Calcium Metabolism in Rat Hepatocytes

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1. The total calcium concentration in rat hepatocytes was $7.9 \mu\text{g-atoms/g}$ dry wt.; 77% of this was mitochondrial. Approx. 20% of cell calcium exchanged with ^{45}Ca within 2 min. Thereafter incorporation proceeded at a low rate to reach 28% of total calcium after 60 min. Incorporation into mitochondria showed a similar time course and accounted for 20% of mitochondrial total calcium after 60 min. 2. The α -adrenergic agonists phenylephrine and adrenaline + propranolol stimulated incorporation of ^{45}Ca into hepatocytes. Phenylephrine was shown to increase total calcium in hepatocytes. Phenylephrine inhibited efflux of ^{45}Ca from hepatocytes perfused with calcium-free medium. 3. Glucagon, dibutyl cyclic AMP and β -adrenergic agonists adrenaline and 3-isobutyl-1-methylxanthine stimulated calcium efflux from hepatocytes perfused with calcium-free medium. The effect of glucagon was blocked by insulin. Insulin itself had no effect on calcium efflux and it did not affect the response to dibutyl cyclic AMP. 4. Incorporation of ^{45}Ca into mitochondria in hepatocytes was stimulated by phenylephrine and inhibited by glucagon and by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The effect of glucagon was blocked by insulin. 5. Ionophore A23187 stimulated hepatocyte uptake of ^{45}Ca , uptake of ^{45}Ca into mitochondria in hepatocytes and efflux of ^{45}Ca into a calcium-free medium.

In rat parotid acinar cells α -adrenergic and cholinergic effectors stimulated ^{45}Ca uptake and inhibited ^{45}Ca efflux from the cells into a calcium-free medium. Methylxanthine inhibitors of cyclic AMP phosphodiesterase and β -adrenergic effectors (which activate adenylate cyclase) had no effect on ^{45}Ca uptake in parotid cells. These agents stimulated ^{45}Ca efflux into a calcium-free medium, inhibited ^{45}Ca uptake into mitochondria in parotid pieces, and stimulated loss of ^{45}Ca from mitochondria in parotid pieces incubated in a calcium-free medium (Kanagasuntheram & Randle, 1976).

The limited amounts of parotid in rats make it rather unsuitable for a study of mechanisms involved in the hormonal regulation of calcium metabolism. Preliminary studies with hepatocytes suggested that the pattern of responses might be similar to those of parotid. Phenylephrine (an α -adrenergic agonist) stimulated ^{45}Ca uptake (S. Foden, cited by Kanagasuntheram & Randle, 1976) and it was known from the work of Friedman & Park (1968) that glucagon may stimulate calcium efflux into a calcium-free medium in perfused rat liver. We describe here the effects of α - and β -adrenergic agonists, glucagon, insulin, dibutyl cyclic AMP and methylxanthines on calcium uptake, calcium efflux and mitochondrial exchangeable calcium in rat hepatocytes.

Experimental

Materials

Collagenase (type II) and bovine plasma albumin (fraction V) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The albumin was defatted as described by Chen (1967), equilibrated with Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing 1.2 mm-CaCl_2 by overnight dialysis, and stored frozen. Hyaluronidase was from Miles Laboratories, Slough, Bucks., U.K. Other reagents and enzymes were from Boehringer Corp. (London) Ltd., London W.5, U.K., or from Sigma, except for the following. Sephadex G-10, from Pharmacia, London W5 5SS, U.K., was acetylated by the method of Reed (1973) and stored in aq. 50% (v/v) ethanol. Ionophore A23187 and glucagon were gifts from Dr. R. L. Hamill and Dr. Mary Root of Eli Lilly and Co., Indianapolis, IN, U.S.A. Ruthenium Red, adrenaline and Trypan Blue were from British Drug Houses, Poole, Dorset, U.K. Isoproterenol was from Kodak, Kirkby, Liverpool, U.K. Phentolamine mesylate was from Ciba Laboratories, Horsham, West Sussex RH12 4AB, U.K. Insulin (six times recrystallized), was a gift from Boots Drug Co., Nottingham, U.K. 3-Isobutyl-1-methylxanthine was from Aldrich

Chemical Co., Gillingham, Dorset SP8 4JL, U.K. Radiochemicals $^{45}\text{CaCl}_2$ and (0.4 Ci/mmol) $[6,6\text{'-}^3\text{H}]\text{-sucrose}$ (2.4 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Preparation of hepatocytes. Hepatocytes were prepared from male albino wistar rats of approx. 300g. Except in a few stated cases there was free access to food and water. The method of preparation was essentially the perfusion method of Berry & Friend (1969) as modified by Cornell *et al.* (1973). The basic perfusion medium was 150ml of Ca^{2+} -free Krebs-Henseleit bicarbonate buffer containing 30mg of collagenase (type II). Initial experiments also included 10mg of hyaluronidase, which was subsequently omitted in preference for digestion with collagenase only. Precise details of digestion are given in the Tables. Perfusion was carried out in a thermostatically controlled cabinet maintained at 37°C , and at a flow rate of 30ml/min. The medium was kept in equilibrium with O_2/CO_2 (19:1) throughout.

The percentage of intact cells could be estimated at this stage by exclusion of Trypan Blue, viewed under an optical microscope.

Incubation and perfusion of hepatocytes. For measurements of spaces and metabolites, cells were incubated at 37°C with shaking (60 cycles/min) in 50ml polythene bottles sealed with serum caps. The bottles were gassed with O_2/CO_2 (19:1) at the beginning of incubation and again if the serum caps were removed during the course of the experiment. The basic medium for incubation or perfusion was bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 1.2mM- CaCl_2 (standardized by atomic absorption spectrophotometry), 1.2% bovine plasma albumin, 25mM-sucrose and 10mM-lactate, gassed with O_2/CO_2 (19:1). The final concentration of cells for incubation was approx. 5mg dry wt./ml. Any further additions are given in the appropriate text, Tables and Figures.

For measurements of Ca efflux a perfusion system was used in a constant-temperature cabinet maintained at 37°C (Kanagasuntheram & Randle, 1976).

Spaces. For measurement of wet and dry weights of hepatocytes, an extracellular correction was applied by using $[6,6\text{'-}^3\text{H}]\text{-sucrose}$ as an extracellular marker. Cells were incubated with $[^3\text{H}]\text{-sucrose}$ (2 $\mu\text{Ci/ml}$). Duplicate samples were centrifuged for 10s in an Eppendorf 3200 centrifuge. The supernatant of one sample was aspirated and retained for assay of radioactivity. The pellet was resuspended in water and transferred quantitatively for assay of radioactivity in methoxyethanol/toluene-based scintillator (Severson *et al.*, 1974). The scintillator was adequate to disrupt the cells. The supernatant of the other sample of the duplicate was aspirated and discarded.

The wet pellet was weighed, dried by vacuum desiccation and weighed again. By applying the extracellular water correction obtained by using $[^3\text{H}]\text{-sucrose}$, estimates for the wet and dry weights were made. A similar procedure was adopted for measurement of ^{45}Ca 'spaces' by incubating hepatocytes with $[6,6\text{'-}^3\text{H}]\text{-sucrose}$ (2 $\mu\text{Ci/ml}$) and $^{45}\text{CaCl}_2$ (0.2 $\mu\text{Ci/ml}$). Radioactivity was assayed in the medium and in all extracts by dual-isotope liquid-scintillation spectrometry, with the use of an external standard for quench corrections.

Preparation of mitochondria for measurement of exchangeable calcium. Hepatocytes were incubated at approx. 8mg dry wt./ml in incubation medium containing 0.5 μCi of $^{45}\text{CaCl}_2/\text{ml}$ (0.2 $\mu\text{Ci/ml}$) $[6,6\text{'-}^3\text{H}]\text{-sucrose}$. At the end of incubation they were packed by centrifuging at 1000g for 15s in an MSE Super Minor centrifuge. The supernatant was aspirated and retained for assay of radioactivity. The mitochondrial preparation medium was modified from that of Hodarnau *et al.* (1973), and contained 192mM-mannitol/59mM-sucrose/2mM-Tris/HCl/0.5% bovine plasma albumin/2mM-EGTA/5 μg of Ruthenium Red/ml (pH 7.4). A sample (1ml) of this was added to the cell pellet followed by immediate disruption of the cells with a Polytron PT10 homogenizer (5s on position 2.5). The homogenate was centrifuged at 4°C for 10min at 2000g and the supernatant removed and centrifuged at 4°C for 10min at 10000g. The pellet was resuspended in about 20ml of the same medium and the low- and high-speed spins were repeated. The final mitochondrial pellet was taken up in 0.1M-Tris buffer (pH 7.5) and disrupted by freezing and thawing (see under 'Analytical methods'). Samples were taken for measurement of radioactivity and for citrate synthase activity.

Mitochondrial exchangeable calcium is given as nmol of Ca/g dry wt. of cells. This is calculated from the specific radioactivity of the extracellular calcium and from values for citrate synthase obtained from mitochondria and intact cells.

Analytical methods. Protein was assayed in extracts of cells and mitochondria by the method of Gornall *et al.* (1949). Correction was made for albumin present in appropriate cases. Glucose was measured spectrophotometrically by the method of Slein (1962). Cells were taken into HClO_4 (5%, v/v) and the supernatant was clarified by centrifuging in an Eppendorf 3200 centrifuge. Samples (10 μl) were taken for assay without neutralization. ATP was assayed by the luciferin/luciferase method (Stanley & Williams, 1969). Similar HClO_4 extracts were used. Citrate synthase was assayed by a modification of the method of Srere *et al.* (1963), as described by Coore *et al.* (1971).

Mitochondrial extracts were prepared by freezing and thawing (liquid N₂) three times. Cell extracts were prepared by freezing and by ultrasonic disintegration in 0.1 M-Tris/HCl buffer, pH 7.4. Ca²⁺ was determined on HNO₃ digests of cells and mitochondria as described by Severson *et al.* (1974). Recordings were made on a Perkin-Elmer atomic absorption spectrophotometer.

Results and Discussion

Composition and hormone responsiveness of rat hepatocytes

Table 1 (lines 1–3) shows concentrations of water, dry solids and protein in representative samples of hepatocytes used in the present study. In other Tables and Figures data are expressed per g dry wt. of tissue. In general, wet weight and protein were measured for each batch of hepatocytes and dry weights were calculated from the conversion factors given in this Table. The data given in Table 1 will allow recalculation per g wet wt. or per g of protein for comparison with other data in the literature. Table 1 (line 5) shows whole-cell citrate synthase;

mitochondrial citrate synthase (results not shown) was 0.1 unit (μmol/min)/mg of mitochondrial protein. The concentration of mitochondrial protein in hepatocytes (line 6, Table 1) was calculated from these citrate synthase data. In measurements of hepatocyte mitochondrial calcium, citrate synthase was used to determine mitochondrial recovery and to calculate concentrations per g dry wt. of cells.

Hepatocyte total calcium in the present study was 2 μg-atoms of Ca/g wet wt. of cells (line 4, Table 1); this agrees with the estimate of 2.3 μg-atoms of Ca/g wet wt. given by Assimacopoulos-Jeannet *et al.* (1977). Mitochondrial total calcium (line 7, Table 1) accounted for 77% of cell calcium; the location of the non-mitochondrial calcium is not known. Approx. 28% of hepatocyte calcium exchanged with extracellular ⁴⁵Ca during 1 h of incubation; most of this exchange occurred in the first 2 min (lines 4 and 8 Table 1; Fig. 1). Approx. 20% of mitochondrial calcium exchanged with extracellular ⁴⁵Ca during 60 min of incubation and the major part of this exchange occurred in the first 2 min (lines 7 and 9 Table 1; Fig. 1). Mitochondrial exchangeable calcium accounted for 55% of hepatocyte exchange-

Table 1. *Some aspects of the composition of rat hepatocytes*

Isolated hepatocytes were prepared by digestion with collagenase only. They were preincubated in basic incubation medium at 37°C for 30 min at 5 mg dry wt./ml. followed by incubation at 37°C for 60 min in similar medium containing [6,6-³H]sucrose (2 μCi/ml) or labelled sucrose and ⁴⁵Ca (0.2 μCi/ml) in experiments measuring exchangeable Ca. For whole-cell measurements rapid separation of cells from medium was achieved by centrifuging for 10 s in an Eppendorf 3200 centrifuge. The supernatant was aspirated and retained for assay of labelled sucrose ± ⁴⁵Ca. Water and solids in the cell pellet were estimated by weighing before and after vacuum desiccation and protein was assayed by the biuret method (Gornall *et al.*, 1949). Extracellular corrections were applied from the [³H]sucrose space. Total cell calcium was measured by resuspending the cell pellets in 0.2 ml of water, assaying one sample for [³H]sucrose and digesting the remainder with HNO₃. Calcium was measured by atomic absorption spectrophotometry. Citrate synthase was assayed by extraction of the cell pellets by freezing and thawing (three times) and by ultrasonic disintegration in Tris buffer (Srere *et al.*, 1963, modified by Coore *et al.*, 1971). For mitochondrial measurements cells were separated from the medium by centrifugation for 15 s in an MSE Super Minor. The supernatant was aspirated and retained, and the cells were resuspended in mitochondrial preparation medium and broken with a Polytron PT10 homogenizer. Mitochondria were isolated and washed by differential centrifugation, resuspended in 0.1 M-Tris/HCl, pH 7.5, and disintegrated by freezing and thawing (three times) for assay of citrate synthase, protein and ⁴⁵Ca and [³H]sucrose. Total Ca was measured by atomic absorption after digestion with HNO₃. For further details, see the Experimental section. Values are means ± S.E.M. for the numbers of observations given in parentheses.

Parameter	Hepatocyte composition based on:		
	g dry wt.	g wet wt.	g of cell protein
Protein concn. (mg)	700 ± 10	190 ± 2.8	—
Dry solids (mg)	—	270 ± 5	—
Water (ml)	2.7 ± 0.16	0.73 ± 0.04	3.9 ± 0.23
Total cell Ca (μg-atoms)	7.9 ± 0.4	2.0 ± 0.1	11.3 ± 0.57
Citrate synthase (units)	35.3	9.6	50.4
Mitochondrial protein (mg)	353	96	504
Mitochondrial total Ca (μg-atoms)	6.1 ± 0.6	1.6 ± 0.16	8.7 ± 0.87
Cell exchangeable Ca (μg-atoms of medium Ca)	2.2 ± 0.04 (94)	0.4 ± 0.02 (94)	3.1 ± 0.07 (94)
Mitochondrial exchangeable Ca (μg-atoms of medium Ca)	1.2 ± 0.05 (10)	0.33 ± 0.01 (10)	1.7 ± 0.07 (10)

Fig. 1. Time course of ^{45}Ca uptake into hepatocytes (whole-cell and mitochondrial fraction)

Rat hepatocytes were prepared with collagenase (no hyaluronidase), preincubated for 20 min in basic medium and incubated for the time shown in basic medium with $[6,6\text{'-}^3\text{H}]\text{sucrose}$ ($2\mu\text{Ci/ml}$) plus ^{45}Ca ($0.2\mu\text{Ci/ml}$). For assay of whole-cell ^{45}Ca , duplicate samples (1 ml) were centrifuged for 10 s in an Eppendorf 3200 centrifuge and the supernatant was aspirated and retained and the pellets were resuspended in water. Radioactivity (^3H and ^{45}Ca) was assayed in medium and cells by dual-isotope liquid-scintillation spectrometry. For assay of mitochondrial ^{45}Ca , cells were separated by centrifuging for 15 s at 1000g, the supernatant was aspirated and assayed for ^3H and ^{45}Ca . The cell pellet was immediately resuspended in mitochondrial preparation medium and disrupted with a Polytron PT10 homogenizer. The mitochondria were separated and washed by differential centrifugation, disrupted by freezing and thawing and assayed for citrate synthase and ^3H and ^{45}Ca . Cell samples were taken for wet weight, protein and citrate synthase determination. Data for total cell exchangeable calcium were assembled in a single experiment. Data for mitochondrial exchangeable Ca were assembled in the course of a number of separate experiments; there were at least two observations at each point. For other details see the Experimental section. ●, Whole-cell exchangeable calcium; ○, mitochondrial exchangeable calcium.

able calcium (lines 8 and 9 Table 1). Data given in lines 4, 7, 8 and 9 of Table 1 indicate that 55% of non-mitochondrial calcium exchanged with extracellular ^{45}Ca .

The rate of gluconeogenesis from 10 mM-L-lactate by a representative sample of hepatocytes ranged from 2.4 to $3.6\mu\text{mol/min per g dry wt.}$ and averaged $2.9 \pm 0.19\mu\text{mol/min per g dry wt.}$ (mean $\pm$ S.E.M. for six observations). The concentration of ATP in a representative sample of hepatocytes averaged

$6.3 \pm 0.15\mu\text{mol/g dry wt.}$ (Table 2). These values for gluconeogenesis rate and ATP concentration are within the range of those generally obtained (e.g. see Wilson *et al.*, 1974; Cornell *et al.*, 1973). Table 2 shows that phenylephrine, isoproterenol, adrenaline, glucagon and dibutylryl cyclic AMP stimulated glucose production by hepatocytes incubated with 10 mM-L-lactate. The purpose of these experiments was to check that the cells were metabolically responsive to these hormones. No attempt was made to differentiate between glycogenolysis and gluconeogenesis. Table 2 also shows that phenylephrine, isoproterenol, adrenaline and glucagon had no effect on hepatocyte ATP concentration.

Effects of hormones, ionophore A23187, dibutylryl cyclic AMP and glucose on ^{45}Ca uptake and total calcium (phenylephrine only) in rat hepatocytes

Time course of ^{45}Ca uptake. In these experiments freshly prepared hepatocytes were preincubated for 30 min in basal medium (which contained 10 mM-L-lactate as substrate) before addition of ^{45}Ca and the start of incubation. This procedure was adopted for two reasons. It is known that hepatocytes do not attain steady rates of gluconeogenesis from lactate for 30 min (Cornell *et al.*, 1974) and it seemed desirable to achieve steady-state rates of metabolism. Also preliminary experiments showed that shorter periods of preincubation resulted in lower and more variable rates of ^{45}Ca uptake (results not shown). The use of this period of preincubation presumably contributes to the higher rates of ^{45}Ca uptake in our experiments as compared with those of Assimacopoulos-Jeannet *et al.* (1977). Inclusion of hyaluronidase during preparation of hepatocytes did not affect the characteristics of Ca^{2+} uptake. It did, however, affect the ability of the cells to retain Ca^{2+} in the Ca^{2+} -efflux studies. The uptake of ^{45}Ca by rat hepatocytes showed a very rapid initial phase (less than 2 min) followed by a much lower and prolonged rate of incorporation (Fig. 1, closed circles). The initial rapid incorporation was $1.6\mu\text{g-atoms of medium calcium/g dry wt. of cells in 2 min.}$ The actual rate was presumably much higher, but measurements over shorter time periods were impracticable. Thereafter incorporation was slow and apparently linear ($0.01\mu\text{g-atom of medium calcium/min per g dry wt.}$). The initial rapid uptake presumably represented exchange between ^{45}Ca in the medium and unlabelled calcium in the cell; it accounted for 20% of cell calcium. The slow incorporation may represent exchange or net uptake; this could not be ascertained as its extent over 60 min ($0.4\mu\text{g-atom/g dry wt.}$) only represented 5% of cell calcium.

This pattern of uptake is very similar to that observed previously in rat adipocytes and rat parotid acinar cells (Severson *et al.*, 1976; Kanaga-

Table 2. Effects of hormones and dibutyl cyclic AMP on glucose production and ATP concentrations of rat hepatocytes
Isolated hepatocytes were prepared with collagenase and hyaluronidase. They were preincubated in basic incubation medium for 30 min at 37°C at 5 mg dry wt./ml and then incubated in similar medium at 37°C for 60 min. In all cases where glucose was measured, sucrose was omitted from the incubation media throughout. Duplicate samples (1 ml) were taken into 20 µl of 70% HClO₄, mixed and cooled to 0°C. The supernatant was clarified by centrifugation in an Eppendorf 3200. Glucose was determined spectrophotometrically on 10 µl samples without neutralization by the method of Slein (1962). ATP was measured by luciferin/luciferase (Stanley & Williams, 1969). Protein and weights of water and dry solids in cell pellets were estimated as in Table 1. For other details see the Experimental section. Values are means ± S.E.M. for the numbers of observations given in parentheses. * $P < 0.01$ versus control. ** $P < 0.01$ versus control on a paired basis. Other differences for experimental versus control $P < 0.05$.

Experimental	Glucose production (µmol/min per g dry wt.)		ATP concn. (µmol/g dry wt.)	
	Control	Experimental	Control	Experimental
Phenylephrine (20 µM)	11.2 ± 1.18 (5)	14.0 ± 0.44 (6)**	6.3 ± 0.15 (4)	6.4 ± 0.07 (4)
Isoproterenol (20 µM)	11.2 ± 1.18 (3)	18.2 ± 3.26 (4)**	6.3 ± 0.15 (4)	6.7 ± 0.07 (4)
Adrenaline (5 µM)	2.9 ± 0.22 (4)	17.2 ± 0.37 (4)*	6.3 ± 0.15 (4)	6.4 ± 0.22 (4)
Glucagon (1.4 µM)	2.9 ± 0.22 (4)	9.8 ± 0.74 (4)*	—	—
(10 nM)	—	—	6.3 ± 0.15 (4)	6.9 ± 0.30 (4)
Dibutyl cyclic AMP (50 µM)	2.7 ± 0.09 (2)	6.2 ± 0.07 (2)*	—	—

Table 3. Effects of hormones, ionophore A23187, dibutyl cyclic AMP and glucose on ⁴⁵Ca uptake by rat hepatocytes
Isolated hepatocytes were prepared with collagenase and hyaluronidase. They were preincubated in incubation medium at 37°C for 30 min at 5 mg dry wt./ml and then incubated for 60 min at 37°C in similar medium containing [6,6'-³H]-sucrose (2 µCi/ml) and ⁴⁵Ca (0.2 µCi/ml). Other additions were made at the end of preincubation. Duplicate samples (1 ml) were centrifuged for 10 s in an Eppendorf 3200. The supernatant was aspirated and retained and the pellet resuspended in water. Samples were then assayed for ³H and ⁴⁵Ca by dual-isotope liquid-scintillation spectrometry. ⁴⁵Ca incorporations were corrected for ⁴⁵Ca in the extracellular fluid by using the sucrose space and assuming the specific radioactivity of the extracellular fluid to be the same as that of the incubation medium. Values are means ± S.E.M. for the numbers of observations given in parentheses. * $P < 0.001$; ** $P < 0.01$; *** $P < 0.05 > 0.02$ versus control. Other differences for experimental versus control $P > 0.05$. For other details, see the Experimental section.

Experiment	Concn. (M)	Ca uptake (µg-atoms of medium Ca/h per g dry wt.)	
		Control	Experimental
Phenylephrine	2×10^{-5}	2.29 ± 0.04 (10)	3.17 ± 0.06 (10)*
Adrenaline, alone	5×10^{-6}	1.72 ± 0.06 (20)	2.71 ± 0.05 (24)*
Propranolol	2×10^{-5}	2.03 ± 0.38 (5)	2.08 ± 0.03 (3)
Adrenaline + propranolol	As above	2.03 ± 0.38 (5)	3.02 ± 0.08 (5)***
Phentolamine	2×10^{-5}	1.04 ± 0.16 (8)	1.07 ± 0.19 (6)
Adrenaline + phentolamine	As above	1.04 ± 0.16 (8)	1.24 ± 0.09 (10)
Isoproterenol	2×10^{-5}	1.86 ± 0.08 (13)	1.69 ± 0.08 (13)
Dimethyl sulphoxide	3.2×10^{-2}	2.06 ± 0.12 (18)	2.01 ± 0.13 (18)
Ionophore A23187 (in dimethyl sulphoxide)	10^{-5}	2.01 ± 0.13 (18)	4.10 ± 0.18 (18)*
Glucagon	$\left\{ \begin{array}{l} 6 \times 10^{-7} \\ 1.5 \times 10^{-6} \end{array} \right\}$	1.88 ± 0.10 (6)	2.01 ± 0.12 (4)
Insulin	10^{-9}	3.59 ± 0.16 (20)	3.29 ± 0.19 (20)
Dibutyl cyclic AMP	5×10^{-5}	2.24 ± 0.07 (4)	2.19 ± 0.18 (4)
Glucose	1.4×10^{-2}	3.46 ± 0.21 (12)	3.32 ± 0.17 (16)

		Total cell Ca (µg-atoms of Ca/g dry wt.)	
		Control	Experimental
Phenylephrine	2×10^{-5}	7.00 ± 0.39 (15)	8.85 ± 0.42 (16)**

suntheram & Randle, 1976). This differs from the slow uptake of ^{45}Ca in hepatocytes reported by Assimacopoulos-Jeannet *et al.* (1977). Possible factors contributing to this difference are the longer period of preincubation in the present studies and the use of a LaCl_3 wash in the experiments of Assimacopoulos-Jeannet *et al.* (1977). We have avoided the use of LaCl_3 in our experiments because of uncertainties with regard to the fraction of cell calcium removed by this agent. In our experiments measurements of mitochondrial exchangeable calcium showed that at least 60% of the ^{45}Ca taken up by the hepatocyte was intramitochondrial, i.e. had entered the cell (Table 1, lines 8, 9). The time course of mitochondrial ^{45}Ca uptake was very similar to that of cell uptake (Fig. 1, open circles).

Effects of ionophore A23187 and α -adrenergic agonists

The bivalent-metal ion ionophore A23187 (Reed & Lardy, 1972) doubled the uptake of ^{45}Ca by hepatocytes in 60 min (Table 3, lines 8, 9). Measurement of mitochondrial ^{45}Ca (Table 4) confirmed that cell

uptake involved entry of ^{45}Ca into the cell.

As shown in Table 3 (line 1) the predominantly α -adrenergic agonist phenylephrine at $20\mu\text{M}$ increased cell uptake of ^{45}Ca by 39% in 60 min. This uptake of ^{45}Ca represented net uptake and not exchange, as shown by measurement of total cell calcium, which increased by 26% (last line, Table 3). A study of the time course of ^{45}Ca uptake with phenylephrine showed that it accelerated the slow phase of calcium uptake; the major deviation from the control occurred between 5 and 25 min of incubation and thereafter the difference remained constant to 60 min (results not shown). A study of the dose-response relationship showed that the effect of phenylephrine developed over the concentration range 0.1 – $10\mu\text{M}$ (results not shown). These effects of phenylephrine were observed with cells prepared with or without hyaluronidase. Adrenaline (a mixed α - and β -adrenergic agonist) also increased ^{45}Ca uptake (Table 3, line 2). This effect of adrenaline was an α -effect; thus it was abolished by the α -blocker phentolamine, but not by the β -blocker propranolol (Table 3, lines 4 and 6). Phentolamine and propranolol had no effects on ^{45}Ca uptake alone (Table 3, lines

Table 4. *Effects of hormones, ionophore A23187, EGTA and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on mitochondrial exchangeable calcium in rat hepatocytes*

Isolated hepatocytes were prepared with collagenase only. They were preincubated in incubation medium at 37°C for 20 min at 10mg dry wt./ml and then, for the times shown in similar medium containing $[6,6\text{'-}^3\text{H}]\text{sucrose}$ ($2.5\mu\text{Ci/ml}$) and ^{45}Ca ($0.5\mu\text{Ci/ml}$) plus (in the experimental series), the additions as shown. At the end of incubation, the cells were separated from the medium by centrifugation for 15 s at $1000g$, and the supernatant was aspirated and retained for assay of radioactivity. The cells were resuspended in 1 ml of mitochondrial preparation medium and disrupted immediately with a Polytron PT10 homogenizer. The mitochondria were isolated and washed by differential centrifugation, and the final pellet was resuspended in 0.1 M-Tris/HCl , pH 7.5, and disrupted by freezing and thawing (three times). The extract was assayed for protein, citrate synthase and for ^3H and ^{45}Ca by dual-isotope liquid-scintillation spectrometry. Samples of the cell suspension were also taken for assay of citrate synthase, wet weight of cells and protein. Experiments marked (s) indicate where hepatocytes were obtained from rats starved overnight. Fed rats were used in all other cases. For further details see the Experimental section. * $P < 0.01$ versus control; † $P < 0.05$ versus control; ** $P < 0.001$ versus control; § $P < 0.001$ for glucagon+insulin versus glucagon alone. Values are means $\pm$ S.E.M. for the numbers of observations given in parentheses.

Experiment	Time (min)	Mitochondrial exchangeable Ca (μmol of medium Ca/g dry wt. of cells)	
		Control	Experimental
Phenylephrine ($10\mu\text{M}$)	25	0.75 ± 0.04 (4)	1.04 ± 0.06 (4)*
	45	1.10 ± 0.03 (4)	1.37 ± 0.01 (4)*
Glucagon (2 nM)	5	0.90 ± 0.02 (4)	0.78 ± 0.03 (4)†
	15	1.17 ± 0.10 (4)	0.65 ± 0.03 (4)*
Glucagon (20 nM)	5	0.95 ± 0.06 (6)	0.84 ± 0.05 (6)
	15	1.22 ± 0.11 (6)	1.06 ± 0.12 (6)
Glucagon (2 nM) (s)	5	1.23 ± 0.05 (10)	0.97 ± 0.03 (10)**
Glucagon (2 nM) + Insulin (4 nM) (s)	5	1.23 ± 0.05 (10)	1.23 ± 0.04 (8)§
A23187 ($5\mu\text{M}$)	20	0.50 ± 0.03 (4)	0.85 ± 0.004 (4)*
	40	0.80 ± 0.05 (4)	1.19 ± 0.02 (4)*
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone ($10\mu\text{M}$)	10	1.05 ± 0.05 (4)	0.40 ± 0.07 (4)*
	20	0.81 ± 0.04 (4)	0.28 ± 0.01 (4)*

3 and 5). Adrenaline also increased total cell calcium by 27%. For both phenylephrine and adrenaline the increase in total calcium (1.85 and 2.10 μg -atoms of medium Ca/g dry wt. respectively) was greater than the uptake of medium calcium based on ^{45}Ca uptake (0.89 and 0.99 μg -atom of medium Ca/g dry wt). The reason for this discrepancy is not known. Mitochondrial ^{45}Ca in hepatocytes was also increased by phenylephrine (Table 4, lines 1 and 2), showing that increased cell uptake involved increased entry of ^{45}Ca into intracellular compartments.

Stimulation of ^{45}Ca uptake by α -adrenergic agonists has been noted in rat parotid acinar cells (Kanagasuntheram & Randle, 1976). Qualitatively similar effects of phenylephrine on ^{45}Ca uptake in hepatocytes has been reported by Keppens *et al.* (1977) and by Assimacopoulos-Jeannet *et al.* (1977).

Effects of isoproterenol, glucagon, insulin, dibutyryl cyclic AMP and glucose

Isoproterenol (10 μM) and adrenaline with the α -blocker phentolamine are predominantly β -adrenergic agonists and they had no detectable effect on ^{45}Ca uptake by hepatocytes after 60 min (Table 3, lines 6 and 7). Insulin (1 nM), glucose (14 mM), glucagon (0.6 or 1.5 μM) and dibutyryl cyclic AMP were also without detectable effect on ^{45}Ca uptake by hepatocytes (Table 3, lines 10–14). These measurements were made after 60 min of incubation and the lack of effect of glucagon at this time is comparable with the results of Pilkis *et al.* (1975), but not with the stimulation of ^{45}Ca uptake reported by Keppens *et al.* (1977); Pilkis *et al.* (1975) and Assimacopoulos-Jeannet *et al.* (1977) have observed stimulation of ^{45}Ca uptake by hepatocytes with glucagon during shorter periods of incubation (1–20 min). In additional experiments (results not shown) we failed to observe any effect of glucagon (7 nM) on ^{45}Ca uptake by hepatocytes at 5 or 20 min of incubation (5 min: control 2.8 ± 0.13 , +glucagon 2.7 ± 0.08 ; 20 min: control 3.1 ± 0.08 , +glucagon 3.1 ± 0.10 ; mean $\pm$ S.E.M. in μg -atoms of medium Ca/g dry wt. of cells for eight observations in each group). We suggest that this difference in results may be due to the very much faster initial uptake of ^{45}Ca by hepatocytes in our study, as discussed above.

Calcium efflux from rat hepatocytes

Calcium efflux from rat hepatocytes was investigated by embedding the cells in a column of acetylated Sephadex G-10 [acetylation substantially eliminates binding of calcium by Sephadex (Kanagasuntheram & Randle, 1976)]. The cells were loaded with ^{45}Ca in a preliminary period of 40 min of perfusion with the isotope and then switched to calcium-free medium. After not less than 15 min (to allow wash-out of extracellular ^{45}Ca), efflux of ^{45}Ca from the cells was monitored by assay of ^{45}Ca in effluent fractions

collected at 1 min intervals. Control experiments showed that more than two-thirds of the calcium efflux is derived from hepatocytes under these conditions. It was important to avoid use of hyaluronidase in the preparation of hepatocytes for efflux studies; cells prepared with hyaluronidase lost most of their ^{45}Ca during the initial 15 min wash period.

Effects of phenylephrine on calcium efflux

These results are shown in Fig. 2. In control perfusions (i.e. buffer alone) efflux of calcium as a function of time was always a smooth curve (see, e.g., Fig. 2, open circles, and Fig. 3, open triangles).

Fig. 2. *Effects of phenylephrine on the efflux of ^{45}Ca from rat hepatocytes*

Hepatocytes were prepared with collagenase only. Approx. 3 mg dry wt. of cells was incorporated into a column of acetylated Sephadex G-10 (see the Experimental section). They were pre-perfused for 30 min with basic incubation medium and then perfused with similar medium containing ^{45}Ca (0.5 $\mu\text{Ci}/\text{ml}$). Perfusion was then switched to calcium-free medium and, after the first 15 min of wash-out, samples were collected at 1 min intervals and assayed for ^{45}Ca by liquid-scintillation spectrometry. O, Control curve, no additions; ●, phenylephrine (to 10 μM), added at A and removed at B.

Addition of phenylephrine ($10\mu\text{M}$) led to prompt inhibition of efflux (within 1–2 min), which was reversed equally promptly on removal of phenylephrine. The inhibition of efflux rate with phenylephrine was approx. 50%. The effects of phenylephrine were qualitatively similar to those seen in perfused parotid cells (Kanagasuntheram & Randle, 1976).

Effects of glucagon, insulin and dibutyryl cyclic AMP on calcium efflux

These results are shown in Fig. 3. Insulin alone at 4 nM ($500\mu\text{i.u./ml}$) had no effect on efflux of ^{45}Ca . Glucagon ($8\mu\text{M}$) produced a prompt increase in the rate of ^{45}Ca efflux (within 1 min) that lasted for 5 min. The loss of calcium with glucagon corres-

ponded to $1.2\mu\text{g-atoms}$ of medium calcium/g dry wt. (assuming that the specific radioactivity of the calcium lost was the same as that of the medium used to load the cells with ^{45}Ca). In other experiments (results not shown) this effect of glucagon was demonstrable at concentrations of 0.1, 10 and 100nM , and $1\mu\text{M}$. As shown in Fig. 3(a), this effect of glucagon ($8\mu\text{M}$) was suppressed by insulin (4 nM). Dibutyryl cyclic AMP (1 mM) also stimulated calcium efflux from hepatocytes (Fig. 3b). The magnitude of the effect was comparable with that of glucagon. The effect of dibutyryl cyclic AMP (1 mM), unlike that of glucagon, was not influenced by insulin (4 nM; Fig. 3b).

Stimulation of ^{45}Ca efflux from perfused liver with glucagon was reported by Friedman & Park (1968).

Fig. 3. Efflux of ^{45}Ca from rat hepatocytes: effects of (a) glucagon, insulin and glucagon+insulin and (b) dibutyryl cyclic AMP+insulin

Hepatocytes were prepared with collagenase only. Approx. 3 mg dry wt. of cells was incorporated into a column of acetylated Sephadex G-10 (see the Experimental section) and pre-perfused for 30 min with basic incubation medium. They were then perfused for 40 min with similar medium + ^{45}Ca ($0.5\mu\text{Ci/ml}$). Perfusion was then switched to calcium-free medium and, after 15 min, samples were collected at 1 min intervals and assayed for ^{45}Ca by liquid-scintillation spectrometry. (a) Δ , Insulin (to 4 nM) added at A; $\bullet$, glucagon (to $8\mu\text{M}$) added at A; $\circ$, glucagon (to $8\mu\text{M}$) + insulin (to 4 nM) added at A. (b) $\bullet$, Dibutyryl cyclic AMP (to 1 mM) added at A; $\circ$, dibutyryl cyclic AMP (to 1 mM) + insulin (to 4 nM) added at A.

Effects of adrenaline, isobutylmethylxanthine and ionophore A23187 on calcium efflux

These results are shown in Fig. 4. Adrenaline (8 μ M) and the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.1 mM), like glucagon and dibutyl cyclic AMP, produced an increase in calcium efflux that lasted for 3 and 5 min respectively (Figs. 4b and 4c).

The effects of ionophore A23187 are shown in Fig. 4(a). The ionophore produced a substantial increase in the rate of 45 Ca efflux that lasted for 6 min. The loss of calcium with ionophore A23187 was similar in magnitude to that seen with glucagon and with dibutyl cyclic AMP.

The effect with adrenaline was immediate, whereas 3-isobutyl-1-methylxanthine showed a 5 min lag before any effect was observed.

Mitochondrial exchangeable calcium in hepatocytes

The procedure for measurement of mitochondrial exchangeable calcium was similar in principle to that developed for adipose tissue and parotid pieces (Severson *et al.*, 1976; Randle *et al.*, 1974; Kanagasutheram & Randle, 1976). To obtain mitochondria from hepatocytes that exhibited good respiratory control, it was necessary to separate hepatocytes by centrifugation and to resuspend in a mannitol/

sucrose medium (see the Experimental section). The cells were then disrupted with a Polytron PT10 homogenizer and mitochondria isolated by differential centrifugation. The respiratory control ratio (Chappell & Hansford, 1969) was 5–6 with 2.5 mM-2-oxoglutarate+0.5 mM-L-malate. The yield of mitochondria was 10–15% (based on citrate synthase). Redistribution of 45 Ca between mitochondrial and extramitochondrial fractions after cell breakage was prevented with EGTA (2 mM) and Ruthenium Red (5 μ g/ml) (Severson *et al.*, 1976). The efficiency of this procedure was such that the uptake of 45 Ca by mitochondria was less than 0.1%/h and 45 Ca loss from mitochondria was less than 5%/h [see Kanagasutheram & Randle (1976) for methods used]. Thus little redistribution of calcium is to be expected during the isolation of mitochondria from the homogenate, which took approx. 45 min. We have no means of ascertaining whether any redistribution occurred during the 30–45 s period involved in separation and resuspension of hepatocytes before homogenization.

The time course of 45 Ca uptake into hepatocyte mitochondria is shown in Fig. 1. It showed the same characteristics as cell uptake of 45 Ca, i.e. a rapid phase (less than 2 min) and a much slower phase. The maximum incorporation in the rapid phase was 1.1 μ g-atoms/g dry wt. of cells in 2 min. The slow

Fig. 4. Efflux of 45 Ca from rat hepatocytes: effects of (a) ionophore A23187, (b) adrenaline and (c) 3-isobutyl-1-methylxanthine. Hepatocytes were prepared with collagenase only. Approx. 3 mg dry wt. of cells was incorporated into a column of acetylated Sephadex G-10 (see the Experimental section). They were pre-perfused for 30 min with basic incubation medium and then perfused for 40 min with similar medium containing 45 Ca (0.5 μ Ci/ml). Perfusion was then switched to calcium-free medium and, after the first 15 min of wash-out, samples were collected every minute and assayed for 45 Ca by liquid-scintillation spectrometry. (a) Ionophore A23187 (to 5 μ M) added at A; (b) adrenaline (to 8 μ M) added at A; (c) 3-isobutyl-1-methylxanthine (to 5 μ M) added at A.

incorporation is more readily appreciated from a comparison of different times of incubation within the same experiment (Table 4, lines 1 versus 2, 3 versus 4, 5 versus 6). The rate of this slow incorporation was 0.02–0.03 μg -atom of medium calcium/min per g dry wt., which is not significantly different from the rate of the slow incorporation in whole cells (0.01 μg -atom of medium calcium/min per g dry wt.). After 60 min, ^{45}Ca incorporation into mitochondria was 18% of total mitochondrial calcium and 55% of cell exchangeable calcium.

Mitochondrial exchangeable calcium was increased by agents that increased whole-cell exchangeable calcium, i.e. by ionophore A23187 (Table 4, lines 9 and 10) and by phenylephrine (Table 4, lines 1 and 2). Uncoupling oxidative phosphorylation with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM) produced the expected decrease in mitochondrial calcium (Table 4, lines 11 and 12). Glucagon (2 or 10 nM) lowered mitochondrial calcium at 5 or 15 min of incubation, although statistical significance was only established with 2 nM-glucagon (lines 3–7 in Table 4). This action of glucagon in lowering mitochondrial calcium was abolished by insulin at 4 nM (Table 4, line 8).

Thus phenylephrine, which increased cell uptake of ^{45}Ca also increased mitochondrial ^{45}Ca . The change in mitochondrial ^{45}Ca (Table 4) could have accounted for 30% of the increase in cell uptake with phenylephrine shown in Table 1. Glucagon, which decreased mitochondrial exchangeable calcium, had no significant effects on cell exchangeable calcium (cf. Table 4 and Table 1). However, the average decrease in mitochondrial exchangeable calcium with glucagon (0.23 μg -atom of medium calcium/g dry wt.) would be difficult to detect in the whole cell (cell exchangeable calcium averaged 2.2 μg -atoms of medium calcium/g dry wt.). Glucagon stimulated efflux of calcium from hepatocytes during perfusion with a calcium-free medium (Fig. 3). This loss of calcium by efflux (1.2 μg -atoms of medium calcium/g dry wt.) was much larger than the decrease in mitochondrial incorporation induced by glucagon (0.23 μg -atom of medium calcium/g dry wt.).

General discussion and conclusion

The present study has shown that hepatocytes contain a substantial amount of calcium (7.9 μg -atoms/g dry wt. or per 2.7 ml of cell water). Much of this calcium (77%) is mitochondrial and, because mitochondria were isolated with EGTA, it is presumably within the inner membrane. No more than 20% of mitochondrial calcium exchanged with extracellular calcium in 60 min. Extramitochondrial calcium accounted for 23% of cell calcium and 55% of it exchanged in 60 min. The distribution of extramitochondrial calcium is at present not known. In particular, we do not know the concentrations of ionizable or diffusi-

ble or bound calcium in cytoplasm or whether hepatocytes contain a tubular system that can concentrate calcium, as in muscle.

Ionophore A23187 increased cell uptake of ^{45}Ca by 2.1 μg -atoms of medium calcium/g dry wt. in 60 min; only 17% of this uptake was accounted for by the mitochondrial fraction. If ionophore A23187 completely equilibrated calcium across the plasma membrane, the minimum uptake would be 2.8 μg -atoms of medium calcium/g dry wt. (assuming uniform distribution in extramitochondrial water, mitochondrial matrix water volume of 1 μl /mg of mitochondrial protein, and ignoring extramitochondrial binding sites). The present data might suggest a considerable degree of equilibration of calcium across the plasma membrane with ionophore A23187. The data might also suggest that the cytoplasmic diffusible calcium in hepatocytes is normally maintained at a much lower concentration than that of extracellular water by an active transport system and that this is by-passed by the ionophore.

The α -adrenergic effectors, phenylephrine or adrenaline + propranolol, stimulated ^{45}Ca uptake into hepatocytes, increased total cell calcium, increased mitochondrial ^{45}Ca and inhibited efflux of ^{45}Ca into a calcium-free medium. These data may indicate that α -adrenergic agonists facilitate entry of calcium into intracellular water by blocking calcium efflux at the plasma membrane.

Glucagon, dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine had no effect on ^{45}Ca uptake in hepatocytes. These agents stimulated efflux of ^{45}Ca into a calcium-free medium. Glucagon inhibited uptake of ^{45}Ca into the mitochondrial fraction in hepatocytes. The effects of glucagon on calcium efflux and on mitochondrial calcium were blocked by insulin, but insulin had no effect on calcium efflux with dibutyryl cyclic AMP. It is suggested that these effects of glucagon are mediated by cyclic AMP and that insulin acts by lowering cyclic AMP. It is known that glucagon increases cyclic AMP and activates cyclic AMP-dependent protein kinase in hepatocytes and that these actions of glucagon are reversed by insulin (Exton *et al.*, 1971; Van der Werve *et al.*, 1977). These observations provide some further support for the suggestion that agents that increase intracellular concentrations of cyclic AMP may release calcium from mitochondria and possibly other intracellular organelles. The degree of inhibition of mitochondrial uptake of calcium (0.26 μg -atom/g dry wt. in 5 min) was small in relation to the stimulation of efflux in a calcium-free medium (1.2 μg -atoms/g dry wt. in 5 min). The mechanisms of action of glucagon, dibutyryl cyclic AMP and isobutylmethylxanthine are not known. Mitochondrial calcium uptake is inhibited by uncouplers of oxidative phosphorylation, but glucagon had no effect on hepatocyte ATP concentration. Cyclic AMP itself

may have no direct effect on mitochondrial calcium uptake, in contradistinction to earlier reports (Schotland & Mela, 1977).

The present studies had the limited objective of ascertaining whether hepatocytes are a useful model system in which to investigate the mechanism of action of α -adrenergic effectors and of agents that modulate intracellular cyclic AMP on calcium metabolism. The results of these studies indicate that they do constitute a useful model system. The metabolic implications of these findings are not without interest. A number of studies have shown that α -adrenergic effectors can activate liver glycogen phosphorylase without any increase in cyclic AMP or activation of cyclic AMP-dependent protein kinase or stable activation of phosphorylase kinase (Sherline *et al.*, 1973; Exton & Harper, 1975; Hutson *et al.*, 1976; Cherrington *et al.*, 1976; Birnbaum & Fain, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Van der Werve *et al.*, 1977). Further studies have shown that effects of α -adrenergic agonists are dependent on external calcium and that ionophore A23187 also activates glycogen phosphorylase provided external calcium is present (Keppens *et al.*, 1977). Our findings with α -adrenergic agonists are consistent with the suggestion that calcium may mediate their effects on phosphorylase.

In order to draw firm conclusions about effects of hormones on cytoplasmic calcium it will be necessary to obtain direct measurements of the cytoplasmic concentration of Ca^{2+} . These measurements are not available. The present study has shown unequivocally that α -adrenergic agonists do increase the flow of ^{45}Ca across the cytoplasmic compartment. The minimum estimate of this increased flow is 11.6ng-atoms/ml of cell water over 25 min (based on mitochondrial exchangeable calcium); the maximum estimate is 11.4ng-atoms/min per ml of cell water (based on the increase in total calcium). The present study has also provided evidence that glucagon can release calcium from intracellular stores. The estimates range from 96ng-atoms of calcium/ml of cell water in 5 min (fall in mitochondrial exchangeable calcium) to 444ng-atoms of calcium/ml of cell water in 5 min (efflux of ^{45}Ca into calcium-free medium).

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