Exogenous phospholipase enzymes mimic effects of phenylephrine on Ca^{2+} transport in hepatocytes

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Phospholipase C from *Clostridium perfringens* induced the release of $^{45}\text{Ca}^{2+}$ from isolated rat hepatocytes incubated at 0.1 mM extracellular Ca^{2+} with a time course similar to that for the action of phenylephrine. Under the conditions of these experiments, no significant damage to the plasma membrane was detected in the presence of phospholipase C. Little $^{45}\text{Ca}^{2+}$ release was induced by bee venom phospholipase A_2 . At 1.3 mM extracellular Ca^{2+} , both phospholipase enzymes stimulated the initial rate of $^{45}\text{Ca}^{2+}$ exchange. Concentrations of phospholipase C comparable with those that stimulated $^{45}\text{Ca}^{2+}$ release increased the rates of glucose release and O_2 utilization by 70 and 20% respectively. An increase in the rate of O_2 utilization but not glucose release was observed after the addition of phospholipase A_2 to hepatocytes. The possible role for a cellular phospholipase C in the mechanism by which phenylephrine stimulates glycogenolysis in the liver cell is briefly discussed.

Stimulation of hepatic glycogenolysis by α -adrenergic agonists has been shown to involve an increase in the cytoplasmic free Ca^{2+} concentration (Murphy *et al.*, 1980; Exton, 1981). A large body of evidence indicates that this is due to the release of Ca^{2+} from the mitochondria and endoplasmic reticulum (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981b; Berthon *et al.*, 1981) and enhancement of Ca^{2+} inflow across the plasma membrane (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978; Barritt *et al.*, 1981b). It has been postulated that metabolites formed during hydrolysis of plasma membrane phosphatidylinositol catalyse the inflow of Ca^{2+} across the plasma membrane (Michell, 1975; Billah & Michell, 1979; Barritt *et al.*, 1981a) (but see Hawthorne, 1982) and its release from intracellular stores (Billah & Michell, 1979; Barritt, 1981; Whiting & Barritt, 1982). In the activation of phosphatidylinositol hydrolysis, the rate-limiting step stimulated by agonists is the reaction catalysed by phosphatidylinositol-specific phospholipase C (Michell, 1975).

The aim of the present experiments was to test the proposal that the action of cellular phospholipase C is required for expression of the effects of α -adrenergic agonists on glycogenolysis in the liver cell. The effects of exogenous phospholipases C and

A_2 on Ca^{2+} release, Ca^{2+} uptake and glucose release in isolated hepatocytes were investigated. The results indicate that exogenous phospholipase C mimics the effects of phenylephrine on these processes and are consistent with a role for cellular phospholipase C in the mechanism by which phenylephrine stimulates glycogenolysis.

Experimental

The isolation of hepatocytes from fed rats, measurement of $^{45}\text{Ca}^{2+}$ release at 0.1 mM- Ca^{2+} and rates of Ca^{2+} exchange at 1.3 mM- Ca^{2+} (using $^{45}\text{Ca}^{2+}$), glucose release, cyclic AMP concentrations, O_2 utilization and cell viability were performed as described previously (Barritt *et al.*, 1981b; Whiting & Barritt, 1982). The incubation medium contained, in a final volume of 3.0 or 6.0 ml, 117 mM-NaCl, 4.7 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 , 20 mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid)/KOH, 30 mg wet wt. of cells/ml and CaCl_2 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. Results are expressed as means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses. The degrees of significance, P , were determined by Student's t test. Values of $P > 0.05$ were considered to be not significant.

Phospholipase C from *Clostridium perfringens* (type IX) and phenylmethanesulphonyl fluoride were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Phospholipase C was dissolved in 10 mM-sodium acetate (pH 6.0) immediately before use. Bee venom phospholipase A₂, which has predominantly phospholipase A₂ activity (Shipolini *et al.*, 1971), was from Calbiochem-Behring Australia Pty., Carlingford, N.S.W., Australia, and was dissolved in water immediately before use.

Results and discussion

The effects of phospholipase enzymes on Ca²⁺ release from isolated hepatocytes were investigated at 0.1 mM extracellular Ca²⁺, conditions under which the effects of agonists on this parameter are most pronounced (Whiting & Barritt, 1982). [Under these conditions, it is proposed that Ca²⁺ released from intracellular organelles by the action of an α -adrenergic agonist is rapidly pumped out of the cell by the plasma membrane Ca²⁺ pumps (Blackmore *et*

Fig. 1. Comparison of the effects of phospholipases C and A with that of phenylephrine on ⁴⁵Ca²⁺ release from isolated hepatocytes

Cell incubations and measurement of the amounts of ⁴⁵Ca²⁺ associated with isolated hepatocytes were performed as described in the Experimental section. Hepatocytes were incubated in the presence of 0.1 mM extracellular ⁴⁵CaCl₂ (added at zero time) in the presence (Δ) or absence (○) of 0.37 mM-dinitrophenol. The final concentrations of the agents tested (added at 30 min as indicated by the arrows) were: (a), no addition; (b–d), 0.5, 1.0 and 1.5 unit of phospholipase C respectively/ml; (e), 0.5 μg of phospholipase A₂/ml; (f), 10 μM-phenylephrine. Results are means ± S.E.M. of three (d and f), four [a (dinitrophenol) and c], five (e) or six [a (no dinitrophenol) and b] experiments. The degrees of significance are: †, *P* < 0.05; *, *P* < 0.02; **, *P* < 0.01; ***, *P* < 0.001. The arrow indicates the point of addition of agent.

Fig. 2. Effects of phospholipases C and A on $^{45}\text{Ca}^{2+}$ exchange by isolated hepatocytes at 1.3 mM extracellular Ca^{2+}

Cell incubations and measurement of the amounts of $^{45}\text{Ca}^{2+}$ associated with isolated hepatocytes were performed as described in the Experimental section. Hepatocytes were incubated in the presence of 1.3 mM extracellular Ca^{2+} for 15 min before the addition of tracer amounts of $^{45}\text{Ca}^{2+}$ at zero min. The additions were: O, none; ●, 1 unit of phospholipase C/ml added 2 min before the $^{45}\text{CaCl}_2$; Δ, 0.5 μg of phospholipase A/ml added 5 min before the $^{45}\text{CaCl}_2$. The results are the means $\pm$ S.E.M. (indicated by the bars) of four experiments.

al., 1979).] Addition of phospholipase C induced a loss of $^{45}\text{Ca}^{2+}$ (Figs. 1a–1d). The time of onset was similar to that for the action of phenylephrine (Fig. 1f), although the maximum amount of $^{45}\text{Ca}^{2+}$ released was less than that released by phenylephrine. A slow re-accumulation of $^{45}\text{Ca}^{2+}$ began after about 3 min exposure to phospholipase C. In the presence of dinitrophenol no significant decrease in $^{45}\text{Ca}^{2+}$ was observed (Figs. 1a and 1c), indicating that the $^{45}\text{Ca}^{2+}$ released by phospholipase C does not come from $^{45}\text{Ca}^{2+}$ bound to extracellular sites, but may arise from the mitochondria and endoplasmic reticulum (Chen *et al.*, 1978; Barritt *et al.*, 1981b). Phospholipase A (0.5 $\mu\text{g}/\text{ml}$) caused a small transient loss of $^{45}\text{Ca}^{2+}$ followed by a substantial accumulation of the isotope (Fig. 1e). At a concentration of 0.1 $\mu\text{g}/\text{ml}$, phospholipase A had a

negligible effect on the amount of $^{45}\text{Ca}^{2+}$ associated with the cells under the conditions of Fig. 1(e).

When tested at 1.3 mM extracellular Ca^{2+} , the concentrations of phospholipases C and A employed in Figs. 1(c) and 1(e), respectively increased the initial rate of $^{45}\text{Ca}^{2+}$ exchange (Fig. 2). Experiments conducted at different concentrations of phospholipase A showed that a substantial increase (70%) in the initial rate of $^{45}\text{Ca}^{2+}$ exchange was observed with 0.25 $\mu\text{g}/\text{ml}$ added 2 min before the addition of $^{45}\text{Ca}^{2+}$. These observations indicate that exogenous phospholipases C and A stimulate the rate of Ca^{2+} inflow to the cell across the plasma membrane, an effect similar to that induced by adrenaline and phenylephrine (Barritt *et al.*, 1981b). The results also indicate that the re-accumulation of $^{45}\text{Ca}^{2+}$ observed at 0.1 mM extracellular Ca^{2+} (Figs. 1c, 1d and 1e) is due to enhanced net Ca^{2+} inflow.

Phospholipase C, but not phospholipase A, increased the rate of glucose release by isolated hepatocytes to values comparable with those obtained in the presence of phenylephrine or adrenaline (Table 1). The stimulation of glucose release was observed within 4 min after the addition of phospholipase C. Maximum stimulation was observed at 1 unit of phospholipase C/ml, a concentration that had a maximal effect on $^{45}\text{Ca}^{2+}$ release (Fig. 1c). No increase in the concentration of cyclic AMP was observed in the presence of phospholipase C. The concentrations of cyclic AMP in the cells plus incubation medium (0.1 mM extracellular Ca^{2+}) under control conditions, or at 2 min after the addition of 1 unit of phospholipase C/ml or 0.1 μM -glucagon were 0.45 ± 0.06 , 0.51 ± 0.04 ($P < 0.5$) and 2.13 ± 0.39 ($P < 0.01$) pmol/mg wet wt. ($n=4$) respectively. Similar values were obtained at 4 min after addition of these agents (results not shown). Phospholipase C stimulated O_2 utilization to the same extent as phenylephrine (Jakob & Diem, 1975; Sugano *et al.*, 1978; Scholz & Schwabe, 1980; Dehay *et al.*, 1981; Reinhart *et al.*, 1982) (Table 1), although at concentrations of the enzyme slightly higher than those that stimulated $^{45}\text{Ca}^{2+}$ release (Figs. 1b–1d). Phospholipase A also gave a marked stimulation of O_2 utilization at 0.1 mM- Ca^{2+} (Table 1). Similar effects of phospholipase A on O_2 utilization were observed at 1.3 mM- Ca^{2+} (results not shown).

The possibility that the effects of phospholipase C on Ca^{2+} movement and glucose release may be due to the action of a contaminating proteinase enzyme was tested by using phenylmethanesulphonyl fluoride, an inhibitor of proteinase activity. In the presence of this inhibitor, the effects of phospholipase C on $^{45}\text{Ca}^{2+}$ release, the initial rate of $^{45}\text{Ca}^{2+}$ exchange, and glucose release were similar to those observed in the absence of the inhibitor, indicating that the observed changes in these

Table 1. *Effects of phospholipases C and A on glucose release and rates of O₂ utilization by isolated hepatocytes*
Rates of utilization and glucose release were measured at 37°C in the presence of 0.1 mM extracellular Ca²⁺ as described in the Experimental section. Rates of O₂ utilization were calculated from the slopes of plots of O₂ concentration as a function of time in the 2 min period after the addition of cells to the incubation medium. Rates of glucose release were calculated from the slopes of linear plots of the amount of glucose present in the extracellular medium as a function of time over a period of 13 min after the addition of cells to the incubation medium. The degrees of significance are: *, $P < 0.01$; **, $P < 0.001$.

Addition	Rate of glucose release ($\mu\text{mol/min per g wet wt.}$)	Rate of O ₂ utilization ($\mu\text{g-atom/min per g wet wt.}$)
None	0.87 ± 0.05 (18)	3.23 ± 0.04 (12)
Phospholipase C (units/ml)		
0.5	0.93 ± 0.06 (4)	3.44 ± 0.23 (3)
1.0	1.47 ± 0.21 (7)**	3.14 ± 0.09 (3)
1.5	1.48 ± 0.15 (5)**	
2.0		3.56 ± 0.11 (5)*
3.0		3.80 ± 0.08 (6)**
4.0		3.92 ± 0.11 (6)**
Phospholipase A ($\mu\text{g/ml}$)		
0.025		3.89 ± 0.35 (3)
0.05		3.86 ± 0.02 (3)
0.10	0.97 ± 0.06 (3)	5.41 ± 0.30 (5)**
0.25		5.26 ± 0.37 (4)*
0.50	1.09 ± 0.12 (5)	5.98 ± 0.41 (4)**
Phenylephrine (40 μM)	1.13 ± 0.08 (14)*	4.07 ± 0.24 (3)**
Adrenaline (1 μM)	1.74 ± 0.16 **	

Table 2. *Effects of phospholipases C and A on lactate dehydrogenase loss and the exclusion of Trypan Blue by isolated hepatocytes*

Hepatocytes were incubated as described in the Experimental section for 5 min in the presence of 0.1 mM-Ca²⁺ before the addition of phospholipase. The quantities of lactate dehydrogenase in the extracellular medium and in the cells plus the incubation medium were determined as described in the Experimental section. The degrees of significance are: *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$; ****, $P < 0.001$.

Addition	Proportion of lactate dehydrogenase outside cells at increasing times after phospholipase addition (%)				Proportion of cells that excluded Trypan Blue at 12 min after phospholipase addition (%)
	1 min	2 min	3 min	5 min	
None	16 ± 1 (9)	21 ± 2 (4)	18 ± 2 (9)	14 ± 2 (5)	88 ± 1 (10)
Phospholipase C (units/ml)					
1.0	17 ± 2 (7)	20 ± 3 (4)	20 ± 2 (6)	21 ± 2 (3)*	83 ± 2 (6)*
1.5	16 ± 1 (6)	24 ± 2 (3)	26 ± 3 (5)*	25 ± 1 (3)***	80 ± 2 (5)****
Phospholipase A (0.5 $\mu\text{g/ml}$)	18 ± 1 (4)		21 ± 2 (4)	22 ± 2 (3)**	85 ± 2 (4)

parameters are unlikely to be due to proteinase activity.

The concentration of phospholipase C (1 unit/ml) that had maximal effect on Ca²⁺ release (Fig. 1c) did not significantly increase the loss of lactate dehydrogenase from hepatocytes during the first 3 min of exposure of the cells to the enzyme (Table 2). Only small changes in cell viability were detected when the cells were exposed to phospholipase C for 5 or 12 min, or to phospholipase A (Table 2). The proportion of lactate dehydrogenase in the extracellular medium in the absence of phospholipase enzymes is within the range reported previously for isolated hepatocytes incubated under similar conditions (Hopgood *et al.*, 1977).

The results have shown that short exposure of

hepatocytes to exogenous phospholipase C induces the release of Ca²⁺ from intracellular stores, Ca²⁺ inflow across the plasma membrane, glucose release and O₂ utilization. These changes were similar to those induced by phenylephrine, and were not associated with significant damage to the plasma membrane. The difference between the concentrations of phospholipase C that affect ⁴⁵Ca²⁺ release and O₂ utilization may reflect different sensitivities of these two parameters to the actions of α -adrenergic agonists.

The present results indicate that metabolites released from the hydrolysis of plasma-membrane phospholipids by an exogenous phospholipase C induce the release of Ca²⁺ from intracellular stores in the liver cell and enhance Ca²⁺ inflow across the

plasma membrane. A change in the phospholipid composition of the plasma membrane may also contribute to enhanced Ca^{2+} inflow. These changes are consistent with the proposal that cellular phospholipase C is involved in the mechanism by which phenylephrine alters Ca^{2+} transport in the liver cell, as proposed by Michell (1975). The results also indicate that cellular phospholipase A_2 may be involved in the agonist-induced stimulation of Ca^{2+} inflow across the plasma membrane.

Since it is likely that exogenous phospholipase C hydrolyses phospholipids located in the outer leaflet of the plasma membrane, action of the products of this hydrolysis at intracellular sites requires the translocation of these compounds across the plasma membrane. Such a translocation is unlikely to be required for the action of cellular phospholipase C since the majority of phosphatidylinositol in the plasma membrane is located in the inner leaflet (Low & Finean, 1977; Higgins & Evans, 1978). Moreover there is evidence to indicate that the cellular phospholipase C that catalyses the agonist-induced hydrolysis of phosphatidylinositol is located in the cytoplasm (Takenawa & Nagai, 1981; Hofmann & Majerus, 1982).

These considerations, together with possible differences in substrate specificity between exogenous and cytoplasmic phospholipases C (Stahl, 1973; Allan *et al.*, 1975; Takenawa & Nagai, 1981; Hofmann & Majerus, 1982) and the possibility that the action of phospholipase C activates α -adrenergic receptors suggest that the results obtained in the present study should be interpreted cautiously in relation to the possible involvement of cellular phospholipase C in the physiological mechanism by which α -adrenergic agonists alter cellular Ca^{2+} transport. Some evidence that the hydrolytic products formed by the action of exogenous phospholipase C from *Bacillus cereus* on neuroblastoma cells are similar to those formed by the action of cellular phospholipase C, and have similar effects on Ca^{2+} influx and cyclic GMP concentrations, has recently been provided (Ohsako & Deguchi, 1981).

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