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On the mechanism by which hormones induce the release of Ca^{2+} from mitochondria in the liver cell

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1. The abilities of dinitrophenol, NaCl, Ruthenium Red and the Ca^{2+}-selective ionophore A23187 to release $^{45}Ca^{2+}$ from isolated hepatocytes and liver mitochondria (incubated at 37°C in the presence of 0.1μM-free Ca^{2+}, Mg^{2+}, ATP and phosphate ions) were compared with the action of adrenaline on $^{45}Ca^{2+}$ release from isolated hepatocytes. The effects of adrenaline were most closely described by those of the ionophore A23187. 2. In isolated hepatocytes, a release of $^{45}Ca^{2+}$ and stimulation of O_2 utilization similar to that induced by adrenaline was observed in the presence of 500 and 20μM-arachidonic acid respectively. The effect of arachidonic acid on $^{45}Ca^{2+}$ release was not specific for this unsaturated fatty acid. 3. Inhibitors of arachidonic acid metabolism, including indomethacin and eicosa-5,8,11,14-tetraynoic acid, did not block the effects of adrenaline on $^{45}Ca^{2+}$ or glucose release from isolated hepatocytes. 4. The ability of adrenaline to stimulate $^{45}Ca^{2+}$ release from isolated hepatocytes was rapidly reversed after the subsequent addition of phenoxybenzamine to the cell suspension, and was completely blocked by 0.5mM-dibucaine. 5. The results are consistent with the action of a Ca^{2+}-selective ionophore in the mechanism by which adrenaline induces the release of Ca^{2+} from mitochondria in the liver cell and indicate that it is unlikely that arachidonic acid or a metabolite of arachidonic acid is involved in this process.

An extensive body of indirect evidence indicates that expression of the effects of α-adrenergic agonists vasopressin and angiotensin on the reaction catalysed by glycogen phosphorylase *b* kinase in the liver cell requires an increase in the concentration of free Ca^{2+} in the cytoplasm (reviewed by Exton, 1980). It has been proposed (Blackmore & Exton, 1981) that a stimulation of Ca^{2+} outflow from mitochondria (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1979*a*; Murphy *et al.*, 1980; Barritt *et al.*, 1981*b*; but see also Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980) is a necessary and early event in the process by which these agonists increase the cytoplasmic Ca^{2+} concentration in the liver cell.

The mechanisms by which the combination of each agonist with its receptor on the plasma membrane leads to stimulation of net Ca^{2+} outflow from the mitochondria are not well understood. It has been proposed that the link between the receptors and mitochondria is provided by an increase in the cytoplasmic concentration of a chemical intermediate, such as Na^+ (Haworth *et al.*, 1980; Hughes *et al.*, 1980), a Ca^{2+} ionophore (Blackmore *et al.*, 1979*a*), an agent that decreases the proton gradient across the mitochondrial inner membrane (Blackmore *et al.*, 1979*a*), a product of phosphatidylinositol hydrolysis (Billah & Michell, 1979), including phosphatidic acid (Barritt *et al.*, 1981*a*) or a metabolite of arachidonic acid (Barritt, 1981*a*), or a change in the ratio of reduced/oxidized pyrimidine nucleotides (Lehninger *et al.*, 1978; Sies *et al.*, 1981). Although a change in the concentration of a metabolite or ion is a reasonable explanation for the effects of agonists on Ca^{2+} release from mitochondria, other possible mechanisms, including the transmission of information through conformational changes in intracellular membranes, have not been eliminated.

The aim of the present experiments was to test some possible mechanisms by which Ca^{2+} may be released from liver cell mitochondria in response to adrenaline. First, the effects of agents that are known to affect membrane Ca^{2+} transport on the release of Ca^{2+} from isolated liver cells and mitochondria were compared with the effect of adrenaline on Ca^{2+} release from isolated hepatocytes in an endeavour to determine which com-

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pounds mimic the action of adrenaline closely. Secondly, the proposal that arachidonic acid or a metabolite of arachidonic acid catalyses the outflow of Ca^{2+} from mitochondria in response to the action of adrenaline on the plasma membrane (Barritt, 1981*a*) was tested. Since activation of a phospholipase enzyme(s) may be involved in this process (Billah & Michell, 1979), the effects of the local anaesthetic dibucaine, an inhibitor of phospholipase activity (Vanderhoek & Feinstein, 1979), on the release of intracellular Ca^{2+} induced by adrenaline were also tested. The results are consistent with the proposal that a Ca^{2+}-selective ionophore may catalyse the agonist-induced release of Ca^{2+} from liver cells, and indicate that this compound is unlikely to be arachidonic acid or a metabolite of arachidonic acid.

Experimental

Preparation of isolated hepatocytes and liver mitochondria

Hepatocytes were isolated from the livers of fed rats by a modification of the method of Berry & Friend (1969) as described previously (Barritt *et al.*, 1981*b*). Mitochondria that sedimented between 3000 and 22500 *g*-min (Prpić *et al.*, 1978) were prepared from the livers of fed rats and their protein content (biuret method) and rates of O_2 utilization were measured as described previously (Hughes & Barritt, 1978). The integrity of the mitochondria was assessed by measuring the ratio of the ADP-stimulated/ADP-depleted rates of O_2 utilization. In the presence of 12.5mM-potassium succinate, this ratio was 6.1 ± 0.3 ($n = 9$).

$^{45}Ca^{2+}$ exchange by isolated hepatocytes

Amounts of $^{45}Ca^{2+}$ exchanged by isolated hepatocytes were measured at 37°C as described previously (Barritt *et al.*, 1981*b*). The incubation medium contained, in a final volume of 6.0ml, 117mM-NaCl, 4.7mM-KCl, 1.2mM-KH_2PO_4, 1.2mM-$MgSO_4$, 24mM-$NaHCO_3$, 20mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulphonic acid)/KOH, 0.1mM-$^{45}CaCl_2$ (0.3MBq), 30mg wet wt. of cells per ml and other additions as indicated. The medium was equilibrated with O_2/CO_2 (19:1, v/v) and the final pH was 7.4. $^{45}Ca^{2+}$ exchange was initiated by addition of the cells to the incubation medium (in the presence of $^{45}Ca^{2+}$), and after 30min (or 20min as indicated in the legend to Fig. 2) the agent under test was added. The quantity of $^{45}Ca^{2+}$ exchanged by the cells at a given time was calculated as described previously (Barritt *et al.*, 1981*b*).

Adrenaline and dibutyryl cyclic AMP were added to the incubation media as aqueous solutions; dinitrophenol, oligomycin and phenoxybenzamine as solutions in ethanol; ionophore A23187 as a solution in dimethyl sulphoxide; and glucagon as a suspension in 1.3% (w/v) $NaHCO_3$. Arachidonic acid was added as a solution in 100mM-Na_2CO_3, as the sodium salt bound to 18% (w/v) bovine serum albumin, or as a solution in ethanol (in experiments with isolated mitochondria). Linolenic acid, eicosa-11-enoic acid and oleic acid were dissolved in 100mM-Na_2CO_3 or bound as the sodium salt to 18% (w/v) bovine serum albumin, and prostaglandins E_1 and E_2 were dissolved in ethanol. Solutions of the unsaturated fatty acids and prostaglandins were prepared under N_2 immediately before use.

$^{45}Ca^{2+}$ exchange by isolated mitochondria

Amounts of $^{45}Ca^{2+}$ exchanged by isolated mitochondria were measured at 37°C as described previously (Barritt, 1981*b*). The incubation medium contained, in a total volume of 6.0ml, 150mM-KCl, 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH, 10mM-nitrilotriacetic acid, 2mM-potassium phosphate, 2mM-potassium succinate, 2mM-sodium ATP, 2mM-$MgCl_2$, 25μM added total Ca^{2+} (0.13μM-free Ca^{2+}), 1.5mg of mitochondrial protein/ml and other additions as indicated. The final pH was 7.4. The mitochondria were incubated with medium (in the absence of $^{45}Ca^{2+}$) for 15min before the addition of $^{45}CaCl_2$ (6kBq). When present, the agent under test was added after a further 16min. The amount of $^{45}Ca^{2+}$ associated with the mitochondria, q_m Bq, was expressed as a fraction, q_m/q_{ao}, of the initial dose of $^{45}Ca^{2+}$, q_{ao} Bq.

Glucose release, O_2 utilization and cyclic AMP

Glucose release from isolated hepatocytes was measured in a medium identical with that employed for the measurement of $^{45}Ca^{2+}$ exchange except that no $^{45}Ca^{2+}$ was present and the concentration of $CaCl_2$ was 0.1 or 1.3mM as indicated in Table 3. After incubation of the cells with the medium for 10min the agent under test was added, samples of the incubation medium (100μl) were removed after a further 1, 5, 9 and 13min and added to 1.0ml of ice-cold Ca^{2+}-free Krebs–Henseleit medium (Krebs & Henseleit, 1932) in 1.5ml plastic centrifuge tubes. The tubes were centrifuged for 10s at 7000 *g* and the concentration of glucose in the supernatant was measured as described previously (Hughes & Barritt, 1978). Rates of glucose release were estimated from the slopes of the linear plots of the amount of glucose present in the extracellular medium as a function of time.

Rates of O_2 utilization by isolated hepatocytes were measured at 37°C as described by Dehaye *et al.* (1981). The incubation medium (6ml final volume) was the same as that employed for the measurement of $^{45}Ca^{2+}$ exchange except that it

contained no $^{45}Ca^{2+}$, 23 mg wet wt. of cells/ml and the indicated concentration of Ca^{2+}. The medium was equilibrated with O_2/CO_2 (19:1, v/v) before use.

For the measurement of cyclic AMP, samples (0.8 ml) of the incubation medium were mixed with 0.6 M-$HClO_4$ (0.4 ml) and treated with KOH to bring the pH to 7.0. After centrifugation, the amount of cyclic AMP present in each extract was measured by the method of Gilman (1970).

Treatment of data

The values given are means ± S.E.M. or the means where only two experiments were performed. The degrees of significance, P, were determined by Student's t test for unpaired samples. Values of $P > 0.05$ were considered to be not significant.

Chemicals

Dibucaine/HCl, $N^6O^{2'}$-dibutyryl cyclic AMP, arachidonic acid, oleic acid, linolenic acid, eicosa-11-enoic acid, prostaglandins E_1 and E_2, dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone, Ruthenium Red, adrenaline, 1-phenylpyrazolidin-3-one (phenidone), indomethacin, ADP, ATP and succinic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; ionophore A23187 was from Calbiochem–Behring Australia Pty., Carlingford, N.S.W., Australia; $^{45}CaCl_2$ was from Amersham Australia Pty., Sydney, N.S.W., Australia; collagenase was from Boehringer-Mannheim Australia Pty., Mount Waverley, Victoria, Australia; and flufenamic acid was from Parke, Davis and Co., Sydney, N.S.W., Australia. Eicosa-5,8,11,14-tetraynoic acid was kindly provided by Dr. W. E. Scott, Hoffman-La Roche Inc., Nutley, NJ, U.S.A. All other reagents were of the highest grade available. Ruthenium Red was recrystallized before use as described previously (Barritt *et al.*, 1981*b*).

Results

Characteristics of Ca^{2+} release from liver cells and mitochondria

The ability of adrenaline and other agonists to release Ca^{2+} from intracellular stores in hepatocytes can be most clearly demonstrated at extracellular Ca^{2+} concentrations below about 0.1 mM (Chen *et al.*, 1978; Blackmore *et al.*, 1979*a*; Barritt *et al.*, 1981*b*). For this reason, a concentration of 0.1 mM-extracellular Ca^{2+} was employed in the present studies. It has previously been shown that under these conditions the loss of $^{45}Ca^{2+}$ that follows the addition of adrenaline to liver cells equilibrated with $^{45}Ca^{2+}$ (Figs. 1*a* and 1*b*) represents a decrease in the amount of Ca^{2+} in intracellular organelles, principally the mitochondria (Barritt *et al.*, 1981*b*).

At a concentration of 1 μM, the Ca^{2+}-selective ionophore A23187 released the same amount of $^{45}Ca^{2+}$ from isolated hepatocytes at the same rate as that released by adrenaline (Fig. 1*c* and Table 1). Dimethyl sulphoxide, the vehicle in which the ionophore was dissolved, had no effect on $^{45}Ca^{2+}$

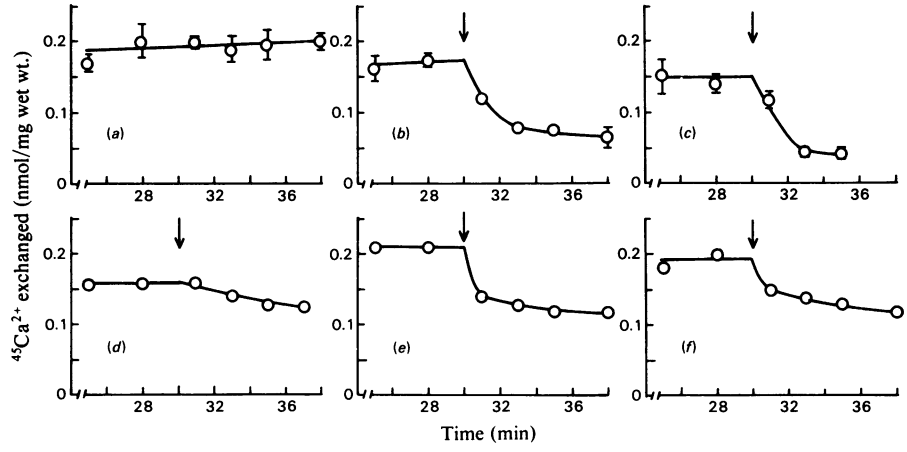

Fig. 1. *Comparison of the effects of ionophore A23187, dinitrophenol, glucagon and dibutyryl cyclic AMP with that of adrenaline on $^{45}Ca^{2+}$ release from isolated hepatocytes*

Cell incubations and measurement of the amounts of $^{45}Ca^{2+}$ associated with isolated hepatocytes were performed as described in the Experimental section. The final concentrations of the agents tested (added at 30 min as indicated by the arrows) were: (*a*), no addition; (*b*), 1 μM-adrenaline; (*c*) 1 μM-ionophore A23187; (*d*), 0.38 mM-dinitrophenol plus 22 μM-oligomycin; (*e*), 0.1 μM-glucagon; (*f*), 0.2 mM-dibutyryl cyclic AMP. Values are means ± S.E.M. of three (*a* and *c*) and five (*b*) experiments or the means of two experiments (*d–f*).

Table 1. *Effects of dibucaine on the release of $^{45}Ca^{2+}$ from isolated hepatocytes induced by adrenaline, dibutyryl cyclic AMP and ionophore A23187*

Cell incubations and measurement of the amount of $^{45}Ca^{2+}$ associated with isolated hepatocytes were performed as described in the Experimental section. Hepatocytes were added to the incubation medium (in the presence of 0.1 mM-$^{45}CaCl_2$) at zero min; dibucaine, when present, was added at 26 min and the agent under test at 30 min. Values are means ± S.E.M. of the numbers of experiments shown in parentheses. The degrees of significance, *P*, for a comparison of a given condition under test with that in the presence of dibucaine alone were determined by Student's *t* test for unpaired samples. The degrees of significance for other comparisons are: $P < 0.001$ for adrenaline plus dibucaine compared with adrenaline; $P > 0.1$ for dibutyryl cyclic AMP plus dibucaine compared with dibutyryl cyclic AMP; $P < 0.1$ for ionophore A23187 plus dibucaine compared with ionophore A23187.

Agent under test	$^{45}Ca^{2+}$ associated with hepatocytes at 34 min (nmol/mg wet wt.)	*P*
No addition	0.184 ± 0.007 (9)	<0.001
Dibucaine (0.5 mM)	0.129 ± 0.008 (10)	
Adrenaline (1 μM)	0.066 ± 0.003 (8)	<0.001
Adrenaline (1 μM) + dibucaine (0.5 mM)	0.112 ± 0.006 (7)	<0.1
Dibutyryl cyclic AMP (0.2 mM)	0.095 ± 0.008 (5)	<0.05
Dibutyryl cyclic AMP (0.2 mM) + dibucaine (0.5 mM)	0.102 ± 0.007 (6)	<0.05
Ionophore A23187 (1 μM)	0.066 ± 0.010 (4)	<0.001
Ionophore A23187 (1 μM) + dibucaine (0.5 mM)	0.046 ± 0.004 (5)	<0.001

release when added alone. The loss of $^{45}Ca^{2+}$ that followed addition of the uncoupling agent dinitrophenol in the presence of oligomycin was much slower (Fig. 1*d*). Similar results (not shown) were obtained after the addition of dinitrophenol alone. Furthermore, in the presence of dinitrophenol, subsequent addition of ionophore A23187 (1 μM) at 34 min rapidly released further $^{45}Ca^{2+}$ (cf. Fig. 1*c*) (results not shown), indicating that the slow release of $^{45}Ca^{2+}$ observed in the presence of dinitrophenol alone is not due to an inhibition of Ca^{2+} outflow across the plasma membrane. The amount of $^{45}Ca^{2+}$ released by glucagon or dibutyryl cyclic AMP was less than that released by adrenaline (Figs. 1*e* and 1*f* and Table 1).

The subsequent addition of the α-adrenergic antagonist phenoxybenzamine rapidly reversed the loss of $^{45}Ca^{2+}$ induced by adrenaline (Fig. 2). In the absence of phenoxybenzamine, the $^{45}Ca^{2+}$ content of the cells remained depressed by the presence of adrenaline during the period of the experiment (results not shown). When added before adrenaline, phenoxybenzamine completely blocked the effect of the hormone on $^{45}Ca^{2+}$ release and had no effect when added alone (results not shown).

When tested on isolated mitochondria at 37°C and in the presence of 0.13 μM-free Ca^{2+}, Mg^{2+}, ATP and P_i, ionophore A23187 caused a rapid release of $^{45}Ca^{2+}$, which was complete within 0.5 min (Figs. 3*a* and 3*b*), whereas the rates of $^{45}Ca^{2+}$ release observed in the presence of dinitrophenol (Fig. 3*c*), carbonyl cyanide *m*-chlorophenylhydrazone (results not shown) and Ruthenium Red (Fig. 3*d*) were much lower. The addition of dimethyl sulphoxide alone did not cause a release of $^{45}Ca^{2+}$. Under the conditions

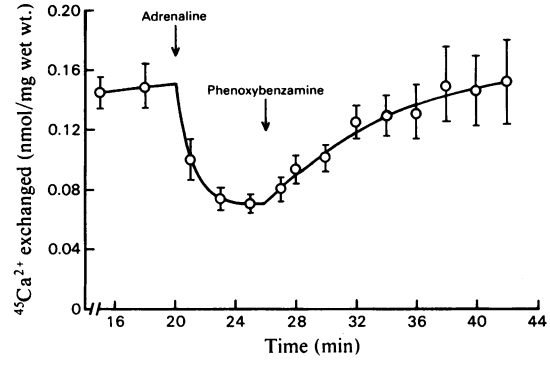

Fig. 2. *Effect of phenoxybenzamine on the amount of $^{45}Ca^{2+}$ associated with isolated hepatocytes after the loss of $^{45}Ca^{2+}$ induced by adrenaline*

Cell incubations and measurement of the amounts of $^{45}Ca^{2+}$ associated with isolated hepatocytes were performed as described in the Experimental section. Adrenaline (1 μM final concentration) and phenoxybenzamine (10 μM) were added at the times indicated by the arrows. Values are means ± S.E.M. of five experiments.

employed in the present experiments (cf. Haworth *et al.*, 1980), the effect of 20 mM-NaCl was small, slower in onset and transient (Fig. 3*e*) when compared with that of ionophore A23187.

Assessment of the possible roles of arachidonic acid and its metabolites in hormone-induced Ca^{2+} release

Arachidonic acid induced a release of $^{45}Ca^{2+}$ from isolated hepatocytes (Figs. 4*a*–4*c*), although a concentration of 500 μM was required to give an

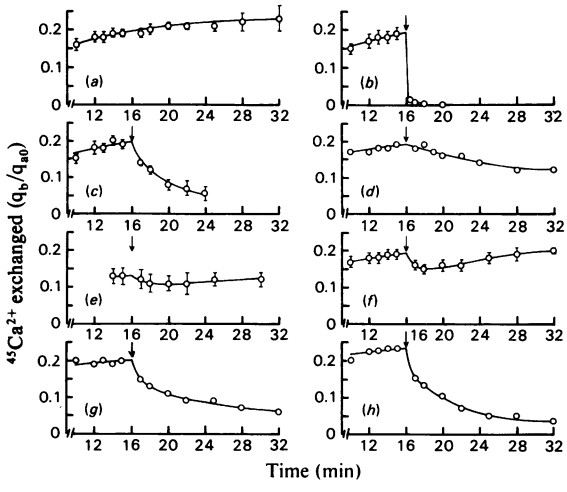

Fig. 3. *Effects of ionophore A23187, dinitrophenol, Ruthenium Red, NaCl and arachidonic acid on the amount of $^{45}Ca^{2+}$ in isolated liver mitochondria incubated at 37°C in the presence of ATP, Mg^{2+} and P_i*

The incubation of mitochondria, measurement of the amount of mitochondrial $^{45}Ca^{2+}$ and expression of the results were as described in the Experimental section. The final concentrations of the agents tested (added at 16min as indicated by the arrows) were: (*a*), no addition; (*b*), 1μM-ionophore A23187; (*c*), 0.6mM-dinitrophenol; (*d*), 0.17μM-Ruthenium Red; (*e*), 20mM-NaCl; (*f*), (*g*) and (*h*), 10μM-, 20μM- and 30μM-arachidonic acid respectively. Values are means ± S.E.M. of four to six (*a*), four (*b* and *f*) or three (*c* and *e*) experiments, the means of two experiments (*d* and *g*) and the results of one experiment (*h*).

effect similar to that of adrenaline (Fig. 4*c*). The effect is not specific for arachidonic acid since some loss of $^{45}Ca^{2+}$ was observed with other unsaturated fatty acids (Figs. 4*d*–4*f*). Arachidonic acid also caused the release of $^{45}Ca^{2+}$ from isolated mitochondria (Figs. 3*f*–3*h*) but the changes were less extensive and the rate of $^{45}Ca^{2+}$ release much lower than that induced by ionophore A23187. Arachidonic acid stimulated the utilization of oxygen by isolated hepatocytes (Table 2), with a concentration of about 25μM giving the same degree of stimulation as that induced by adrenaline (Jakob & Diem, 1975; Sugano *et al.*, 1978; Reinhart *et al.*, 1980; Scholz & Schwabe, 1980; Dehaye *et al.*, 1981). These effects were observed at both 0.1 and 1.3mM extracellular Ca^{2+}. Near maximal stimulation was given by 40μM-arachidonic acid (Table 2). This unsaturated fatty acid also increased the rate of glucose release from isolated hepatocytes to rates comparable with those observed in the presence of adrenaline and ionophore A23187 (Table 3), but caused only a very small increase in the amount of cyclic AMP present (Table 4).

No inhibition of the release of $^{45}Ca^{2+}$ from isolated hepatocytes induced by 1μM-adrenaline was observed in the presence of 10–100μM-indomethacin, 20–50μM-flufenamic acid or 200μM-acetylsalicylic acid [inhibitors of prostaglandin formation (Whittle, 1979; Partington *et al.*, 1980)] or 15–30μM-eicosa-5,8,11,14-tetraynoic acid or 300μM-1-phenylpyrazolidin-3-one [inhibitors of both the lipoxygenase and cyclo-oxygenase pathways of arachidonic acid metabolism (Whittle, 1979)] when these inhibitors were added 10min

Table 2. *Effects of adrenaline and arachidonic acid on rates of O_2 utilization by isolated hepatocytes*

Rates of O_2 utilization were measured at 37°C as described in the Experimental section. The agent under test, dissolved in 50–100μl of water (adrenaline) or 100mM-Na_2CO_3 (arachidonic acid), was added 6min after addition of the cells to the medium. The rate of O_2 utilization was calculated from the slope of the plot of O_2 concentration as a function of time in the following 2min period. Control incubations received 50–100μl of water or 100mM-Na_2CO_3. Values are means ± S.E.M. of the number of experiments given in parentheses.

Extracellular Ca^{2+} concentration (mM)	Agent under test	Rate of O_2 utilization (μg-atom/min per g wet wt. cells)	*P*
1.3	No addition	3.18 ± 0.21 (13)	
	Adrenaline (1μM)	4.38 ± 0.34 (6)	<0.01
	Arachidonic acid		
	20μM	4.26 ± 0.14 (3)	<0.05
	40μM	5.85 ± 0.96 (4)	<0.001
	100μM	5.67 ± 0.32 (3)	<0.001
	180μM	6.54 ± 1.06 (4)	<0.001
0.1	Control	3.83 ± 0.17 (6)	
	Adrenaline (1μM)	5.01 ± 0.29 (8)	<0.01
	Arachidonic acid		
	25μM	4.95 ± 0.19 (6)	<0.002
	100μM	7.37 ± 1.04 (6)	<0.01

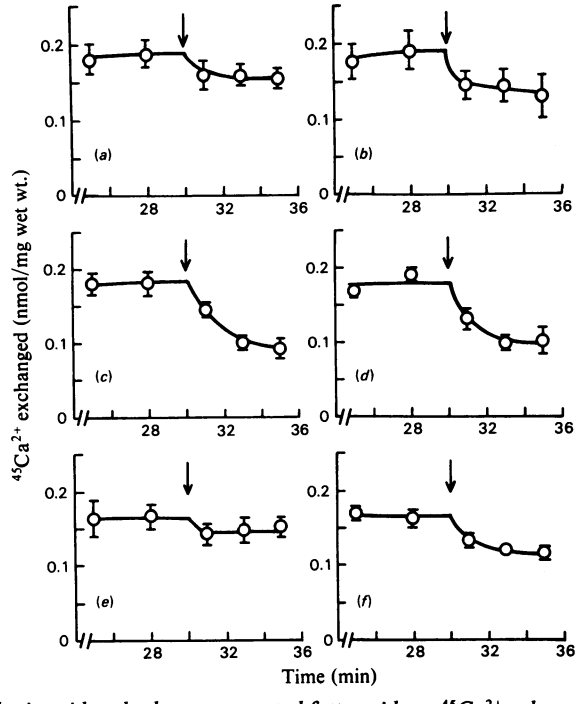

Fig. 4. *Effect of arachidonic acid and other unsaturated fatty acids on $^{45}Ca^{2+}$ release from isolated hepatocytes*
Cell incubations, the preparation of solutions of unsaturated fatty acids and measurement of the amounts of $^{45}Ca^{2+}$ associated with isolated hepatocytes were performed as described in the Experimental section. The unsaturated fatty acids were added at 30min (indicated by the arrow). The final concentrations were: (*a*), 100μM-arachidonic acid ($C_{20:4}$); (*b*), 250μM-arachidonic acid; (*c*), 500μM-arachidonic acid; (*d*), 500μM-linolenic acid ($C_{18:3}$); (*e*), 500μM-eicosa-11-enoic acid ($C_{20:1}$); (*f*), 500μM-oleic acid ($C_{18:1}$). Values are means of three (*b*, *d–f*), four (*a*) and six (*c*) experiments.

Table 3. *Effects of arachidonic acid on the release of glucose from isolated hepatocytes*
Cell incubations and measurement of the rates of glucose release were performed as described in the Experimental section. Arachidonic acid was dissolved in 100mM-Na_2CO_3. Values are means ± S.E.M. of the number of experiments given in parentheses.

Extracellular Ca^{2+} concentration (mM)	Agent under test	Rate of glucose release (μmol/min per g wet wt.)	*P*
1.3	No addition	0.81 ± 0.16 (5)	
	Arachidonic acid		
	250μM	1.00 ± 0.13 (6)	>0.1
	500μM	1.63 ± 0.12 (3)	<0.02
	Adrenaline (1μM)	1.87 ± 0.17 (3)	<0.01
	Ionophore A23187 (1μM)	1.04 ± 0.16 (3)	>0.1
0.1	No addition	1.17 ± 0.12 (6)	
	Arachidonic acid (500μM)	1.98 ± 0.27 (6)	<0.02
	Adrenaline (1μM)	2.08 ± 0.08 (6)	<0.001

before adrenaline (results not shown). Furthermore, the stimulation of glucose release by 1μM-adrenaline was not inhibited by 25–50μM-indomethacin or 10–100μM-eicosa-5,8,11,14-tetraynoic acid added 10min before the adrenaline. Prostaglandins E_1 and E_2 (1–50μM) induced only a small release of $^{45}Ca^{2+}$ from isolated hepatocytes in comparison with the effects of adrenaline or ionophore A23187. Prostaglandin E_1 (10μM) caused only a small and transient loss of $^{45}Ca^{2+}$ from isolated mitochondria in comparison with the effects of ionophore A23187 (results not shown).

Table 4. *Effects of hormones, arachidonic acid and dibucaine on the amount of cyclic AMP in suspensions of isolated hepatocytes*

Cells incubations and measurement of the total amount of cyclic AMP present in the incubation mixture were performed as described in the Experimental section. The incubation medium contained the same components as those present for the measurement of $^{45}Ca^{2+}$ exchange (0.1 mM extracellular $CaCl_2$) except that $^{45}Ca^{2+}$ was omitted. Hepatocytes were added to the incubation medium at 0 min, dibucaine, when present, at 1 1 min, and the agent under test at 15 min. Samples of the incubation medium were removed for the measurement of cyclic AMP at 2 and 4 min after addition of the agent under test. Values are means ± S.E.M. of five experiments. The degrees of significance, *P*, for a comparison of a given condition under test with the value for the control (no addition), determined by Student's *t* test for unpaired samples, are given in parentheses. The degrees of significance for comparison of adrenaline + dibucaine with adrenaline are $P < 0.02$ and $P < 0.05$ at 2 and 4 min respectively.

	Amount of cyclic AMP (nmol/mg wet wt.)	
Agent under test	2 min after addition of agent under test	4 min after addition of agent under test
No addition	0.49 ± 0.02	0.49 ± 0.02
Glucagon (0.1 μM)	4.5 ± 0.7 ($P < 0.001$)	5.6 ± 0.8 ($P < 0.001$)
Arachidonic acid (500 μM)	0.68 ± 0.06 ($P < 0.05$)	0.57 ± 0.04 ($P > 0.1$)
Dibucaine (0.5 mM)	0.52 ± 0.05 ($P > 0.1$)	0.54 ± 0.06 ($P > 0.1$)
Adrenaline (1 μM)	1.30 ± 0.19 ($P < 0.01$)	0.97 ± 0.09 ($P < 0.001$)
Adrenaline + dibucaine	0.66 ± 0.06 ($P < 0.05$)	0.66 ± 0.06 ($P < 0.05$)

Effects of dibucaine on hormone-induced Ca^{2+} release

At a concentration of 0.5 mM, dibucaine completely blocked the effect of adrenaline on $^{45}Ca^{2+}$ release (Table 1). Some $^{45}Ca^{2+}$ was lost in the presence of dibucaine alone (Table 1). When the concentration of dibucaine was decreased, its ability to block the action of adrenaline and induce a loss of $^{45}Ca^{2+}$ in the absence of the hormone was impaired. At concentrations of dibucaine of 1.0 or 2.0 mM, a much larger loss of $^{45}Ca^{2+}$ was observed in the absence of adrenaline (results not shown). Dibucaine did not block the release of $^{45}Ca^{2+}$ induced by ionophore A23187 and did not appear to inhibit the action of dibutyryl cyclic AMP (Table 1).

Adrenaline caused a small increase in the total amount of cyclic AMP present in the incubation mixture (Cherrington *et al.*, 1976) compared with that induced by glucagon (Table 4). Although dibucaine had no effect on the amount of cyclic AMP present in the absence of hormones, it decreased slightly the amount present in the presence of adrenaline (Table 4).

Discussion

The rapid onset of the re-uptake of $^{45}Ca^{2+}$ by hepatocytes after the addition of phenoxybenzamine to cells previously exposed to adrenaline indicates that occupation of the receptor by adrenaline (and hence continual generation of a signal from the agonist–receptor complex) is required to maintain the effect of the agonist on the release of Ca^{2+} from the mitochondria. The rates of $^{45}Ca^{2+}$ release from isolated mitochondria induced by dinitrophenol and Ruthenium Red, an inhibitor of Ca^{2+} uptake by mitochondria (Moore, 1971), and the rate of release of $^{45}Ca^{2+}$ from hepatocytes induced by dinitrophenol are each slower than the rate of release of $^{45}Ca^{2+}$ from hepatocytes induced by adrenaline. These observations, together with the results for the effect of NaCl on $^{45}Ca^{2+}$ release from isolated mitochondria, indicate that a metabolite that is solely either an uncoupler of mitochondrial oxidative phosphorylation or an inhibitor of Ca^{2+} inflow to mitochondria, or Na^+ is unlikely to cause the release of Ca^{2+} from mitochondria in hepatocytes in the presence of adrenaline.

The action of adrenaline on $^{45}Ca^{2+}$ release from isolated hepatocytes was mimicked most closely by the Ca^{2+}-selective ionophore A23187. This result is consistent with the idea that a Ca^{2+} ionophore may catalyse the release of Ca^{2+} from mitochondria that follows the combination of adrenaline with its receptor on the plasma membrane. However, the possibility that Ca^{2+} efflux from the mitochondria is stimulated by some other mechanism that is rapid in onset cannot be eliminated. Furthermore, it is possible that the rapid release of $^{45}Ca^{2+}$ observed in the presence of adrenaline may be due to a stimulation by this hormone of Ca^{2+} outflow across the plasma membrane by a mechanism that acts in addition to the stimulation of Ca^{2+} outflow that is induced by a rise in the cytoplasmic Ca^{2+} concentration. This possibility is considered to be unlikely in view of the rapid release of $^{45}Ca^{2+}$ induced by ionophore A23187 and the results of steady-state kinetic studies of the effects of adrenaline on Ca^{2+} transport in isolated hepatocytes (Barritt *et al.*, 1981*b*).

Results obtained in the present experiments indicate that a metabolite of arachidonic acid, or arachidonic acid itself, is unlikely to be involved in the agonist-induced release of Ca^{2+} from mitochondria in the liver cell, as proposed previously (Barritt, 1981*a*). The main observations, which are consistent with this conclusion, are as follows. (*a*) The concentrations of arachidonic acid required to induce a release of $^{45}Ca^{2+}$ from hepatocytes and isolated mitochondria are considerably higher than those required to induce a given response in platelets and pancreatic acinar cells for which there is evidence that a metabolite of arachidonic acid mediates the action of a particular agonist (Hamberg *et al.*, 1975; Marshall *et al.*, 1980, 1981). (*b*) The concentration of arachidonic acid that induced the same amount of $^{45}Ca^{2+}$ release from isolated hepatocytes as adrenaline was 25-fold higher than that which gave the same degree of stimulation of O_2 utilization as adrenaline. (*c*) The effect of arachidonic acid on $^{45}Ca^{2+}$ release is not specific for this unsaturated fatty acid (cf. Roman *et al.*, 1979). (*d*) The effect of arachidonic acid on $^{45}Ca^{2+}$ release from isolated mitochondria develops much more slowly than that of ionophore A23187. (*e*) Inhibitors of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism did not block the effects of adrenaline on $^{45}Ca^{2+}$ or glucose release from hepatocytes, whereas these agents have been shown to inhibit the response of some other cell types to agonists (Partington *et al.*, 1980; Marshall *et al.*, 1980; Nemeth & Douglas, 1980). (*f*) Two metabolites of arachidonic acid, prostaglandins E_1 and E_2, did not induce the release of $^{45}Ca^{2+}$ from hepatocytes.

Unsaturated fatty acids (Orly & Schramm, 1975) and some local anaesthetics (Gordon *et al.*, 1980) have been shown to modify the activity of adenylate cyclase. However, the values obtained for the amounts of cyclic AMP present under the conditions employed in the present experiments indicate that it is unlikely that an increase in the intracellular concentration of cyclic AMP accounts for the effects of arachidonic acid on $^{45}Ca^{2+}$ release, O_2 utilization or glucose release. Moreover, the data indicate that a decrease in the concentration of cyclic AMP makes only a small, or negligible, contribution to the mechanism by which dibucaine completely blocks the stimulation by adrenaline of $^{45}Ca^{2+}$ release. Whereas adrenaline alone caused a small increase in the concentration of cyclic AMP, the effect of adrenaline on $^{45}Ca^{2+}$ release was completely blocked by the α-adrenergic antagonist phenoxybenzamine, indicating that the increase in cyclic AMP is unlikely to be responsible for the release of $^{45}Ca^{2+}$.

The observation that dibucaine, which has been shown to inhibit phospholipase A_2 activity in platelets (Vanderhoek & Feinstein, 1979), blocked the effects of adrenaline on $^{45}Ca^{2+}$ release from hepatocytes provides some evidence that the action of a phospholipase enzyme(s) is involved in the process by which α-adrenergic agonists induce the release of $^{45}Ca^{2+}$ from mitochondria as proposed for the action of these agonists by Billah & Michell (1979). However, an alternative explanation that dibucaine displaces adrenaline from its receptor cannot be excluded [cf. the effects of verapamil (Blackmore *et al.*, 1979*b*) and trifluoperazine (Blackmore *et al.*, 1981; Reinhart *et al.*, 1981)].

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