 ml of serum-free medium for 5 min at 37°C. The reaction was stopped and intracellular cAMP was extracted by adding HCl at 0.1 M final concentration. After 30 min, the supernatants were collected and their pH adjusted to 6.4. cAMP was determined by radioimmunoassay using a rabbit anti-cAMP antiserum raised in the laboratory [13].

2.5. *5'-Nucleotidase assay and cell protein*

Enzyme activity was measured on intact cells in culture Cells were exposed to the agents tested during 48 h since we reported previously that such a lag-time was necessary for induction of 5'-nucleotidase activity [9,10]. At the end of this incubation period, the cells were washed first with a 30 mM Tris-HCl buffer (pH 7.4) containing (mM) 130 NaCl, 0.25 EDTA, 0.125 EGTA and 5.5 glucose. They were then washed with the incubation medium made of 30 mM Tris-HCl buffer (pH 7.4) containing (mM) 130 NaCl, 5 $MgCl_2$ and 5.5 glucose. The substrate, 5'-AMP, was added at 3 mM concentration and the enzymatic activity was determined as previously described [1]. Enzyme activity was expressed as nmol inorganic phosphate formed per minute and per mg of cell protein. After appropriate digestion with 1 M NaOH, cell protein was determined according to [14] using bovine serum albumin as standard.

2.6. *Calculations and statistical analysis*

Means ± S.E. are given throughout. Results of competitive inhibition binding studies were analysed according to [15]. This transformation allows the Hill coefficient and the IC_{50} (concentration of inhibitor providing 50% inhibition of [^{3}H]NECA binding) to be estimated. The equilibration dissociation constant of the agonists or antagonists (K_{DI}) was calculated according to [16] using the following formula: $K_{DI} = IC_{50}/(1 + D/K_{DD})$ where D is the concentration of radioactive ligand and K_{DD} its apparent dissociation constant (calculated from Scatchard transformation of saturation binding studies). Statistical significance was estimated by using Student's t-test or analysis of variance for repeated data as appropriate. Correlation between two parameters was estimated by regression analysis.

3. RESULTS

3.1. *[^{3}H]NECA binding studies*

[^{3}H]NECA binding to rat mesangial cells was studied at 4°C as a function of time at a concentration of 90 nM. A plateau was reached within 60–120 min of incubation. Nonspecific binding represented 25% of total binding at equilibrium. Under these conditions, addition of 1 mM unlabeled NECA produced a rapid dissociation of the ligand–receptor complex. There remains no specific radioactivity bound 10 min later (Fig. 1). The slopes of the two logarithmically transformed association and dissociation curves were used to calculate the kinetic parameters of the binding process. The dissociation (k_{-1}) and association (k_{+1}) rates were −0.458 min^{-1} and 0.00459 $nmol^{-1} \cdot l^{+1} \cdot min^{-1}$, respectively. The apparent dissociation constant (K_D) value obtained as the ratio k_{-1}/k_{+1} was 0.1 μM. When rat mesangial cells were incubated for 60 min with increasing concentrations of [^{3}H]NECA, the amount of [^{3}H]NECA bound

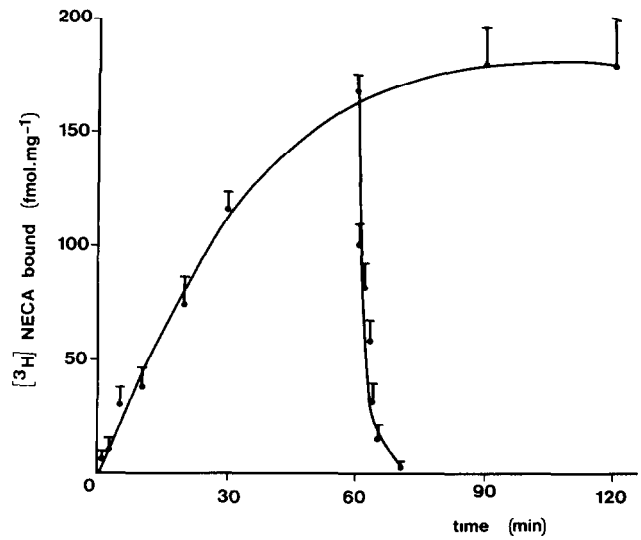

Fig. 1. Time course of specific [^{3}H]NECA binding to rat mesangial cells. Specific [^{3}H]NECA binding was studied at 4°C from 1 to 120 min after addition of the radiolabeled ligand. Reversibility of [^{3}H]NECA binding to rat mesangial cells was assessed by addition of 1 mM unlabeled NECA at equilibrium and incubation for 1–10 min. Means ± S.E. of 4 determinations are given.

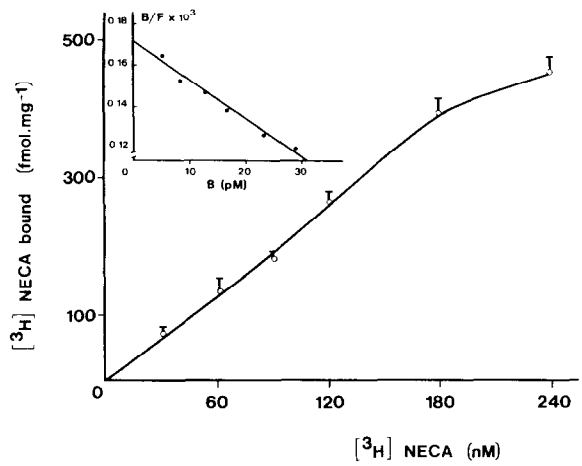

Fig. 2. Concentration dependency of [^{3}H]NECA binding to rat mesangial cells. Specific [^{3}H]NECA binding to rat mesangial cells at 4°C for 60 min is shown. Means ± S.E. of 4 determinations are given. Scatchard analysis of the data (inset) indicates a single class of binding sites with a K_D value of 0.53 μM and a B_{max} of 1,317 fmol/mg.

followed a curvilinear ascending curve. The Scatchard plot of the data was linear, which suggests the presence of one class of NECA binding sites (Fig. 2). The maximum binding (B_{max}) and the K_D value derived from the Scatchard analysis were 1,317 fmol/mg and 0.53 μM, respectively. The latter value is thus greater than that calculated from the kinetic studies. The maximum binding corresponds to around 700,000 sites per cell. Competitive inhibition of [^{3}H]NECA binding by unlabeled NECA, other adenosine agonists and adenosine antagonists was studied over a large range of concentrations (10 nM–1 mM). Total inhibition of binding was obtained with 100 μM NECA whereas it could not be reached with the other agents. Hill transformation of the data allowed calculation of the Hill coefficients and of the concentrations of analogs providing 50% inhibition of maximum binding (Table I). The inhibition constants (K_{DI}) were then obtained according to [15]. K_{DI}

Table I

Characteristics of binding of adenosine agonists and antagonists to A_2 adenosine receptors in rat mesangial cells

Agent	IC_{50} (μM)	n	K_{DI} (μM)
NECA	0.75	−0.73	0.66
2-cADO	1.05	−0.31	0.92
R-PIA	22.8	−0.24	19.9
CHA	52.9	−0.31	46.4
PD 115,199	3.53	−0.53	3.1
PD 116,948	36.9	−0.33	32.4

Competitive inhibition curves were transformed according to Hill to obtain IC_{50} and n values. K_{DI} values were calculated according to the following formula: $IC_{50}/(1 + [D]/K_{DD})$ in which $[D]$ is the concentration of [^{3}H]NECA and K_{DD} the apparent dissociation constant for [^{3}H]NECA calculated from the data shown in Fig. 3. IC_{50} = concentration for 50% inhibition of binding; n = Hill coefficient; K_{DI} = equilibrium dissociation constant for inhibitor.

for NECA (0.66 μM) was close to the K_D value calculated from Scatchard analysis (0.53 μM). The order of inhibitory potency of the adenosine analogs studied was NECA > 2-cADO > PD 155,199 > R-PIA > PD 116,948 > CHA. There was a clear difference between the high inhibitory potency of A2 agonists (NECA, 2-cADO) and antagonist (PD 155,190) and the low inhibitory potency of A1 agonists (R-PIA, CHA) and antagonist (PD 116,948).

3.2. *cAMP productio*

All the adenosine analogs studied stimulated cAMP accumulation in rat mesangial cells. The effect was dose-dependent, being more marked at 10 μM than at 1 μM. The order of stimulatory potency was NECA > 2-cADO > CHA. The A_2 antagonist PD 155,190 inhibited adenosine-dependent cAMP accumulation. When studied at 10 μM in the presence of 1 μM of agonist, it only inhibited significantly NECA. When studied at 100 μM in the presence of 10 μM of agonist, it exhibited equivalent inhibitory activities on the three agonists studied (Fig. 3).

3.3. *5′-Nucleotidase activity*

After 48 h incubation, all the adenosine analogs tested produced an increase of 5′-nucleotidase activity between 2 and 2.5 times basal value when they were used at 10 μM. Difference in potencies of the various adenosine analogs was apparent only at the lowest concentrations (0.1 and 1 μM). At 0.1 μM, NECA exhibited its maximum effect with an increase of 2.5 times basal value. Studied at the same concentration 2-cADO, R-PIA and CHA produced stimulations of 1.7, 1.46 and 1.18 times basal value, respectively. At 1 μM, 2-cADO and NECA had identical maximum effects while R-PIA and CHA were less potent (Fig. 4). In contrast, guanosine (10 μM) diminished 5′-nucleotidase activity (82.0 ± 5.1% of basal value; $P < 0.05$) showing that stimulation of 5′-nucleotidase activity was only a characteristic of adenosine-related compounds.

4. DISCUSSION

Mesangial cells are both the source [9,10] and the target [17,18] of adenosine, the former event depending on 5′-nucleotidase activity. Olivera et al. [17] showed recently that 1 μM NECA increased by 68% the cAMP levels of forskolin-stimulated rat mesangial cells, which suggested that rat mesangial cells possess functional A_2 adenosine receptors in addition to the previously described A_1 adenosine receptors [18]. However, binding studies were lacking in this report allowing no comparison to be made between the concentrations of agonists needed for competitive inhibition at the receptor sites and those stimulating cAMP accumulation. In the present study, 0.75 μM NECA provided 50% inhibition of binding. Values in the same range were observed for

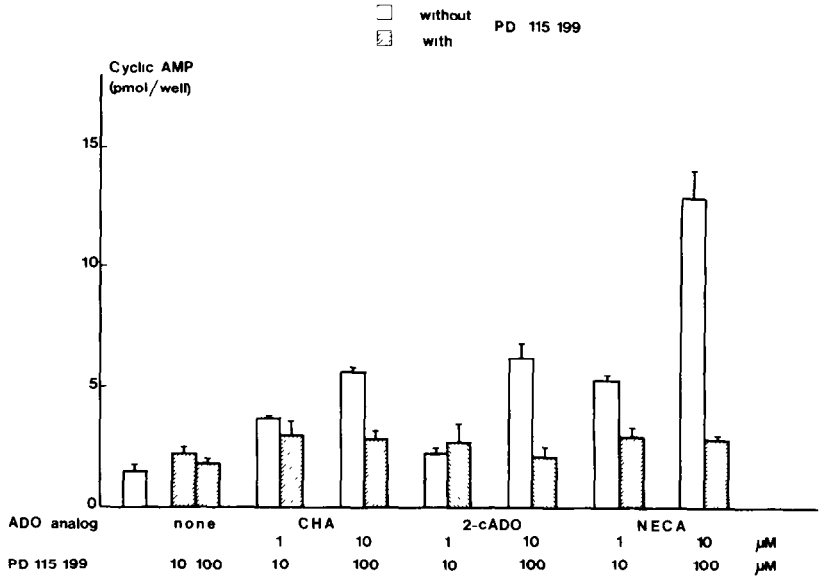

Fig. 3. cAMP accumulation in rat mesangial cell. Mesangial cells were exposed for 5 min to adenosine analogs and PD 155,199, an A_2 antagonist, separately or in combination. Values are means of 4 determinations. Results were analysed by two-way analysis of variance (agonist, antagonist) for repeated data. The effects of both types of agents were significant ($P < 0.001$) and interacted significantly ($P < 0.001$).

stimulation of cAMP (3.7 times basal value at 1 μM) and 5'-nucleotidase activity (2.5 times basal value at 0.1 and 1 μM). The K_D value for [^{3}H]NECA (0.53 μM) found in saturation binding studies with rat mesangial cells is close to those already reported with other preparations, 0.24, 0.3 and 0.46 μM in rat type II pneumocytes [19], human placenta [20,21] and rabbit alveolar macrophages [22], respectively. It is also noteworthy that the concentrations of NECA needed for cAMP and 5'-nucleotidase activity stimulations are lower than or equal to those of adenosine in rat kidney which Miller et al. found at 6 μM under control conditions and up to 15–26 μM after renal artery occlusion [23]. Another argument for assuming that [^{3}H]NECA binding sites are true A_2 adenosine receptors is the demonstration of the same order of potency for the drugs competing for binding and those stimulating cAMP (NECA > 2-cADO > CHA) in agreement with what has been reported for A_2 receptors in a variety of preparations [19–22]. Taken together, these results strongly suggest that rat mesangial cells possess functional A_2 adenosine receptors. Such receptors are likely to also exist in vivo and thus to influence glomerular physiology since adenosine stimulated cAMP accumulation in rat freshly isolated glomeruli [24].

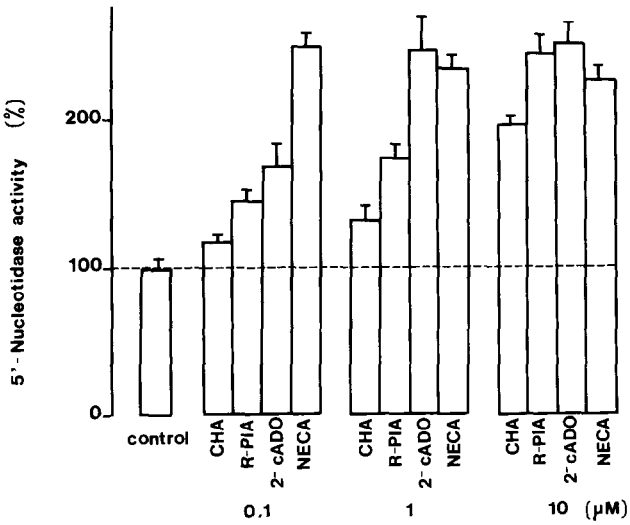

Fig. 4. Effect of adenosine analogs on 5'-nucleotidase activity of rat mesangial cells. Mesangial cells were cultured for 48 h with the adenosine analogs indicated at three increasing concentrations (0.1, 1 and 10 μM). Values (percent of control) are means ± S.E of 4 determinations. Basal 5'-nucleotidase activity was 68 nmol · min^{-1} · mg^{-1}. Results were analysed by one-way analysis of variance. The effect of the four agonists was significant ($P < 0.001$) at the three concentrations studied.

Table II

Effect of adenosine agonists on [^{3}H]thymidine incorporation in rat mesangial cells (percent of basal value)

Agent tested	Concentration (μmol/l)		
	0.1	1	10
NECA	99.5 ± 4.5	90.5 ± 4.1	80.5 ± 3.6
2-cADO	95.5 ± 4.3	86 ± 3.9	60.5 ± 2.7
R-PIA	97.5 ± 4.4	80 ± 3.6	54 ± 2.4
CHA	98.5 ± 4.4	78 ± 3.5	50 ± 2.3

Means ± S.E. of four values are shown. Basal [^{3}H]thymidine incorporation was 33,915 ± 1,479 cpm/well. Results were analyzed by one-way analysis of variance. The effects of the four agonists were significant ($P < 0.01$) at 1 and 10 μmol/l.

The present study also demonstrates that adenosine via its A_2 receptors stimulates 5'-nucleotidase activity. We have already reported the effect of cAMP on 5'-nucleotidase activity. Stimulation of enzyme activity was obtained after exposure of rat mesangial cells for 24 h to forskolin, PGE_2 or isoproterenol which activate adenylate cyclase or to 3-isobutyl-1-methyl xanthine or Ro 20-1724 which inhibit cAMP degradation [8]. Therefore, it is very likely that the mechanism whereby A_2 analogs stimulate 5'-nucleotidase activity is the increase in intracellular cAMP. We showed in the same report that protein synthesis was a prerequisite for the stimulation of 5'-nucleotidase activity. Similar data were observed by Colombi and Le Hir in rat renal fibroblasts [25]. Implication of A_2 receptors in 5'-nucleotidase activity control may also be inferred from the values of the concentrations of NECA needed to stimulate this enzyme (0.1–10 μM) which were in the same range as those increasing cAMP accumulation. In addition, the order of potency observed for 5'-nucleotidase activity stimulation (NECA > 2-cADO > CHA) was also in accordance with that found for [^{3}H]NECA binding inhibition and cAMP stimulation.

In conclusion, our data clearly demonstrate that expression of 5'-nucleotidase is upregulated by adenosine, the product of the reaction, which implies a positive feed-back loop. The physiological meaning of this event could be an adaptation of the enzyme to the amount of extracellular adenine nucleotides available. These nucleotides may either result from local ATP degradation during ischemia or AMP release from cells in which cAMP production has been stimulated.

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