EVIDENCE THAT PHOSPHATIDIC ACID STIMULATES THE UPTAKE OF CALCIUM BY LIVER CELLS BUT NOT CALCIUM RELEASE FROM MITOCHONDRIA

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1. Introduction

The stimulation of glycogen phosphorylase activity in liver cells by α-adrenergic agonists, vasopressin and angiotensin II is mediated in part, by an increase in the concentration of calcium in the cell cytoplasm (reviewed [1,2]). The release of calcium from the mitochondria appears to be an important source of this additional cytoplasmic calcium ([2–4], but see [5,6]). ^{45}Ca-exchange experiments conducted in this laboratory have provided evidence that adrenaline or phenylephrine stimulates the inflow of calcium across the plasma membrane (cf. [8,9]) to a small compartment of cellular exchangeable calcium, tentatively identified as cytoplasmic calcium, as well as the release of calcium from the mitochondria and possibly the endoplasmic reticulum [7].

The mechanisms by which α-adrenergic agonists, vasopressin and angiotensin alter cellular calcium transport have not yet been described. Each of these agonists has been shown to stimulate phosphatidylinositol hydrolysis [10,11], and it has been proposed that this may be a step in the process by which the agonists modify cellular calcium-transport systems [10,11]. Evidence that phosphatidic acid, the formation of which is increased during the stimulation of phosphatidylinositol hydrolysis [12], can act as a calcium ionophore [13–15] and catalyses the calcium-dependent release of rubidium from slices of rat parotid gland [15] and contraction of smooth muscle cells [16] has been presented. These observations raised the possibility that phosphatidic acid may act as a calcium ionophore which mediates the actions of agonists on calcium transport in the liver cell. We now present results of experiments designed to test this proposal.

2. Materials and methods

The isolation of parenchymal cells from the livers of fed rats and the measurement of ^{45}Ca-exchange by isolated hepatocytes under steady-state conditions were done as in [7]. Fits of exponential equations and compartment configurations (see section 3) to the data were performed using the non-linear iterative curve-fitting procedures in [7]. Degrees of significance between the values of a given parameter for untreated and phosphatidic acid-treated cells were assessed using a *t*-test [17]. The rate of glucose release (assayed as in [19]) from hepatocytes incubated under the same conditions as those employed for ^{45}Ca-exchange studies was measured over a 10 min period following addition of the agent under test to the incubation medium.

The isolation of liver mitochondria, measurement of their integrity and concentration [18], measurement of ^{45}Ca-exchange at 37°C and estimation of the free extramitochondrial calcium concentration [7] were done as described.

The sodium salt of dipalmitoyl L-α-phosphatidic acid (P-4013, Sigma, St Louis, MO) was dissolved in warm water or incubation medium by extensive use of a vortex mixer. Samples of the resulting milky solution (or fine suspension) were added to the incubation media to give the final concentrations indicated.

The calcium ionophore A23187 was a gift from Dr R. L. Hamill (Lilly Research Labs.) and was used as a solution in dimethylsulphoxide. All other reagents were obtained from the sources in [7].

3. Results

Exposure of isolated liver cells to phosphatidic acid for 15 min before the addition of tracer quantities

of $^{45}CaCl_2$ increased the amount of ^{45}Ca associated with the cells (fig.1). Dose–response experiments showed that the quantity of ^{45}Ca exchanged by the cells at 10 min was 0.34 ± 0.01 nmol/mg wet wt cell (mean ± SEM, $n = 4$) in the absence of phosphatidic acid and 0.32 ± 0.03, 0.36 ± 0.01 and 0.40 ± 0.01 nmol/mg in the presence of 1, 10 and 100 μM phosphatidic acid, respectively. A significant increase ($P < 0.05$) was observed only in the presence of 100 μM phosphatidic acid.

Kinetic analysis showed that an equation which contains a constant term and two exponential terms [7] is the simplest equation which is consistent with

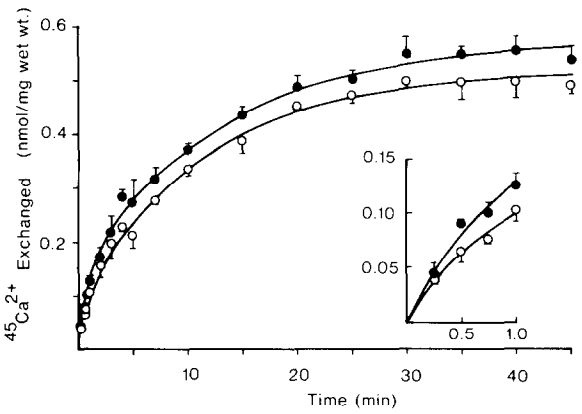

Fig.1. Effect of phosphatidic acid on ^{45}Ca-exchange in isolated hepatocytes incubated in the presence of 1.3 mM calcium. The inset shows the quantities of ^{45}Ca exchanged within the first minute. The composition of the complete incubation medium is described in [7]. Hepatocytes were added to the incubation medium in the presence of 1.3 mM $CaCl_2$ and absence (○) or presence (●) of 100 μM phosphatidic acid at 37°C, and incubated for 15 min before the addition of tracer quantities of $^{45}Ca^{2+}$ (0.5 MBq) at 0 min. Separation of the cells from the incubation medium and measurement of the amount of ^{45}Ca associated with the cells were done as in section 2. The data are the means ± SEM for 4–6 separate determinations at each time point. The lines were drawn using values of the constants for a fit of a 3 compartment closed system (see text) to the data. It was found that in the presence of phosphatidic acid but absence of hepatocytes some ^{45}Ca entered the $HClO_4$ layer after centrifugation of samples of the incubation medium through silicon oil [7], presumably as a result of the formation of a ^{45}Ca–phosphatidic acid precipitate. The quantities of ^{45}Ca were found to be equivalent to 0.007 ± 0.006, 0.006 ± 0.004, 0.005 ± 0.002, 0.017 ± 0.013 and 0.028 ± 0.007 nmol/mg wet wt cells (mean ± SEM, $n = 5$) at 5, 10, 15, 20 and 30 min after the addition of ^{45}Ca. Corrections for this contribution to ^{45}Ca associated with the cells were made, and the corrected values are those shown in the figure.

the data obtained for both untreated cells and cells treated with phosphatidic acid (fig.1; values of the kinetic constants not shown). A significant increase ($P < 0.05$) in the value of the plateau of the curve of best fit from 0.52 ± 0.01 nmol exchangeable calcium/mg wet wt for untreated cells to 0.57 ± 0.02 for cells treated with phosphatidic acid was observed. In agreement with [7], a three compartment closed system (the medium (1) and two kinetically-distinct compartments of exchangeable calcium, (2) and (3), associated with the cell) was found to be consistent with the data (fig.1, table 1). Phosphatidic acid induced an increase of 1.7-fold in the quantity of exchangeable calcium in compartment (2) but little change in that in compartment (3). The largest absolute change in calcium flux was an increase of 0.03 nmol . min^{-1} . mg wet wt^{-1} in R_{21}, the flux between medium and cellular compartment (2).

The effect of phosphatidic acid on the uptake of calcium (measured by atomic absorption spectroscopy) by hepatocytes at 1°C was also investigated (fig.2). Evidence that calcium uptake measured in this manner represents the inflow of calcium to the cell across the plasma membrane has been presented [20]. Phos-

Table 1

Effect of phosphatidic acid on the values (± SD) of the calcium fluxes and compartment sizes obtained for a fit of a 3 compartment closed system to ^{45}Ca-exchange data for isolated hepatocytes incubated in the presence of 1.3 mM intracellular calcium

Kinetic parameter	Untreated cells	Phosphatidic acid-treated cells
Fluxes (nmol . min^{-1} . mg^{-1})		
R_{21}	0.18 ± 0.04	0.21 ± 0.02
R_{32}	0.046 ± 0.006	0.034 ± 0.007
Compartment sizes (nmol . mg^{-1})		
Q_2	0.11 ± 0.03	0.20 ± 0.03[a]
Q_3	0.41 ± 0.03	0.38 ± 0.02
Weighted sums of squares	1.3×10^{-3}	0.43×10^{-3}

The series configuration of three compartments of exchangeable calcium was fitted to the data of fig.1 (4–6 expt) as in section 2. The quantity of exchangeable calcium present in compartment (i) is represented by Q_i, and the flux of calcium to compartment i from compartment j by R_{ij}. The quantity of exchangeable calcium in compartment (1) Q_1, was 42 nmol . mg^{-1}. The degree of significance [17] between the values of a given parameter for untreated and phosphatidic acid-treated cells was [a] $P < 0.05$

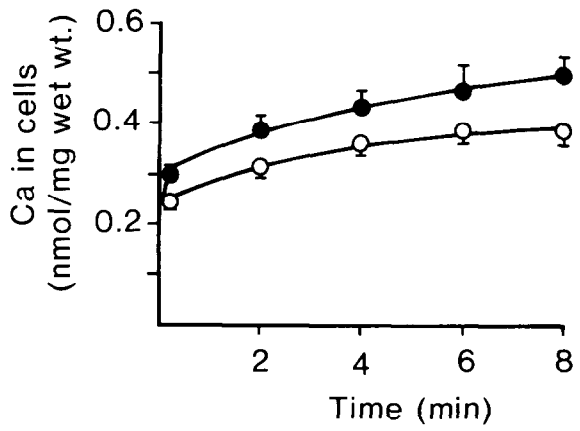

Fig.2. Effect of phosphatidic acid on the uptake of calcium by hepatocytes incubated at 1°C. The composition of the incubation medium was that in fig.1. Hepatocytes preisolated in a calcium-free medium and kept in a calcium-free medium at 0°C [7] were equilibrated with incubation medium in the absence of added calcium, and in the absence (○) or presence (●) of 100 μM phosphatidic acid for 3 min at 1°C. Calcium uptake was initiated by the addition of $CaCl_2$ (1.3 mM final conc.) at $t = 0$ min. At the times indicated, the amount of calcium in the cells was measured as in [7]. Each data point is the mean ± SEM of 6 separate expt.

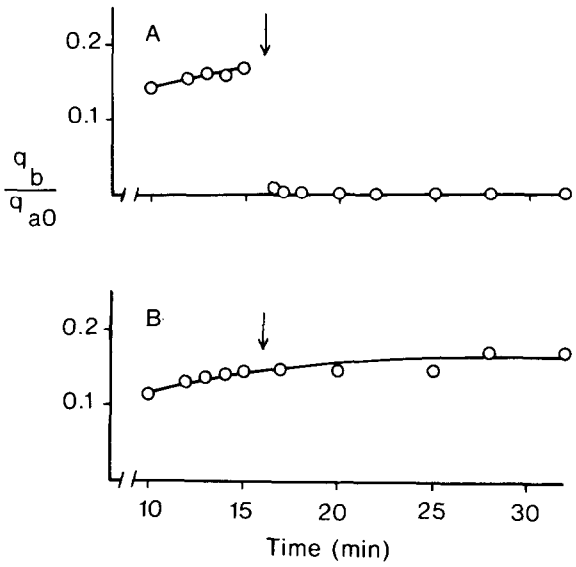

Fig.3. Effects of the calcium ionophore A23187 (A) and phosphatidic acid (B) on the quantity of ^{45}Ca associated with isolated mitochondria incubated at 37°C in the presence of 0.14 μM free calcium (25 μM added total calcium). The composition of the complete incubation medium is described in [7]. After an equilibration period of 15 min, ^{45}Ca (24 kBq) was added (0 min). After 16 min (indicated by the arrow), 5 μM (final conc.) ionophore A23187 (A) or 20 μm phosphatidic acid (B) was added. Estimation of the concentration of free calcium, separation of the mitochondria from the incubation medium and estimation of the quantity of ^{45}Ca associated with the mitochondria, q_b, expressed as a fraction of the initial dose of ^{45}Ca, $q_{a,o}$, were done as in section 2. The data shown represent those obtained from 1 of 5 expt which gave similar results.

phatidic acid increased the rate of calcium uptake (measured as the slope of the line over 2–8 min) from 0.011 ± 0.003 (mean ± SEM, $n = 5$) nmol min^{-1} mg wet wt^{-1} to 0.023 ± 0.003 ($P < 0.05$). Phosphatidylcholine, added as a solution in chloroform, had no effect on the rate of calcium uptake (not shown). In similar experiments conducted with human red blood cells, phosphatidic acid was found to have no effect on the slope of the curve ($n = 4$; not shown). Control experiments conducted in the absence of cells, or in the presence of red blood cells, showed that the rapid increase in calcium uptake observed at 0.25 min in the presence of phosphatidic acid (fig.2) is due to the precipitation of some calcium as a calcium–phosphatidic acid complex.

Since the data indicate that phosphatidic acid may increase the quantity of calcium in the cell cytoplasm, the effect of this agent on the release of glucose from isolated hepatocytes was tested. The rate of glucose release was increased from 0.69 ± 0.08 (mean ± SEM, $n = 4$) for control cells to 1.09 ± 0.07 ($P < 0.05$) in the presence of 100 μM phosphatidic acid, compared with 1.77 ± 0.16 ($P < 0.05$) in the presence of 10^{-6} M adrenaline.

When added to hepatocytes which had been equilibrated with ^{45}Ca at 100 μM extracellular calcium, 80 μM phosphatidic acid induced a small decrease in the amount of ^{45}Ca associated with the cells (not shown). The magnitude of this decrease was 35 ± 21% ($n = 3$) of that induced by adrenaline [7] and in contrast to the effect of the hormone, which was maintained for at least 30 min [7], was transient in nature (complete within 5 min).

When compared with the ionophore A23187 (fig.3A), phosphatidic acid at 1–100 μM did not induce the release of calcium from isolated mitochondria which had been allowed to exchange ^{45}Ca at 37°C in the presence of 0.14 μM free calcium, Mg^{2+}, ATP and P_i (fig.3B). Furthermore, when 100 μM phosphatidic acid was added at the beginning of the equilibration period, no change in the shape of the ^{45}Ca-exchange curve was observed (not shown).

4. Discussion

Taken together, the results of the ^{45}Ca-exchange experiments and the measurement of calcium uptake at 1°C indicate that phosphatidic acid induces a small increase in the rate of calcium uptake by liver cells. The effect of phosphatidic acid on ^{45}Ca-exchange clearly differs from that of adrenaline [7]. Whereas the hormone was found to stimulate the initial rate of ^{45}Ca-exchange and cause a small decrease in the plateau of the exchange curve [7], phosphatidic acid increased both the initial rate of exchange and plateau of the exchange curve.

The concentration of phosphatidic acid (100 μM) required to induce an effect on calcium inflow is high in comparison with the concentration of the calcium ionophone A23187 (~5 μM) which induces changes in calcium transport in the liver cell [21]. However, concentrations of ~100 μM phosphatidic acid were required to give substantial effects on ^{86}Rb efflux from rat parotid gland slices [15]. The effect of phosphatidic acid on the initial rate of ^{45}Ca exchange was less than that induced by adrenaline under the same conditions [7]. Nevertheless, it is concluded that the data are consistent with the proposal that an increase in the concentration of phosphatidic acid induced by the action of an agonist on the liver cell may mediate the stimulation, by the agonist, of calcium inflow to the cell.

The results of experiments conducted with both isolated mitochondria and isolated hepatocytes provide no evidence to support the proposal that phosphatidic acid mediates the adrenaline-induced release of calcium from mitochondria. This conclusion is subject to the reservations that:

(i) Phosphatidic acid generated within the liver cell as a result of the stimulation of phosphatidylinositol hydrolysis may interact with mitochondria in a manner which differs from that for phosphatidic acid under the conditions employed in the present experiments;

(ii) The nature of the 2 acyl groups on phosphatidic acid may influence its action on mitochondrial calcium efflux.

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