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Some hormonal effects on myocardial phosphate efflux

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The effects of 23 agonists on the rates of cellular ^{32}P efflux and lactate dehydrogenase (LDH) release were tested in a perfused rat heart preparation which had been prelabelled *in vitro* with [^{32}P]P_i. Some 13 compounds produced detectable changes at high doses within 10 min, and in most cases a polyphasic response was observed. Six classes of compound gave rise to substantial effects, as follows. (1) Catecholamines and glucagon produced a transient initial stimulation of P_i efflux, followed by a long-term inhibition of P_i transport and an increased rate of LDH release. These effects were clearly different from the response seen after treatment with dibutyryl cyclic AMP, which had a slower, stimulatory, effect on P_i output in doses which gave rise to a pronounced inotropic effect, and produced a marked increase in both coronary flow and LDH release. (2) Carbachol also gave rise to a large transient stimulation of P_i efflux, which was followed by smaller sustained increase in P_i output without any obvious effect on LDH release. Dibutyryl cyclic GMP had no effect on P_i efflux or LDH release. (3) Insulin decreased the rate of P_i efflux, although the loss rate partially recovered towards the control value after prolonged exposure to the hormone. Insulin had no obvious inotropic effects and produced no change in the rate of LDH release. (4) Corticosteroids increased the rate of P_i efflux, although the loss rate partially declined towards the control value with prolonged exposure to the hormones. Corticosteroids produced a very slight inotropic response, and large doses sometimes increased the rate of LDH release from the tissue. Aldosterone slightly stimulated P_i output. (5) A small, transient and somewhat variable stimulation of P_i efflux was observed with vasopressin and angiotensin, whereas tri-iodothyronine was slightly inhibitory, but adenosine, histamine, spermidine, des-Asp^1-angiotensin, prolactin, parathyroid substances, calcitonin and somatostatin had no significant effects under our experimental conditions. (6) Ouabain stimulated P_i efflux in doses that had no detectable inotropic effect. It is suggested that P_i efflux involves the electroneutral transport of NaH_2PO_4 across the cardiac plasmalemma and that many of the hormonal effects might be explained by changes in the intracellular [Na^+] and pH in addition to changes in the intracellular [P_i].

P_i plays a central role in cellular energy metabolism. In addition to its direct role in oxidative phosphorylation, the cellular P_i pool is well placed to influence the detailed metabolic pattern within each tissue as a result of its involvement in reactions such as glycogen phosphorylase, glyceraldehyde phosphate dehydrogenase and succinic thiokinase. P_i also has an allosteric effect on several important regulatory enzymes, including glycogen phosphorylase and phosphofructokinase.

Abbreviation used: LDH, lactate dehydrogenase.

In the short term, intracellular [P_i] in mammalian muscle varies with energy supply and demand, largely as a result of the breakdown and resynthesis of phosphocreatine, since changes in ATP concentration tend to be small under normal physiological conditions. In the longer term the cellular [P_i] must reflect the balance between the uptake and loss of P_i and other phosphorus metabolites across the plasmalemma. This process is comparatively slow in cardiac muscle. We have previously measured P_i-uptake rates in the range 0.4–0.8 μmol/min per g dry wt. in perfused rat

hearts, so that many hours are required to exchange the whole of the metabolically active organophosphate pool (Medina & Illingworth, 1980). Intracellular [P_i] (4–8 mM) is higher than the normal extracellular value, and the energy for P_i uptake is apparently derived from the pre-existing transmembrane Na^+ gradient.

The factors which control the rate of P_i transport across the cardiac plasmalemma have not yet been subjected to intensive study. This transport system is an obvious candidate for involvement in both intracellular and extracellular control systems. The present paper describes preliminary tests on a range of substances with important effects on cardiac function and metabolism in order to identify those classes of compound which are suitable for detailed investigation.

Methods

Male Sprague–Dawley rate (300–600 g) were decapitated without prior use of heparin or anaesthetics. The hearts were removed and perfused via the aorta (Langendorff, 1895) at 35°C by using a non-recirculating system with Krebs bicarbonate medium (Krebs & Henseleit, 1932) containing 1 mM-pyruvate as substrate. No albumin was present in the perfusion medium. All hearts were paced electrically at 5 Hz and the aortic pressure was 13.3 kPa (100 mmHg). Each preparation was initially loaded with [^{32}P]P_i (1.5–3 mCi/litre) for 20–30 min and then rinsed free of loading buffer for a further 10–15 min before the first measurements were taken. Effluent radioactivity, lactate dehydrogenase content and coronary flow rate were then continuously monitored with the apparatus shown in Fig. 1.

Unless otherwise stated in the Results and discussion section, control readings were taken during the first 10 min observation period before the heart was switched to medium containing the agonist under investigation. The effects were normally followed for at least 10 min before the heart was returned to the control medium, and observations continued for a final 10–20 min period. At the end of each perfusion the heart was freeze-clamped and finely powdered under liquid N_2 before sonication at room temperature with 5 ml of 50 mM-sodium phosphate buffer (pH 7.4), with a Dawe 'Soniprobe' type 1130A for 30 s at full power. This treatment was designed to disrupt the tissue completely, and release as much as possible of the remaining lactate dehydrogenase activity and the intracellular phosphorus metabolites into the soluble fraction. Insoluble material was removed by centrifugation at 30000 *g* for 15 min, and the supernatant diluted with perfusion medium so that the total cardiac soluble radioactivity (1:500 dilution) and lactate dehydrogenase content (1:5000 and 1:12500 dilution) could be measured on four successive 15 ml samples by using the flow-through method under the conditions previously used for the coronary effluent. All the experiments reported here were repeated with at least three separate hearts, and the individual graphs selected for publication were typical of the group as a whole.

The radiochemical purity of the coronary effluent was assessed in a separate series of experiments in which the fluid leaving the heart was collected directly into liquid N_2, with vigorous stirring to accelerate the rate of freezing. The granular product from each 1 min collection interval was weighed and finely powdered under liquid N_2 before storage at −20°C before assay. Powdered effluent (15 g) was mixed with 5 ml of 600 mM-$HClO_4$, thawed and promptly divided into two 10 ml samples. One sample was immediately shaken for 30 s with 5 ml of 100 mM-Na_2MoO_4 in 600 mM-$HClO_4$ and 15 ml of ethyl acetate at 0°C to extract the phosphomolybdate complex into the organic phase, and the other sample was first hydrolysed at 100°C for 15 min and cooled before an identical extraction step.

The radioactivity present in the bright-yellow organic phase was measured by Čerenkov counting at about 10% efficiency. Alternatively, the ^{32}P could be back-extracted from the ethyl acetate into 15 ml of 1 M-NaOH and measured by Čerenkov counting in the colourless aqueous layer at 30% efficiency.

The hearts used for radiochemical-purity measurements were freeze-clamped at the end of the effluent collection, powdered and stored exactly like the effluent samples. Portions (200 mg) of frozen heart powder were extracted with 10 ml of 600 mM-$HClO_4$ at 0°C and promptly diluted to 42 ml with cold perfusion medium. Precipitated protein was removed by brief centrifugation, and the supernatant was treated with Na_2MoO_4 and ethyl acetate as described above.

Results and discussion

The effects of glucagon on ^{32}P efflux, LDH losses and coronary flow are shown in Fig. 2. Exposure to adrenalin (11 μM) and noradrenaline (6 μM) produced a similar sequence of changes. All three compounds had an immediate inotropic effect and increased the coronary flow rate. In each case there was a brief episode of very rapid ^{32}P efflux, which was superseded by a pronounced inhibition of P_i output. All three agonists slowly increased LDH losses to about 6 times the control rate. The stimulation of LDH release by catecholamines was studied by Opie *et al.* (1979), and there are similar reports from other laboratories. In our experi-

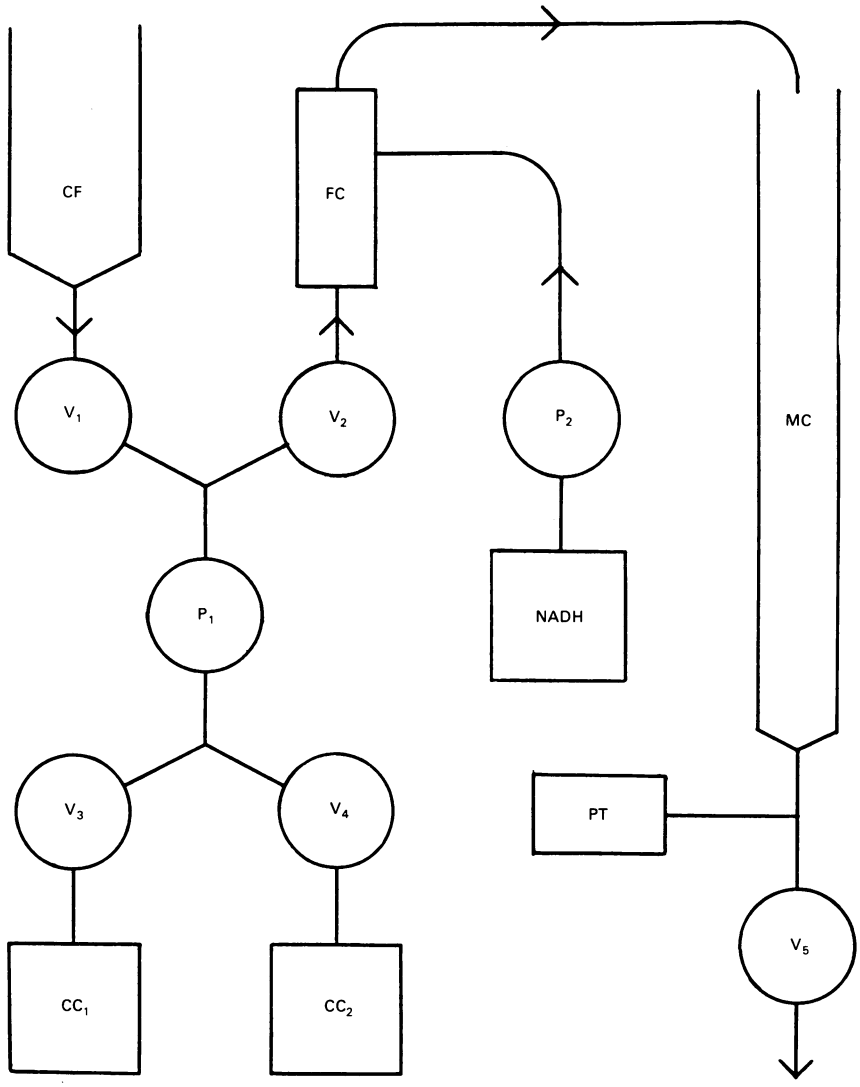

Fig. 1. *Coronary effluent monitoring equipment*

Coronary-venous effluent was collected in the funnel (CF), divided into fractions by the solenoid valve V_1 and processed in a cyclical fashion whereby radioactivity measurements in counting chamber 1 (CC_1) were overlapped with the emptying and re-filling of chamber 2 (CC_2), and vice versa. The pump P_1 was reversible and was used for all coronary-effluent transfers while the solenoid valves V_3 and V_4 selected the appropriate counting chamber. Each chamber contained a Centronics B12H all-glass halogen-quenched Geiger tube which was partially immersed in the spent perfusion medium. The counting efficiency was about 10% and the counting period was adjusted between 20 and 40s in order to accommodate variations in coronary flow rate without overfilling the chambers, although each individual experiment utilized a single counting interval. After counting, the liquid was pumped from either CC_1 or CC_2 via the solenoid valve V_2 into a fluorimeter cuvette (FC) for the lactate dehydrogenase assay before it finally passed into the flowmeter chamber (MC), where each fraction was weighed with a pressure transducer (PT). The solenoid valve V_5 was used to control the emptying of the flowmeter chamber. Coronary effluent entered the fluorimeter cuvette from below and overflowed at the top, thereby flushing the previous cuvette contents before each assay. The speed of pump P_1 was sufficient to transfer the whole of each effluent fraction and blow air bubbles when fluid transfer was complete. The bubbles were used to mix the cuvette contents with 0.5ml of 100 μM-NADH solution injected by pump P_2 immediately before each assay. The NADH concentration after mixing was 20–25 μM. Pyruvate was already present in the perfusion medium at a residual concentration of about 0.75mM and the cuvette temperature was 30°C. Fluorimetric assays on the previous counter fraction were overlapped with the second half of each new counter cycle while the other counting chamber was being re-filled.

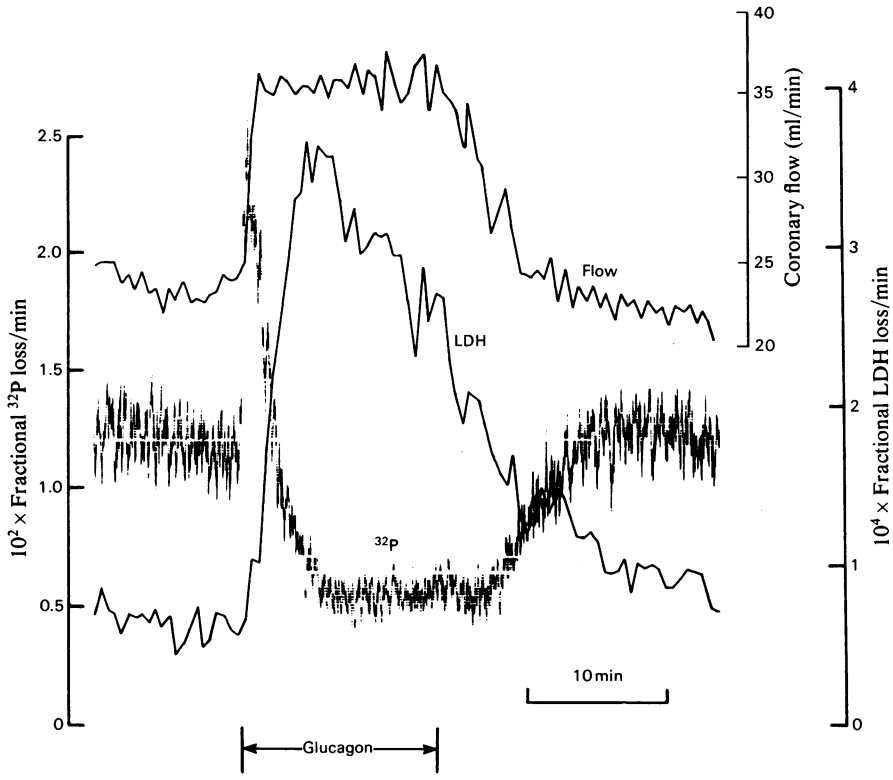

Fig. 2. *Effect of glucagon on cardiac ^{32}P efflux, LDH losses and coronary flow*
This experiment was performed on a Langendorff heart preparation. The effluent ^{32}P and LDH activities were divided by the total cardiac ^{32}P and LDH contents measured under the same conditions at the end of the experiment. Glucagon (2munits/ml) was infused for 15min as indicated by the arrows.

ments the effects produced by glucagon and the catecholamines were apparently reversible on return to the control medium.

The phosphomolybdate extractions showed that the effluent radioactivity was almost exclusively in the form of P_i. Under control conditions 97% of the radioactivity could be extracted into ethyl acetate without prior hydrolysis, and even after 5min perfusion with 75nM-isoprenaline (which doubled cardiac work output and coronary flow) 84% of the radioactivity could be extracted without prior hydrolysis. In contrast with this, less than 40% of the ^{32}P in the cardiac muscle samples could be directly extracted into the organic phase, and the remainder required hydrolysis. This shows that ATP and phosphocreatine were reasonably stable under our storage and extraction conditions, despite the presence of active cardiac enzymes in the frozen muscle samples, and the relatively high storage temperature.

The LDH losses presumably resulted from cellular damage at high work outputs, and it is conceivable that the transient episode of rapid ^{32}P efflux could arise through a similar mechanism. The temporal separation between the two peaks might be attributed to the need for more extensive lesions before LDH escape became possible. The total additional loss of ^{32}P over basal values during the transient episode of rapid efflux was about 5% of the total cardiac ^{32}P content. This may be compared with the total LDH losses during hormonal exposure, which were about 0.7% of the total cardiac enzyme content. Moreover, the phosphomolybdate extractions showed that the initial efflux peak produced by catecholamine treatment consisted almost exclusively of P_i, whereas a mixture of organophosphates would have been expected if cellular lysis made a significant contribution. In the light of these observations, it seems to us unreasonable to attribute the whole of the transient ^{32}P peak to cellular damage, and an additional explanation is therefore required.

We have previously demonstrated (Medina & Illingworth, 1980) that increases in cardiac work output can also produce transient episodes of rapid ^{32}P efflux. This effect was associated with increased LDH output, as shown in Fig. 3, but once again the fractional ^{32}P losses were much larger

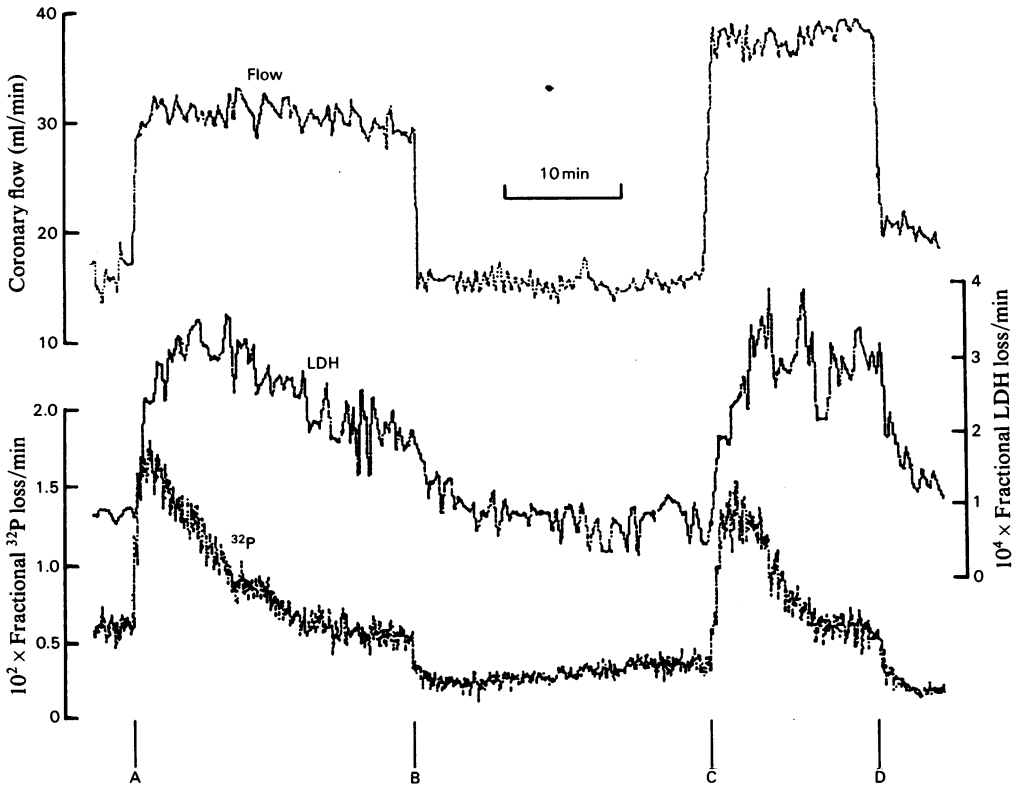

Fig. 3. *Effect of changes in work load on cardiac ^{32}P efflux, LDH losses and coronary flow*
This experiment was performed on a 'closed aorta' working heart preparation as described by Strong *et al.* (1979). After labelling, the heart was equilibrated for 20min at an atrial filling rate of 15ml/min before the start of the recording. This produced an initial aortic pressure of 10.7kPa (80mmHg). At point 'A' the atrial filling rate was increased to 30ml/min so as to yield an aortic pressure of 21.3kPa (160mmHg) until point 'B', when the atrial filling rate was restored to the control value of 15ml/min. The aortic pressure returned to 10.7kPa and was maintained at this value until point 'C', when a second work increase was initiated by raising the filling rate to 36ml/min. On this occasion the aortic pressure reached 22.7kPa (170mmHg) and was maintained near this value until point 'D', when control conditions were re-established.

than the fractional escape of LDH. Although the rate of ^{32}P efflux declined after extended periods at high work output, it never fell below the basal efflux rate under control conditions. This may be contrasted with the effects of glucagon and the catecholamines, where a true inhibition of ^{32}P efflux was eventually observed after prolonged exposure. Moreover, the reverse transition from high to low work load always inhibited ^{32}P efflux, whereas removal of the hormones increased the rate of efflux back to control values.

In a series of control experiments with a working heart preparation it was possible to demonstrate that the effects of high work load were primarily dependent on the peak systolic pressure developed by the heart, and that 'volume loading' at a constant aortic pressure had little effect on ^{32}P efflux. Other control experiments where the left ventricle was artificially drained showed that high coronary flow rates induced by high aortic pressures had no effect on ^{32}P output in the absence of left-ventricular pressure development.

In view of the similarities between the catecholamines and glucagon, it was decided to test the effect of dibutyryl cyclic AMP (N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate). Although this substance produced the expected inotropic response, it did not fully mimic the effects of either catecholamines or glucagon. Low doses of dibutyryl cyclic AMP increased both LDH output and coronary flow, but had no measurable effect on ^{32}P efflux (Fig. 4*a*). Higher doses of dibutyryl cyclic AMP produced a larger and more immediate effect on LDH release and coronary flow, and also increased ^{32}P efflux (Fig. 4*b*). In all cases the responses to dibutyryl cyclic AMP were clearly slower than the responses to variations in work load, catecholamines or glucagon, but this may

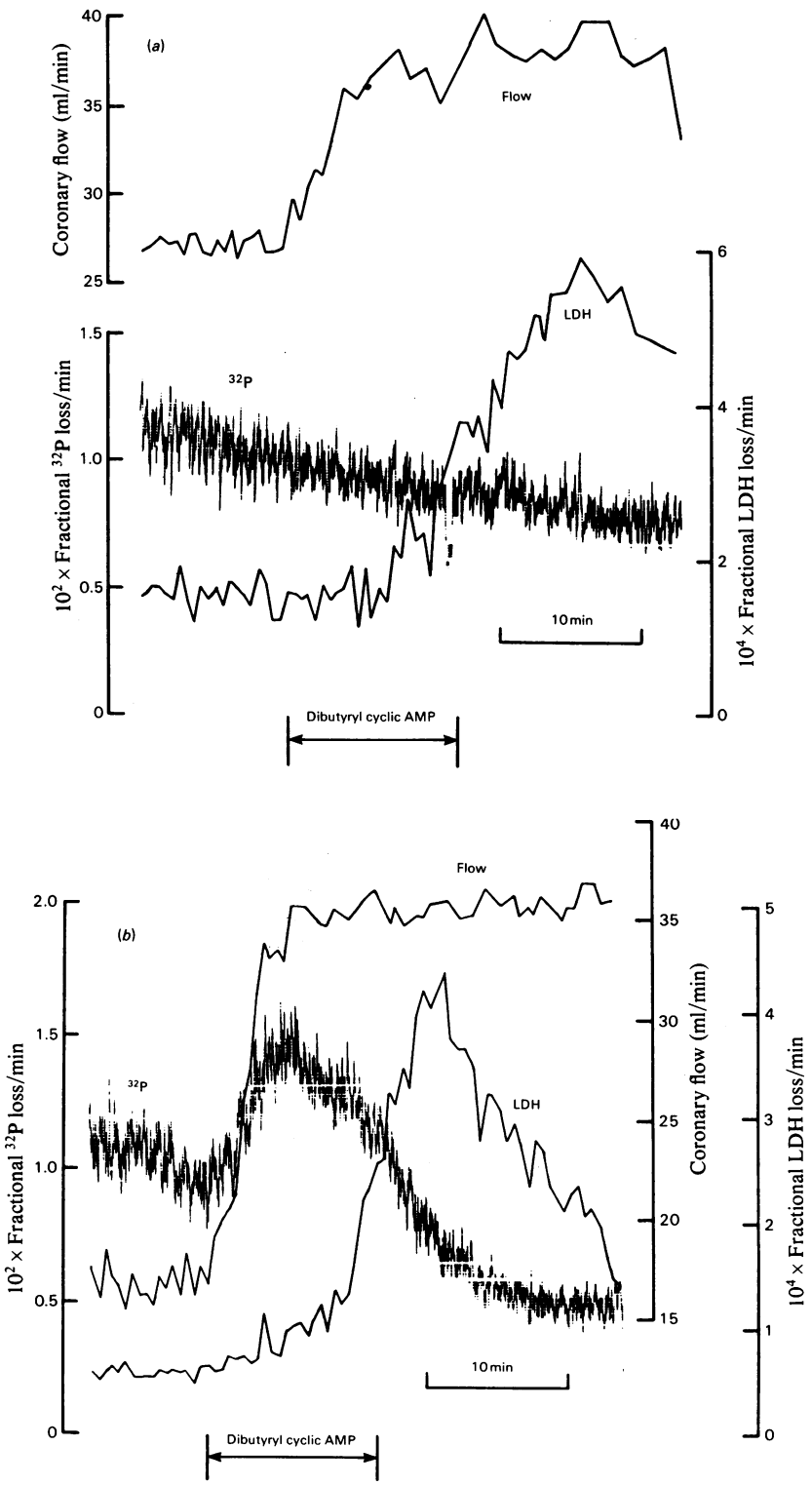

Fig. 4. *Effect of dibutyryl cyclic AMP on cardiac ^{32}P efflux, LDH losses and coronary flow*
Dibutyryl cyclic AMP (*a*, 120 μM; *b*, 193 μM) was infused for 12 min as indicated by the arrows. The other details were the same as in Fig. 2.

simply reflect the speed with which dibutyryl cyclic AMP was able to enter the cells. It seems reasonable to attribute some of the effects of dibutyryl cyclic AMP to the associated increase in work output, but this is not sufficient to explain the results with either catecholamines or glucagon.

Exposure to carbachol produced the expected negative inotropic effects, and with high doses contractions almost ceased for a short period. Nevertheless, the introduction of carbachol caused a transient episode of rapid ^{32}P efflux (5% of total cardiac content), which resembled the effect seen with the catecholamines and glucagon. Continued perfusion with carbachol resulted in a final ^{32}P efflux rate which was higher than the control value (Fig. 5), in contrast with the long-term inhibition produced by inotropic agents. Carbachol treatment had little or no effect on the rate of LDH output, which implies that the transient losses of ^{32}P were unconnected with cell lysis.

It has been suggested (George *et al.*, 1970) that some of the effects of acetylcholine might be mediated by cyclic GMP in cardiac muscle, and we therefore examined the effect of $N^2,O^{2'}$-dibutyryl-guanosine 3':5'-cyclic monophosphate (40 μM) on our preparation. This compound had no detectable effect on ^{32}P efflux, LDH release or contractile performance over a 20 min infusion period. This negative result might have been expected from our previous finding that sodium nitroprusside was without effect on ^{32}P efflux (Medina & Illingworth, 1980). Nitroprusside activates the soluble guanylate cyclase in cardiac muscle (Katsuki *et al.*, 1977) and produces a substantial increase in cyclic GMP in rat hearts, without any corresponding effect on protein kinase activity or contractile performance (Lincoln & Keely, 1981).

Insulin decreased the rate of cardiac ^{32}P efflux (Fig. 6), but in this case there were no obvious initial transient effects. If the insulin-exposure period was extended to 30 min, ^{32}P efflux partially recovered towards the control value before the hor-

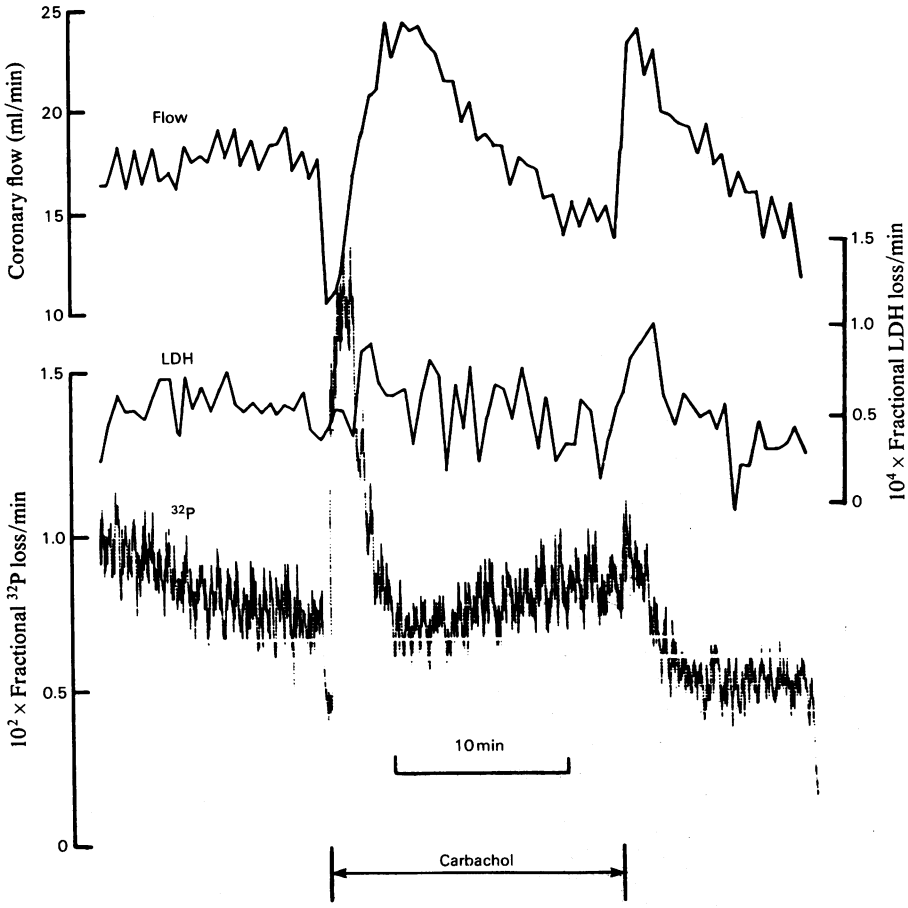

Fig. 5. *Effect of carbachol on cardiac ^{32}P efflux, LDH losses and coronary flow*
Carbachol (55 μM) was infused for 15 min as indicated by the arrows. The other details were the same as in Fig. 2.

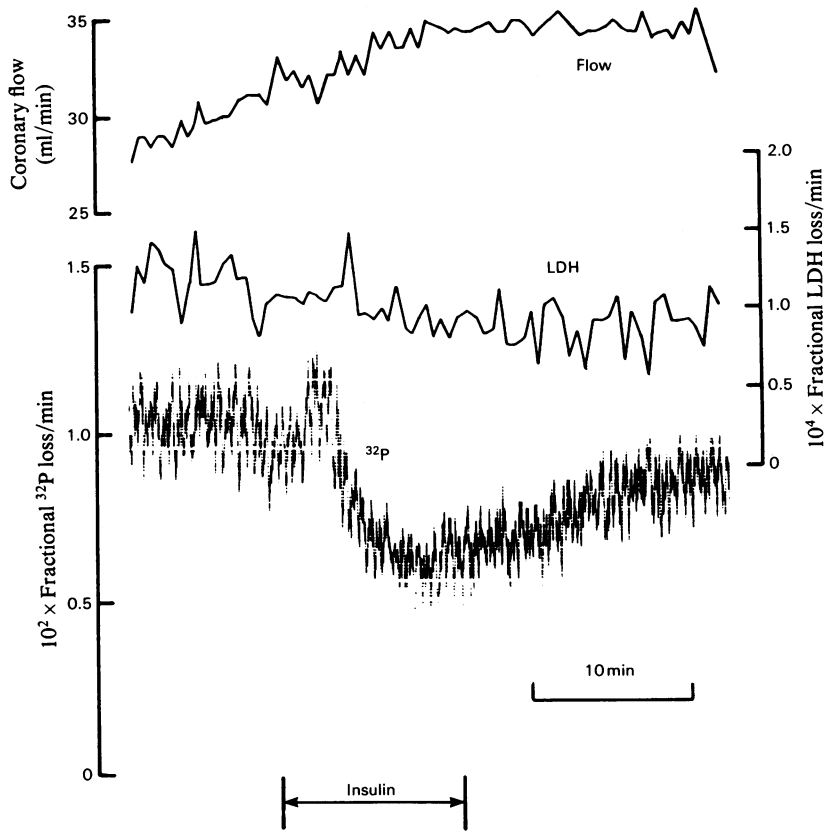

Fig. 6. *Effect of insulin on cardiac ^{32}P efflux, LDH losses and coronary flow*
Insulin (8 munits/ml) was infused for 12 min as indicated by the arrows. The other details were the same as in Fig. 2.

mone was removed. Insulin produced a slight vasodilation, but it showed no inotropic action, and it had a slightly inhibitory effect on the rate of LDH release. It is unlikely that the response to insulin could result from the metabolism of P_i to form glucose 6-phosphate within the tissue, since glucose was absent from the perfusion buffer, and the cardiac energy source was 1 mM-pyruvate. A similar biphasic effect of insulin on ^{32}P efflux was seen in other experiments when the perfusion buffer contained 10 mM-glucose.

Cortisol and corticosterone (2–200 μg/ml) produced an immediate stimulation of ^{32}P release from the tissue (Fig. 7), and in some cases LDH output also rose after a few minutes' delay. Corticosteroids had a very slight inotropic effect. ^{32}P output declined towards control values during continued exposure to the steroids, and withdrawal of the hormones gave rise to a 'mirror image' response which resembled the effects produced by changes in work load (Fig. 3). Aldosterone (0.07–10 μg/ml) resembled the corticosteroids in its effects, but the response was very small.

Vasopressin (Sigma, V5501: 0.1–10 munits/ml) and angiotensin (Sigma, A9525: 1–125 ng/ml) produced a transient and somewhat variable stimulation of ^{32}P efflux, but the rate returned to basal values after a few minutes' exposure to the hormone. Tri-iodothyronine (29–120 ng/ml) slightly inhibited ^{32}P output. Adenosine (2.5 μM), histamine (71–1400 ng/ml), spermidine (1–3 mM), prolactin (0.3 μg/ml), parathyroid substance (Sigma, P0892: 125–800 munits/ml), calcitonin (14–171 munits/ml), des-Asp^1-angiotensin (Sigma, A0903: 71–2500 pg/ml) and somatostatin (14–40 ng/ml) did not produce any significant effects on ^{32}P efflux under our experimental conditions.

Transient episodes of supranormal P_i efflux have been previously observed during glucose-induced insulin release from pancreatic islets (Freinkel *et al.*, 1978), and it may therefore be suspected that the initial P_i-efflux peak in Figs. 2, 3, 5 and 7 reflects some generalized change in phosphorus metabolism which will be found in other tissues responding to a sudden external stimulus. An unusually rapid turnover of inositol phospholipids has

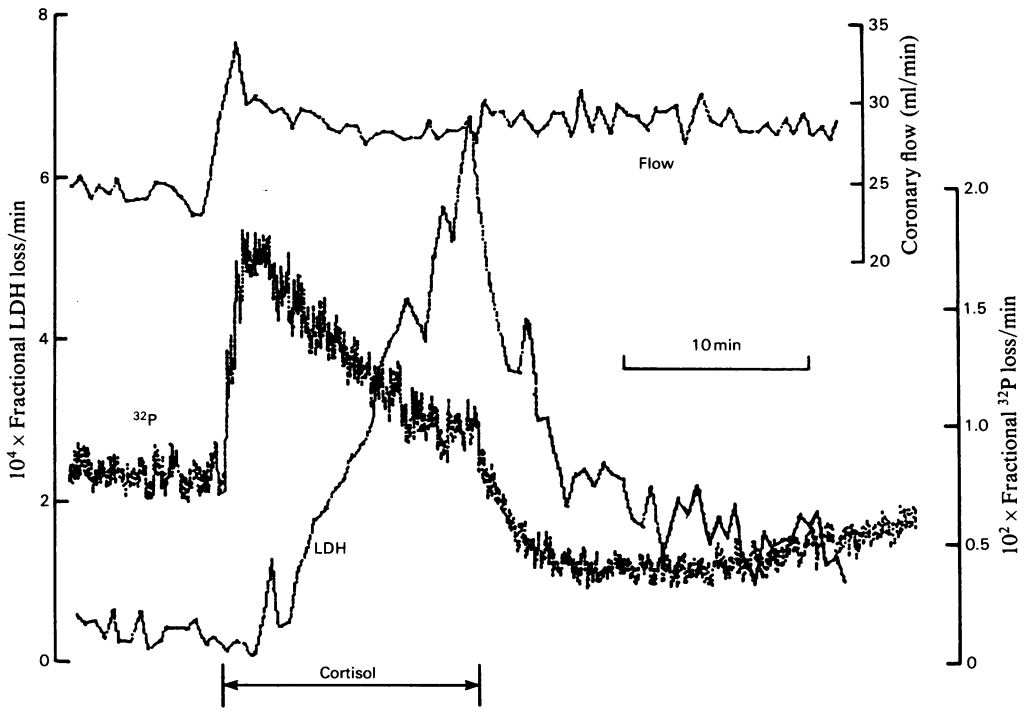

Fig. 7. *Effect of glucocorticoids on cardiac ^{32}P efflux, LDH losses and coronary flow*
Cortisol (200 μg/ml) was infused for 14 min as indicated by the arrows. The other details were the same as in Fig. 2.

been demonstrated in many tissues subjected to hormonal stimulation (Michell, 1975), although the size of the phosphatidylinositol pool is not sufficient to account for the substantial quantities of P_i (up to 5 μmol/g dry wt.) which may be lost during the initial transient response. Our results strongly suggest that most of the radioisotope leaving the heart is always in the form of P_i, although it is difficult to rule out the possible intracardiac breakdown of escaping organophosphates in view of the high activity of alkaline phosphatase in the capillary endothelium (Hudlická, 1982) and 5′-nucleotidase in both the capillary endothelium and the cardiac plasmalemma (Rubio *et al.*, 1973).

In addition to the ventricular cardiomyocytes, which constitute the bulk of the preparation, perfused hearts also contain atrial muscle, connective tissue, nerve endings, vascular smooth muscle and the endothelial cells lining the coronary vascular bed. It is conceivable that one or more of these minor cell populations might be partly responsible for the transient efflux peaks, although their small mass must preclude any substantial contribution to the sustained responses. On the whole we think it unlikely that there was any large contribution from the vascular system, since there was little correlation between the changes in ^{32}P or LDH efflux and alterations in vascular tone, whether these were produced by chemical agents or by changes in perfusion pressure in the absence of ventricular pressure development.

Our previous work (Medina & Illingworth, 1980) suggested that at least part of the P_i flux across the cardiac plasmalemma was linked to the simultaneous movement of Na^+, as is known to be the case in several other tissues. The simplest hypothesis consistent with our data involved the slow, reversible, electroneutral transport of NaH_2PO_4 across the plasmalemma, with a single carrier mediating both uptake and export processes. This implies that cardiac glycosides such as ouabain should accelerate P_i efflux, since these compounds elevate the intracellular [Na^+] by inhibiting the $Na^+ + K^+$-dependent ATPase (Grupp *et al.*, 1984). This is indeed the case: 25 μM-ouabain (which under our experimental conditions had little or no inotropic effect) increased ^{32}P efflux by about 50%.

Conversely, interventions which lower the intracellular [Na^+] should depress the P_i-efflux rate, as we have previously observed with low-Na^+ perfusion media (Medina & Illingworth, 1980). Wasserstrom *et al.* (1982) have reported that catecholamines decrease the intracellular [Na^+] in dog heart by stimulating the Na^+ pump, and this may well explain the long-term inhibition of P_i efflux observed after catecholamine or glucagon treatment. Positive inotropic agents might otherwise be

expected to raise the internal [P_i] (and hence elevate the efflux rate) as a result of increased ATP utilization. In practice all the inotropic agents tested actually produced a fall in the long-term efflux rate after the initial transient peak.

Changes in the intracellular pH are also known to modulate P_i-efflux rates, which fall as the intracellular pH rises (Medina & Illingworth, 1980). It has been suggested that insulin stimulates the $Na^+ + K^+$-dependent ATPase in target cells and accelerates Na^+/H^+ exchange across the plasmalemma (Moore, 1983). The expected fall in the intracellular [Na^+] and rise in intracellular pH have been demonstrated in rat soleus (Moore *et al.*, 1983) and frog skeletal muscle (Fidelman *et al.*, 1982). Both effects should decrease the rate of P_i efflux, as we in fact observed after insulin exposure (Fig. 6), but at present we have no explanation for the subsequent partial recovery.

Insulin binding to cell-surface receptors is thought to be followed by internalization of the receptors and their subsequent degradation (Knutson *et al.*, 1983). In other tissues this 'down-regulation' of insulin receptor numbers normally takes several hours, and it therefore seems unlikely that it could account for the self-limiting effect of insulin on cardiac P_i export which we observed in our experiments.

Our data suggest that corticosteroids may produce rapid changes in the intracellular ionic composition which would be the converse of those produced by insulin. Despite the extensive literature relating to aldosterone, there are relatively few reports on the action of corticosteroids on intracellular [Na^+] in non-renal tissue. However, Streeten & Moses (1968) have shown that physiological doses of cortisol rapidly stimulate Na^+ uptake (but not efflux) by erythrocytes from adrenalectomized dogs, and it would be of interest to examine the effect of corticosteroids on cardiac Na^+ fluxes. It is possible that all the hormonal effects described in the present paper may eventually be explained by changes in the intracellular [Na^+], [H^+] and [P_i], so that there would be no need to postulate a direct hormonal effect on the P_i-transport system itself. There are some difficulties with this simple theory, since alterations in the intracellular pH are known to have an immediate effect on contractile performance (Poole-Wilson & Langer, 1975) and any change in the intracellular [Na^+] would be expected to alter myocardial contractility as a result of the operation of the Na^+/Ca^{2+}-exchange system (Langer, 1982). These expected variations in contractility were not always observed in our experiments.

Insulin, cortisol, and especially glucagon, catecholamines and carbachol all produced significant long-term changes in cardiac P_i efflux, which might be expected to cause a substantial alteration in the intracellular [P_i] and a major redistribution of phosphorus metabolites within about 30min. Long-term changes in cardiac glycogen and other metabolites could well be mediated through such a mechanism. It may be significant that catecholamines and carbachol had opposite effects on the long-term P_i-efflux rate, since these compounds produce antagonistic effects on cardiac function in the intact animal. The difference between the responses to insulin and cortisol is interesting for the same reason. It is obviously necessary to determine whether any of the metabolic effects of these important hormones and drugs can be attributed to their actions on P_i fluxes, or whether the changes in P_i efflux are secondary to a pre-existing metabolic action.

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