Biochem. J. (1975) **152**, 429–432
Printed in Great Britain

The Release of Adenosine 3′:5′-Cyclic Monophosphate from the Isolated Perfused Rat Heart

By JOHN A. O'BRIEN
Department of Biochemistry, University of Edinburgh, Medical School, Edinburgh EH8 9AG, U.K.

and RICHARD C. STRANGE
Department of Clinical Chemistry, University of Edinburgh, Edinburgh EH3 9YW, U.K.

(*Received* 10 *September* 1975)

Although basal release of cyclic AMP from isolated perfused rat hearts was not measurable, isoprenaline induced substantial release of the nucleotide, suggesting that *in vivo* the myocardium can contribute to plasma cyclic AMP. Anoxia also increased the amount of cyclic AMP released, but insulin and nicotinate alone or in combination had no effect.

Several tissues can release the nucleotide cyclic AMP both in the basal state and after stimulation by hormones (Broadus *et al.*, 1970). Although the liver is an important source of extracellular cyclic AMP (Liljenquist *et al.*, 1974), intravenous injection of isoprenaline to hepatectomized rats results in a large increase in plasma cyclic AMP concentrations (Strange & Mjøs, 1975). This suggests that extrahepatic tissues can also be sources of the nucleotide. Leucocytes release cyclic AMP in the presence of isoprenaline (Øye, 1973), and, if changes in the extracellular concentration of cyclic AMP reflect changes in intracellular concentrations, catecholamine-sensitive tissues such as adipose tissue, skeletal muscle and the myocardium are other possible sources.

Although the catecholamines have inotropic, glycogenolytic and lipolytic effects on the myocardium, all of which are associated with changes in the intracellular concentration of cyclic AMP (Williamson, 1966; Sobel & Mayer, 1973; Entman, 1974), release of the nucleotide by this tissue has not been reported. We now describe experiments with an isolated perfused rat heart preparation in which the effect of isoprenaline on the ability of the heart to release cyclic AMP into the perfusing medium was investigated. The effects of anoxia, insulin, caffeine and nicotinate on this process are also described.

Materials and methods

Perfusion system. Hearts were taken under ether anaesthesia from fed male Wistar rats (200–250g body wt.) of a local strain. The hearts were perfused at 50mmHg pressure for 90min with a Krebs bicarbonate medium (Krebs & Henseleit, 1932) in which the concentrations of Ca^{2+} and Mg^{2+} had been halved. The medium was equilibrated with CO_2+O_2 (5:95) at 37.5°C. In the perfusion system (Fisher & O'Brien, 1972), infusate is introduced into recirculating perfusate whose volume is kept constant by withdrawal at a rate equal to infusion. The instantaneous rate of uptake or release of a substance by the heart is therefore given by the equation:

$$v = i(a-x) - P\frac{dx}{dt}$$

where v is the rate of uptake or release, i is the rate of infusion, a is the concentration of the substance in the infusate, x is the concentration of the substance in the perfusate, P is the volume of recirculating perfusate and t is the time. In our experiments P was 6.5ml and i was 0.5ml/min, so the $t_{\frac{1}{2}}$ for the approach to a steady state of a substance added or removed at a constant rate was 9min. Glucose (5.5mmol/l) and, when used, insulin (2 units/l), nicotinate (1mmol/l) and caffeine (1mmol/l) were present in the infusate and perfusate from the initiation of perfusion. Isoprenaline was infused only after 40min of perfusion. A solution of isoprenaline (95μmol/l in sodium metabisulphite, 0.1%, w/v) was introduced (3.1μl/min) into the lead carrying infusate into the perfusate so that the infusate concentration of isoprenaline was 0.57μmol/l. The theoretical concentration of isoprenaline in the perfusate therefore rose to 0.57μmol/l with a $t_{\frac{1}{2}}$ of 9min, but, because of oxidation in the medium, the effective concentration was less. However, changes in the mechanical performance of the hearts were maximal within one half-time after the initiation of perfusion. Anoxia was established by replacing the gas mixture of the recirculating perfusate with CO_2+N_2 (5:95) and replacing the oxygenated infusate with one equilibrated with CO_2+N_2 (5:95) but otherwise identical.

The perfusate, which was being continuously withdrawn from the system, was collected for

analysis over 2min periods with 4min or 8min intervals.

Assessment of the hearts. In the system described the time-course of glucose utilization by hearts perfused with glucose (5.5mmol/l) and insulin (2units/l) was characterized by a constant uptake of approx. 300μmol/h per g dry wt. and in the absence of insulin by a decrease of more than 20% in the first 40min of perfusion to a constant rate of approx. 100μmol/h per g dry wt. (Fisher & O'Brien, 1972). Conformation to these patterns of utilization in the absence of isoprenaline was taken as evidence of metabolic stability. Higher rates were regarded as evidence of hypoxic metabolism and were rejected. The inclusion of nicotinate or caffeine in the perfusate did not influence the rate or pattern of glucose utilization.

The mechanical performance of the hearts was assessed from the constancy of the heart rate, the magnitude and stability of the change of pressure and the rate of change of pressure in the perfusate as measured by a pressure transducer (Devices Instruments Ltd., Welwyn Garden City, Herts., U.K.) attached as close as possible to the cannula.

Analytical methods. The concentration of cyclic AMP in samples of perfusate was measured by using a protein-binding method (Strange & Mjøs, 1975). No interference occurred when ATP, ADP, AMP (10nmol/incubation) or cyclic GMP (10pmol/incubation) was added to the assay mixture. The lowest amount of standard used in the assay was 0.15pmol, which corresponds to a concentration of 3nmol/l. All perfusate samples were assayed in triplicate with a coefficient of variation of 6%. Cyclic AMP was shown to be stable in the perfusate by measuring the concentration of nucleotide in samples immediately after collection and after incubation at 37°C for up to 3h.

Glucose concentrations were measured by using an automated glucose oxidase method with gum guaiacum resin as chromogen (Fisher & O'Brien, 1972).

Results

Basal rate of cyclic AMP release. When the criteria of metabolic and mechanical stability described above were satisfied, the concentration of cyclic AMP in the perfusate was usually less than the lowest standard used in the assay method (3nmol/l). Thus, in 120 samples of perfusate taken during the first 40min of perfusion from 21 hearts perfused by glucose alone (six hearts), or glucose in combination with insulin (three hearts), nicotinate (three hearts) or caffeine (six hearts), the concentration of cyclic AMP in the perfusate exceeded 3nmol/l in only 25 of the samples (maximum concentration found 4.5nmol/l). There was no correlation between the occurrence of measurable amounts of cyclic AMP in the perfusate and any particular condition of perfusion. Similarly, in some experiments where perfusion with glucose (three hearts) or with glucose and caffeine (three hearts) was continued for 90min without the introduction of isoprenaline, the concentration of cyclic AMP in the perfusate did not usually equal or exceed 3nmol/l. The average rate of infusion in our experiments was 0.49±0.02(S.D.)ml/min and the average dry weight of the hearts used was 0.129±0.013(S.D.)g. A constant perfusate cyclic AMP concentration of 3nmol/l thus corresponds to a rate of release of 0.7nmol/h per g dry wt. The basal rate of cylic AMP release did not therefore exceed this value.

Effects of isoprenaline, insulin, nicotinate and caffeine. After initial perfusion for 40min with glucose (three hearts) or with glucose and caffeine (three hearts), isoprenaline was introduced into the perfusion medium. As shown in Fig. 1, for two representative hearts, measurable amounts of cyclic

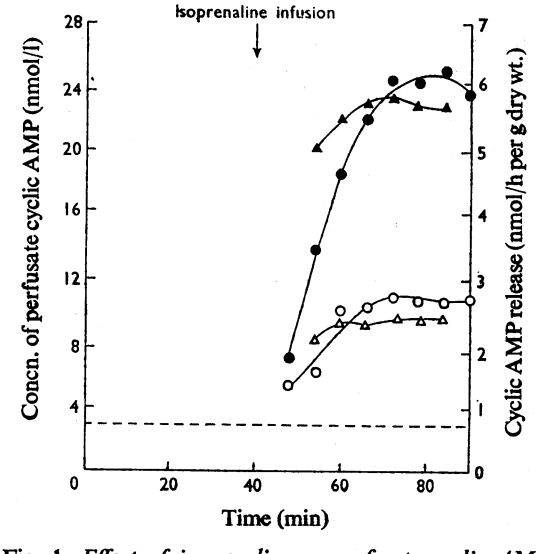

Fig. 1. *Effect of isoprenaline on perfusate cyclic AMP concentrations and rate of nucleotide release from the isolated perfused rat heart*

After perfusion for 40min with glucose (5.5mmol/l) or with glucose and caffeine (1mmol/l), isoprenaline was introduced into the perfusate. The data, from two representative experiments, show the concentration of cyclic AMP in the perfusate and the rate of release of the nucleotide from the heart perfused with glucose alone (○ and △ respectively) and for the heart perfused with glucose and caffeine (● and ▲ respectively). The broken line corresponds to the lowest measurable perfusate cyclic AMP concentration.

Table 1. *Effect of isoprenaline on cyclic AMP release*

Rat hearts were initially perfused with glucose (5.5 mmol/l), and with caffeine (1 mmol/l), insulin (2 units/l) and nicotinate (1 mmol/l) as indicated. After 40 min, isoprenaline (0.57 μmol/l) was infused into the perfusing medium and the rate of cyclic AMP release was measured 30 min later. Results show means±S.E.M. with the numbers of hearts used in parentheses.

	Release of cyclic AMP (nmol/h per g dry wt.)
Glucose	1.9±0.3 (3)
Glucose+caffeine	4.7±0.6 (3)*
Glucose+insulin	2.8±0.7 (3)
Glucose+nicotinate	2.1±0.2 (3)
Glucose+insulin+nicotinate	1.8±0.2 (3)

* $P<0.02$; difference compared by Student's t test.

AMP appeared in the medium within 10 min. The time-course of the rate of cyclic AMP release for these hearts (Fig. 1) derived from the time-course of cyclic AMP concentrations shows that the rate of release tends to become constant within 30 min of the start of the isoprenaline infusion. The rate of cyclic AMP release after 30 min of infusion with isoprenaline is shown in Table 1 for hearts perfused with medium containing in addition to isoprenaline glucose, glucose and caffeine, glucose and insulin, glucose and nicotinate, and glucose, insulin and nicotinate. With isoprenaline and glucose alone the rate of nucleotide release was 1.9±0.3 nmol/h per g dry wt. When caffeine was also included this rate was significantly ($P<0.02$) increased to 4.7±0.6 nmol/h per g dry wt. Insulin and nicotinate alone or in combination had no significant effect on the rate of release of cyclic AMP elicited by isoprenaline.

Effect of anoxia on cyclic AMP release. The possibility that anoxia may stimulate cyclic AMP release was raised by the observation that in three experiments, in which the rates of glucose utilization in the initial 40 min of perfusion suggested hypoxic metabolism, the concentration of perfusate cyclic AMP exceeded 3 nmol/l in all samples. Three hearts were therefore exposed to a 10 min period of anoxia after 40 min of perfusion. The concentration of perfusate cyclic AMP at the end of this period was 5.9±0.2 (S.E.M.) nmol/l, which indicates an increase in the rate of cyclic AMP release over the basal rate of at least 1.3 nmol/h per g dry wt. Perfusate cyclic AMP concentrations subsequently fell during aerobic perfusion to reach values below 3 nmol/l 25 min after reoxygenation. When three hearts were exposed to anoxia and isoprenaline after 40 min of perfusion, the perfusate cyclic AMP concentration rose to 5.9±1.6 (S.E.M.) nmol/l at the end of the anoxic period. Thereafter the amount of the nucleotide released was not different from that released from hearts exposed to isoprenaline alone. Anoxia therefore caused a rate of cyclic AMP release similar to that induced by isoprenaline, but the effects of the isoprenaline and anoxia were not additive.

Discussion

Under basal conditions the aerobic perfused rat heart does not usually release cyclic AMP in amounts that can be measured by the assay system described. The basal nucleotide release from these hearts cannot therefore be quantified, although the finding that some perfusate samples contained measurable amounts of cyclic AMP suggests that under basal conditions a low rate of release does occur. Treatment of the isolated heart with isoprenaline results in an increased release of nucleotide, and the presence of caffeine significantly enhances this effect. This suggests that after administration of isoprenaline *in vivo* the myocardium can contribute to cyclic AMP concentrations in plasma. The lack of effect of insulin on both basal and isoprenaline-stimulated cyclic AMP release from the isolated heart is consistent with the failure of insulin to affect either basal or catecholamine-stimulated myocardial cyclic AMP concentrations (Laraia & Reddy, 1969). If the rate of nucleotide release is determined by the intracellular nucleotide concentration the absence of an effect of nicotinate on myocardial cyclic AMP release is reasonable, since nicotinate has not been reported to affect cyclic AMP concentrations in muscle tissues.

The ability of the liver to release cyclic AMP both *in vivo* (Liljenquist *et al.*, 1974) and *in vitro* (Exton *et al.*, 1972) is well established and, in contrast with the myocardium basal release of the nucleotide, is readily measurable. Administration of adrenaline to the isolated perfused rat liver caused a sixfold increase in the rate of nucleotide release but a less than twofold increase in tissue cyclic AMP concentrations (Exton *et al.*, 1971, 1972). It has therefore been suggested that the rate of hepatic release of cyclic AMP is a better index of the amount of metabolically active nucleotide in the cell than is the total tissue cyclic AMP concentration, which may consist of both diffusible cyclic AMP and metabolically inactive, non-diffusible, cyclic AMP (Exton *et al.*, 1972).

Similar basal concentrations of cyclic AMP (range 0.3–0.8 nmol/g wet wt. of tissue) have been reported for the heart and liver. The small basal rate of myocardial nucleotide release indicates either that the amount of diffusible intracellular nucleotide is smaller in the heart than in the liver or that the myocardial membrane is less permeable to cyclic AMP than is the liver membrane. Since exogenous cyclic AMP is glycogenolytic in the liver (Exton *et al.*, 1972), but has failed to reproduce the cardiac actions

of the catecholamines (Entman, 1974), the latter seems more likely. Isoprenaline stimulates cyclic AMP release from the isolated heart by at least 2.7-fold, and increases intracellular cyclic AMP by between 2- and 6-fold (Øye & Langslet, 1972; Christian *et al.*, 1969). There is therefore no evidence that in the heart an active diffusible pool of cyclic AMP increases out of proportion to an increase in the total amount of intracellular nucleotide.

Measurement of extracellular cyclic AMP concentrations in samples of perfusate from single hearts may allow monitoring of the time-course of changes in intracellular cyclic AMP concentrations. This approach would be complicated if the release of cyclic AMP is a saturable process or is affected by changes in membrane permeability. Saturation of the process in the presence of isoprenaline alone is unlikely because the addition of caffeine further enhances the rate of release. Any change in membrane permeability to cyclic AMP would be most likely to occur during the anoxic experiments. However, the anoxia-induced increase in nucleotide release probably results from increased intracellular cyclic AMP concentrations, which occur after ischaemia in the isolated rat heart and which can be prevented by the inclusion of a β-blocker (Shahab *et al.*, 1972). This suggests that the rise in intracellular and extracellular cyclic AMP concentrations after anoxia is a result of the release of endogenous catecholamines from sympathetic nerve-endings. Further, the effects of anoxia and isoprenaline are not additive. The rate of cyclic AMP release from the myocardium is therefore probably determined primarily by the intracellular nucleotide concentration.

We thank Dr. O. Mjøs for helpful criticism and Mrs. P. Taylor, Miss M. Jamieson and Mr. W. Ramsay for skilled technical assistance.

Broadus, A. E., Kaminsky, N. I., Hardman, J. G., Sutherland, E. W. & Liddle, G. W. (1970) *J. Clin. Invest.* **49**, 2222–2236

Christian, D. R., Kilsheimer, G. S., Pettet, G., Paradise, R. & Ashmore, J. (1969) *Adv. Enzym. Regul.* **7**, 71–82

Entman, M. L. (1974) *Adv. Cyclic Nucleotide Res.* **4**, 163–193

Exton, J. H., Robinson, G. A., Sutherland, E. W. & Park, C. R. (1971) *J. Biol. Chem.* **246**, 6166–6177

Exton, J. H., Lewis, S. B., Ho, R. J. & Park, C. R. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 91–101

Fisher, R. B. & O'Brien, J. A. (1972) *Q. J. Exp. Physiol.* **57**, 176–191

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Laraia, P. J. & Reddy, W. J. (1969) *Biochim. Biophys. Acta* **177**, 189–195

Liljenquist, J. E., Bomboy, J. D., Lewis, S. B., Sinclair-Smith, B. C., Felts, P. W., Lacy, W. W., Crofford, O. B. & Liddle, G. W. (1974) *J. Clin. Invest.* **53**, 193–204

Øye, I. (1973) *Scand. J. Clin. Invest.* **32**, 189–191

Øye, I. & Langslet, A. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 291–300

Shahab, L., Wollenberger, A., Krause, E. G. & Genz, S. (1972) in *Effect of Acute Ischaemia on Myocardial Function* (Oliver, M. F., Julian, D. G. & Donald, K. W., eds.), pp. 97–108, Churchill-Livingstone, Edinburgh and London

Sobel, B. E. & Mayer, S. E. (1973) *Circ. Res.* **32**, 407–414

Strange, R. C. & Mjøs, O. D. (1975) *Eur. J. Clin. Invest.* **5**, 147–152

Williamson, J. R. (1966) *Mol. Pharmacol.* **2**, 206–220