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RELEASE OF ^{3}H-PURINES FROM [^{3}H]-ADENINE LABELLED RABBIT KIDNEY FOLLOWING SYMPATHETIC NERVE STIMULATION, AND ITS INHIBITION BY α-ADRENOCEPTOR BLOCKADE

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1 Rabbit kidneys were isolated and perfused with Tyrode solution. Release of ^{3}H-purines was studied after labelling of the adenine-nucleotide stores with [^{3}H]adenine (more than 60% uptake during a single passage).

2 One hour after labelling the spontaneous ^{3}H-outflow amounted to 0.1 to 0.2% of the total tissue content per minute. The release rate was enhanced following nerve stimulation (3 to 10 Hz), or brief infusion of noradrenaline (0.1 to 2.4 μg i.a.). Release of radioactivity was also enhanced by angiotensin II, by interruption of perfusion flow for 0.5 to 2 min and by hypoxia (5 to 25% O_2).

3 The release of tracer induced by nerve stimulation or noradrenaline was markedly reduced or abolished by phenoxybenzamine, which also inhibited the vasoconstrictor response. The release following angiotensin II, ischaemia and hypoxia could not be antagonized by this α-adrenoceptor antagonist.

4 The radioactivity in the kidney was predominantly in nucleotide form, while that released was composed mainly of nucleosides, of which adenosine predominated.

5 The results indicate that in the rabbit kidney vasocontriction, arterial clamping or reduced perfusion oxygen tension, cause release of adenosine and related compounds. In view of the reported actions of adenosine on noradrenaline effects and release in the kidney a possible physiological role is discussed.

Introduction

Adenosine is known to increase renal vascular resistance (Thurau, 1964; Scott, Daugharty, Dabney & Haddy, 1965, Hashimoto 1971). From the results of experiments in the dog and rat it has been concluded that adenosine might play a role in the regulation of renal blood flow and glomerular filtration rate (Ono, Inagaki & Hashimoto, 1966; Tagawa & Vander, 1970; Haddy & Scott, 1971; Ueda, 1972; Osswald, Schmitz & Heidenreich, 1975). In addition to its direct vascular effects, adenosine potentiates the vasoconstrictor effect of noradrenaline (NA) (Hashimoto, 1971; Hedqvist & Fredholm, 1976) and sympathetic nerve stimulation (Hedqvist & Fredholm, 1976). Furthermore, adenosine inhibits NA release in a number of different tissues, including the rabbit kidney (Hedqvist & Fredholm, 1976). Adenosine might therefore influence kidney function both directly and by modulating adrenergic neurotransmission.

Support for the opinion that adenosine might be physiologically important also derives from experiments in which release of adenosine has been studied. Thus, Haddy & Scott (1971) showed that a vasoactive substance with properties similar to adenosine was released into the venous effluent following 1 min occlusion of the renal artery. Osswald, Schmitz & Kemper (1977) recently reported significant elevation of renal adenosine content following ischaemia in the rat kidney. The tissue content of inosine and hypoxanthine was also increased.

In rabbit kidney ischaemia produced a parallel reduction of tissue adenine nucleotide content and increase in oxypurine secretion (Buhl & Jörgensen, 1975; Buhl, 1976a).

In view of our previous finding that adenosine might modulate kidney neurotransmission, we were particularly interested in the possibility that nerve stimulation might release purine compounds from the kidney, as previously shown in e.g. intestine and adipose tissue (Su, Bevan & Burnstock, 1971; Fredholm, 1976). Our results suggest that adenosine is released

from the rabbit kidney by nerve stimulation, and that the release is, at least in part, a consequence of hypoxia induced by vasoconstriction. Some of these results have been reported in a preliminary form (Fredholm & Hedqvist, 1976).

Methods

Rabbits of either sex (weighing 2 to 3 kg) were anaesthetized with sodium pentobarbitone, 40–50 mg/kg intravenously. The left kidney was isolated and perfused with Tyrode solution as described previously (Hedqvist & Fredholm, 1976). The kidney was perfused at a constant rate of 10 ml/min. The perfusion pressure was measured with a Statham (P23 AC) pressure transducer and recorded on a Grass Model 5 Polygraph. The nerve was placed on platinum wire electrodes and stimulated with pulses of supramaximal intensity (1 ms duration, 10 V) at rates of 1 to 10 Hz for 0.25 to 2 min by means of a Grass S4 stimulator. NA or angiotensin II was administered by close intra-arterial infusion. In some experiments, phenoxybenzamine was added to the perfusion medium. Hypoxia was induced by changing the composition of the gases bubbling through the perfusion medium from 95% O_2, 5% CO_2 to 5 to 25% O_2, 5% CO_2 balance N_2.

[2-^{3}H]-adenine (50 μCi, 0.56 Ci/mmol, obtained from the Radiochemical Centre, Amersham) was infused intra-arterially for a period of 10 min. Samples of the infusion solution, and of the venous effluent during and 30 min after infusion were taken to calculate the uptake of [^{3}H]-adenine; 60 to 90 min after the [^{3}H]-adenine infusion the experiments were started and continuous 1 min (10 ml) samples were collected of the venous effluent. An aliquot (usually 1 ml) was taken for the determination of total radioactivity. The remaining volume was subjected to various chromatographic steps to determine the composition of the radioactivity released from the kidney.

At the end of the experiment the kidney was frozen by means of a Wollenberger clamp precooled in liquid nitrogen. The kidney was stored at −80°C for up to 5 weeks. The tissue was then crushed between cooled metal blocks and the pulverised tissue homogenized in 0.4 M perchloric acid. After neutralization with 4 M KOH and 1 M Tris-base the extract was subjected to ion-exchange chromatography.

Ion-exchange chromatography

Three ml of a 1:3 slurry of Dowex 1 × 2 (200 to 400 mesh) (Serva Feinbiochemica, Heidelberg), extensively washed, was poured into a small plastic column (0.7 cm diameter); 2 ml samples were applied. The column was eluted in sequence with (a) 5 ml 50 mM Tris-HCl pH 7.4 followed by 5 ml redistilled water, (b) 5 ml 0.01 M HCl and (c) 5 ml 2 M HCl. As confirmed in pilot experiments, nucleosides and bases appear in the first fraction, adenosine 5'-phosphate (AMP) and most of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the second fraction, adenosine 5'-pyrophosphate (ADP) plus adenosine 5'-triphosphate in the third fraction. In a few experiments the effluents were chromatographed on long (0.7 × 25 cm) columns of Dowex 1 × 2, eluted by a concave gradient of HCl as shown in Figure 1.

PEI-cellulose thin-layer chromatography

PEI-cellulose sheets were obtained from Merck (Darmstadt); 20 μl sample (unpurified effluent, concentrated eluate from the ion-exchange chromatography or venous effluent concentrated by adsorption on acid-washed Norit A and deadsorption with 50% NH_4OH (sp. gr. 1.18) in ethanol), was applied to the plate in a band of 2 cm width. The chromatogram was developed in 5 mM HAc and 0.5 M LiCl essentially as described by Böhme & Schultz (1974).

Radioactivity was measured in a liquid scintillation counter. Water containing samples were counted in a scintillator composed of 3.9 g PPO, 0.06 g *bis*-MSB per litre of toluene: Triton X-100 (2:1, v/v). Radioactivity on thin layers was first eluted in 1 ml 0.1 M HCl before counting in the same liquid scintillator.

Results

Labelling of kidney nucleotide stores by [^{3}H]-adenine

The uptake of [^{3}H]-adenine infused into the kidney was substantial. On the average 64.5% of the radioactivity was taken up by a single passage (s.d. 10.4%). In three experiments the kidney was freeze-clamped 60 min after the infusion of [^{3}H]-adenine. An aliquot of the tissue extract was subjected to ion-exchange chromatography. As shown in Figure 1 the bulk of the radioactivity was present in nucleotides of which ATP dominated. As verified by selective inorganic ion coprecipitation (not shown) cyclic AMP was not appreciably labelled.

Release of radioactivity from the labelled kidney

Basal release. Soon after the infusion of [^{3}H]-adenine the rate of appearance of radioactivity in the venous effluent approached a constant value, which varied between 0.1 and 0.2% of the tissue content per min (Figure 2). As determined by ion-exchange and thin-layer chromatography the bulk of the radioactivity was in the form of nucleosides (see below).

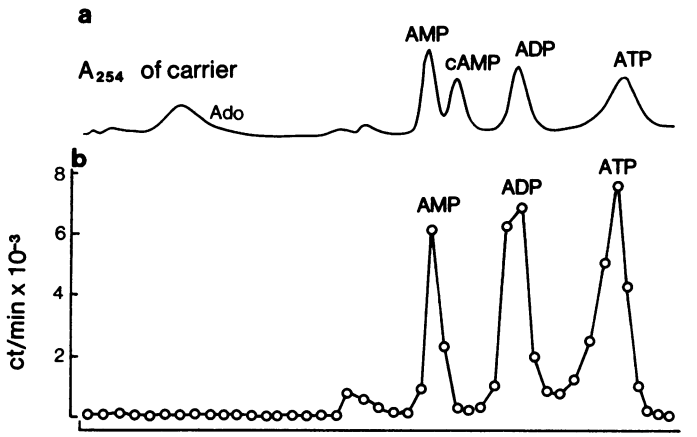

Figure 1 Distribution of radioactivity in kidney following labelling with [^{3}H]-adenine. After labelling and following 30 min perfusion the kidney (9.5 g) was rapidly frozen by means of a Wollenberger clamp. The tissue was crushed between metal blocks precooled in liquid nitrogen; 96 mg of the tissue powder was homogenized in 1 ml 0.4 M perchloric acid. 0.8 ml of the supernatant was neutralized with 4 M KOH and 1 M Tris. The neutralized extract was chromatographed on Dowex 1 × 2 (Cl^-) 200–400 mesh (0.7 × 25 cm). Elution with water followed by a concave HCl gradient from 0.003 to 0.4 M HCl. (a) Is a tracing from the continuous u.v.-absorption determination indicating the elution of carriers; (b) shows the radioactivity distribution; 4 other experiments gave essentially similar results. Ado = adenosine; cAMP = cyclic AMP.

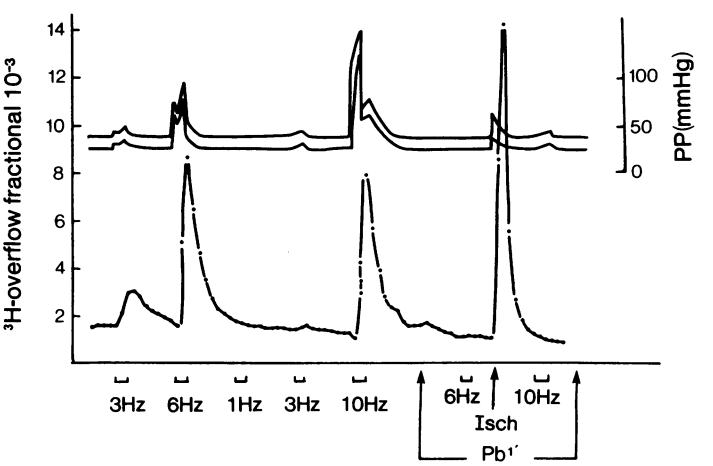

Figure 2 Release of ^{3}H-radioactivity from rabbit kidney; 26.8 μCi was taken up by the kidney. The upper panel shows the perfusion pressure (PP) in mmHg. The lower panel shows the fractional release of radioactivity ($\times 10^{-3}$) per min.

Effect of nerve stimulation. Stimulation of the nervous supply of the kidney caused an enhanced release of radioactivity. This is illustrated in Figure 2. Stimulation, with frequencies higher than 1 Hz always caused release of radioactivity (Table 1). The release was not quite constant during the experiment. As seen in Figure 2 there was sometimes a clear tachyphylaxis. However, in other experiments this was not so marked. On the average stimulation no. 2 released 73 ± 7% of the radioactivity released during the first stimulation (n = 6). There was a clear relationship between the magnitude of the induced vasoconstrictor-response and the release of radioactivity (Figure 3). The release was apparently dependent on the total number of pulses delivered. Over the range 3 to 10 Hz the release was 201 ± 42 pCi/pulse (mean ±

s.e.), which corresponds to 1–5 × 10^{-5} of the total tissue content per pulse. Since there was, in some experiments, pronounced tachyphylaxis and since the release of radioactivity was clearly dependent upon the response of the tissue, a detailed analysis of the frequency-response relationship did not appear to be meaningful. However, there were no statistically significant differences in the fractional release per shock over the frequency range 3 to 10 Hz. The release of radioactivity by nerve stimulation was abolished (or in one experiment markedly reduced) by treatment with phenoxybenzamine (Figure 2, Table 1).

The material released following nerve stimulation was mainly in the form of nucleosides and bases, as judged by ion-exchange chromatography (Figure 4a). Thin layer chromatography on PEI-cellulose revealed that most of the radioactivity co-chromatographed with adenosine, while inosine and adenine were minor components (Figure 5).

Effects of noradrenaline and angiotensin

Since there was a relationship between the vasoconstrictor effect of nerve stimulation and the release of radioactivity (Figure 3) other vasoconstrictor agents were also tried. NA (0.4 and 2.4 μg i.a.) always induced release of radioactivity. There was a similar relationship between the amount of radioactivity released and the magnitude of the vasoconstriction induced, as noted for nerve stimulation (Figure 3). The effect of NA was abolished by phenoxybenzamine (Table 1). The radioactivity released was mainly recovered as nucleosides (Figure 4b) of which adenosine dominated (Figure 6). Interestingly, release of AMP was also found (Figure 4b, Figure 6). The release of adenosine and AMP preceded the release of inosine. The time course suggests a substrate-product relationship.

In a total of ten trials, before and after phenoxybenzamine, angiotensin II (0.2 to 2.6 μg) caused release of radioactivity (Table 1 and Figure 3). Also in this case most of the radioactivity was found to be adenosine-like (not shown).

Finally, ATP and adenosine were tested. Both drugs given in a dose of 0.2 to 0.4 μg induced vasoconstriction and release of radioactivity (Figure 3). The effect of ATP was seen also in the presence of 10^{-5} M dilazep, a concentration which almost completely inhibits adenosine uptake into cells (Pohl &

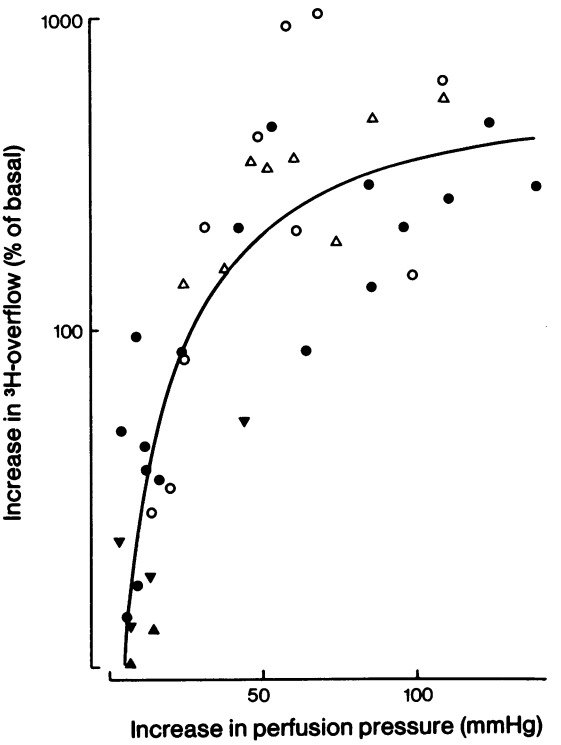

Figure 3 Relationship between ^{3}H-overflow and increase in perfusion pressure induced by different stimuli. Each point represents results from one separate experiment. (●) Nerve stimulation; (○) noradrenaline; (△) angiotensin II; (▼) ATP; (▲) adenosine. The increase in ^{3}H-overflow is expressed as % (peak release rate ×100/prestimulatory release rate). Note that the y-axis represents a logarithmic scale.

Table 1 Effect of different procedures on release of radioactivity from the perfused rabbit kidney

	Before Pb[1]	*After Pb*[1]
Nerve stimulation		
1–1.5 Hz	1/6	
2–2.5 Hz	2/2	
3 Hz	7/7	
6 Hz	9/9	0/4
10 Hz	4/4	1/3[2]
Noradrenaline		
0.4 μg	3/3	
2.4 μg	7/7	0/3
Angiotensin II		
0.2–2.6 μg	7/7	3/3
Ischaemia hypoxia		
	2/2	
	3/3	4/4

Results are given as number of experiments with evident release over total number of experiments. [1]Phenoxybenzamine (Pb) 3 μM. [2]Even in the experiment in which release was found it was very much lower than during the control period. There was also a vasoconstrictor response, suggesting incomplete α-blockade.

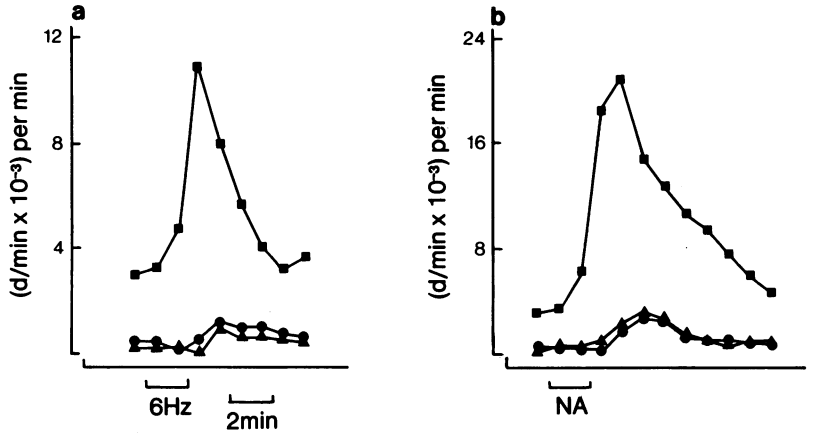

Figure 4 Release of ^{3}H-radioactivity by (a) nerve stimulation and (b) noradrenaline (NA, 2.6 μg/2 min). The radioactivity was separated into three fractions: (■) nucleosides, (●) AMP, and (▲) ADP, ATP by ion exchange chromatography. For details see Methods.

Brock 1974), suggesting that the release is not due simply to displacement of tissue stores.

Effects of ischaemia and hypoxia

The effect of interruption of perfusion flow was tested twice in the absence and twice in the presence of phenoxybenzamine. After only 30 s interrupted perfusion flow there was an enhanced release of adenosine-like material. This was accompanied by an enhanced perfusion pressure (Figure 2). If the ischaemic period was prolonged to 2 min the increase in perfusion pressure was further enhanced as was the release of adenosine-like radioactivity. Similarly, in two experiments hypoxia induced release of adenosine-like material (Figure 5).

Discussion

Our results show that adenosine is rapidly taken up by the rabbit kidney. A very efficient mechanism must exist for capture of kidney purines since more than 60% of the labelled adenine was taken up and retained by the tissue by a single passage. The radioactivity taken up was incorporated into the nucleotide pool. One hour after labelling most of the radioactivity was found in the ATP fraction, but the labelling of ADP and AMP was also substantial. There was very little radioactivity in the nucleoside fractions (which also contains unmetabolized adenine). Very soon after labelling the rate of overflow of radioactivity approached a steady state of 0.1 to 0.2% of the total tissue content per minute. Kidney nucleotide stores are approximately 3 μmol/g (*e.g.* Buhl, 1976b). Release of 0.1 to 0.2%/min in a 10 g kidney would imply release of 30 to 60 nmol/min. This figure may be compared with the reported values for kidney adenosine content of 7.6 nmol/g in the dog and 5.1 nmol/g in the rat (Thomas, Rubio & Berne, 1975; Osswald *et al.*, 1977). These two groups have thus found adenosine levels of about 60 nmol per kidney of the size used in our experiments. In view of the high activity of adenosine deaminase in the rabbit kidney (Fredholm, Hedqvist & Vernet, 1978) as well as the high rate of adenosine uptake and phosphorylation in this tissue (Buhl, 1976b and present data) the rate of adenosine formation must be quite high to maintain an adenosine concentration of the magnitude reported. Thus the figure derived from the present results and the assumption of homogeneous labelling of nucleotide stores may not be completely unrealistic. Even so the present data lend themselves better to qualitative than to quantitative conclusions.

Following nerve stimulation the rate of overflow of adenosine-like radioactivity increased several fold. The release of radioactivity was essentially abolished by the α-adrenoceptor antagonist, phenoxybenzamine. This finding suggests that most of the radioactivity is released from non-neuronal elements. The finding that noradrenaline was as effective as nerve-stimulation in inducing release of radioactivity also suggests that the site of release is mainly post-junctional. All the agents which produced renal vasoconstriction induced release of adenosine-like material. Moreover, the magnitude of the release appeared to be a function of the magnitude of the vasoconstrictor response. It is therefore possible that the effective stimulus for release was the vasoconstriction *per se*, i.e. that the constriction of the vascular elements is accompanied

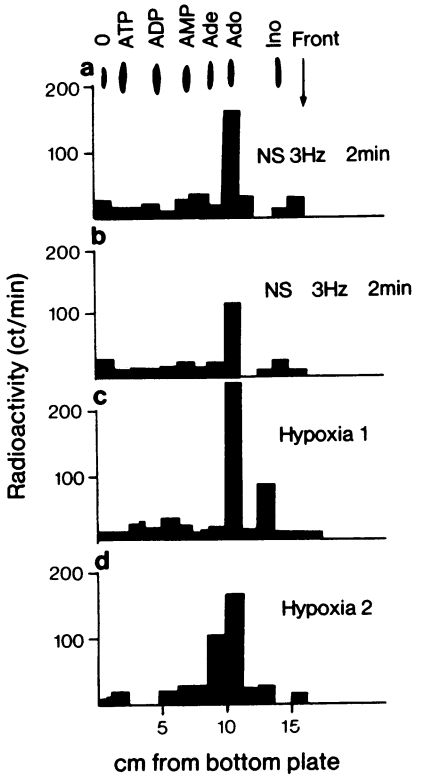

Figure 5 PEI-cellulose chromatography of perfusates of rabbit chromatography. The position of marker compounds is indicated above. (a) Perfusate 2 min after nerve stimulation (NS) 3 Hz for 2 min. (b) Perfusate at end of NS 3 Hz for 2 min (another stimulation). (c) Perfusate after 8 min perfusion with buffer bubbled with 10% O_2. (d) Perfusate after 12 min perfusion with buffer bubbled wtith 10% O_2. Ado = adenosine; Ade = adenine; Ino = inosine.

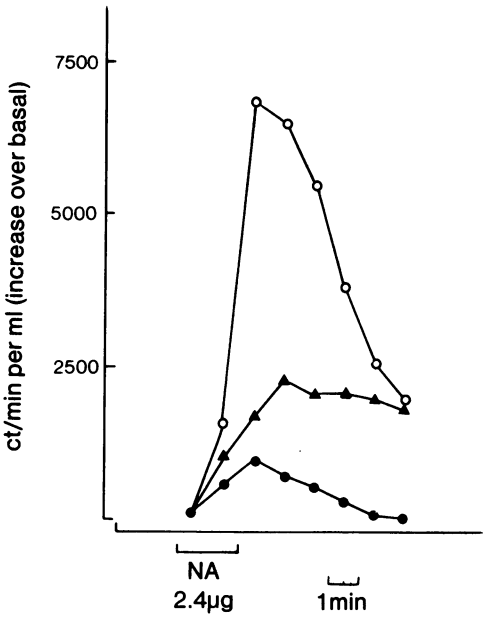

Figure 6 Release of radioactivity (increase over basal = 1,100 ct/min) during and following noradrenaline (NA) 2.4 µg. The radioactivity was separated on PEI-cellulose: (○) adenosine; (▲) inosine; (●) AMP.

by release of adenosine-like materials. Another possibility is that the vasoconstriction has caused a regional hypoxia even though perfusate flow was kept constant, and that this is the stimulus for release of adenosine-like material. The finding that interruption of perfusate flow or hypoxia were effective stimuli for release of adenosine-like material could be taken as evidence for the latter hypothesis.

Adenosine is a vasoconstrictor in the rabbit kidney (see Introduction). A vasoconstrictor effect is observed with only 0.1 µM in the perfusate and a 30% increase in perfusion pressure was found at 1 µM adenosine (Hedqvist & Fredholm 1976). The release of adenosine following ischaemia is well documented in the dog and rat (Haddy & Scott 1971; Thomas *et al.*, 1975; Osswald *et al.*, 1977). The present data indicate that it occurs also in the rabbit. It is of interest that ischaemia was always associated with a vasoconstriction. This vasoconstriction amounted to a 30 to 60% increase in perfusion pressure. Hypoxia similarly increased perfusion pressure. It is possible that the vasoconstriction induced by ischaemia and hypoxia may have been caused by adenosine (*cf.* Haddy & Scott, 1971; Thomas *et al.*, 1975). The findings of Osswald *et al.* (1977) that the postischaemic, transient vasoconstriction is reduced or eliminated by theophylline, a known adenosine antagonist in the kidney (Osswald, 1975; Hedqvist, Fredholm & Ölundh, 1976; 1978) further supports this idea.

Besides its direct effects on the renal vasculature, adenosine potentiates the vasoconstriction induced by nerve stimulation (Hedqvist & Fredholm 1976). In view of the present finding that adenosine is released by nerve stimulation it is possible that adenosine contributes to the vasoconstrictor effect of nerve stimulation. The finding that theophylline, which is an antagonist of the adenosine actions, reduces the vasoconstrictor response to nerve stimulation (Hedqvist *et al.*, 1976; 1978) is compatible with a role of adenosine. Further data are however, necessary to determine the validity of this hypothesis.

Finally, we recently demonstrated that adenosine could diminish the release of noradrenaline from

several mammalian tissues including rabbit kidney (Hedqvist & Fredholm, 1976). The present finding that nerve stimulation may induce release of adenosine may indicate that adenosine could be a modulator of neurotransmission via a prejunctional site of action. However, much work with substances that enhance or decrease the release and effects of adenosine is needed before the physiological importance of adenosine in this context is known.

In conclusion, the present results demonstrate that several stimuli which cause kidney vasoconstriction are capable of enhancing the rate of ^{3}H-purine release from [^{3}H]-adenine prelabelled kidneys. The bulk of the released radioactivity co-chromatographs with adenosine. The technique employed makes quantitative conclusions hazardous, but the results suggest that the amounts released may be high enough to be of significance. The results are compatible with the theory that adenosine plays a role as mediator of postischaemic or hypoxic vasoconstriction. Furthermore, the results are compatible with the theory that adenosine plays a role as modulator of neurotransmission pre- and postjunctionally.

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