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How far does phospholipase C activity depend on the cell calcium concentration?

A study in intact cells

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The dependence of phospholipase C activity on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in intact liver cells treated with the Ca^{2+}-mobilizing hormone vasopressin, or not so treated. Phospholipase C (PLC) activity was estimated from the formation of [^{3}H]inositol trisphosphate (InsP_3) and the degradation of [^{3}H]phosphatidylinositol 4,5-bisphosphate (PtdInsP_2). The $[Ca^{2+}]_i$ of the cells was clamped from 29 to 1130 nM by quin2 loading. This wide concentration range was obtained by loading the hepatocytes with a high concentration of the Ca^{2+} indicator in low-Ca^{2+} medium or by using the Ca^{2+} ionophore ionomycin in medium containing Ca^{2+}. In resting cells, in which $[Ca^{2+}]_i$ was 193 nM, treatment with 0.1 μM-vasopressin which stimulates liver PLC maximally, tripled InsP_3 content and raised $[Ca^{2+}]_i$ to 2 μM within 15 s. Lowering $[Ca^{2+}]_i$ partially decreased cell InsP_3 content as well as the ability of vasopressin to stimulate InsP_3 formation maximally. At 29 nM, the lowest Ca^{2+} concentration obtained in isolated liver cells, basal InsP_3 content was 64% of that measured in control cells. Addition of vasopressin no longer affected $[Ca^{2+}]_i$, but significantly increased InsP_3 by 200%, although less than in the controls (300%). The maintenance of the greater part of the PLC response at constant $[Ca^{2+}]_i$ indicated that, in the liver, InsP_3 formation does not result from an increase in $[Ca^{2+}]_i$. The effects of lowering $[Ca^{2+}]_i$ were reversible. When low cell$[Ca^{2+}]_i$ was restored to a normal value, resting InsP_3 content and the ability of vasopressin to stimulate InsP_3 formation maximally by 300% were also restored. Raising $[Ca^{2+}]_i$ from 193 to 1130 nM had little effect on the InsP_3 content or the vasopressin-mediated increase in InsP_3. In agreement with the stimulation of PLC activity by vasopressin, cell [^{3}H]PtdInsP_2 and total PtdInsP_2 were degraded by application of this hormone for 15 s. In contrast, when $[Ca^{2+}]_i$ was lowered to 29 nM, basal [^{3}H]PtdInsP_2 and total PtdInsP_2 were increased by about 30%, [^{3}H]PtdInsP_2 was further increased by vasopressin, but total PtdInsP_2 was not changed. These results show that, in intact hepatocytes, PLC is little affected by $[Ca^{2+}]_i$ concentrations above 193 nM, but is partially dependent on Ca^{2+} below that value. They suggest that, in addition to activating PLC activity, vasopressin might stimulate PtdInsP_2 synthesis, presumably via phosphatidylinositol-phosphate kinase, and that this pathway might predominate in cells with low $[Ca^{2+}]_i$.

INTRODUCTION

In the cells of liver and many other tissues, phosphoinositide metabolism is involved in coupling the receptors occupied by Ca^{2+}-mobilizing hormones (Michell, 1975, 1983). Inositol 1,4,5-trisphosphate (InsP_3), formed by the cleavage of phosphatidylinositol 4,5-bisphosphate (PtdInsP_3) by phospholipase C (PLC), functions as a second messenger by permeabilizing the endoplasmic reticulum to Ca^{2+} (Burgess *et al.*, 1984; Joseph *et al.*, 1984). This process, combined with hormone-mediated Ca^{2+} influx, allows the internal free Ca^{2+} ($[Ca^{2+}]_i$) to increase, and to stimulate the glycogenolytic cascade (Binet *et al.*, 1985; for review see Williamson *et al.*, 1985; Putney, 1986). The Ca^{2+}-dependency of PLC activation by vasopressin and other Ca^{2+}-mobilizing hormones has been a matter of controversy since Creba *et al.* (1983) reported that PtdInsP_2 hydrolysis after addition of vasopressin was independent of Ca^{2+}, whereas Rhodes *et al.* (1983) reported that Ca^{2+} was essential for PtdInsP_2 hydrolysis induced by vasopressin. In all these studies, the role of Ca^{2+} in phosphoinositide metabolism was investigated either by incubating the hepatocytes in media containing EGTA, in order to eliminate the effect of the Ca^{2+} influx, or by treating them with the Ca^{2+} ionophore A23187. However, the cytosolic Ca^{2+} concentration was not determined. The development of the quin2 technique, which allows not only the monitoring of $[Ca^{2+}]_i$, but also its clamping to fixed values in suspensions of small cells, led us to re-examine this point in liver. This technique has been successfully applied to intact cells to establish the relationships in these cells between $[Ca^{2+}]_i$ on the one hand and membrane ion transport on the other (Berthon *et al.*, 1985; Poggioli *et al.*, 1985; for review, see Rink & Pozzan, 1985).

Abbreviations used: Ins$P$, InsP_2, InsP_3, inositol mono-, bis- and tris-phosphates respectively; PtdIns$P$, phosphatidylinositol 4-monophosphate; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; quin2/AM, quin2 tetra-acetoxymethyl ester.

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The Ca^{2+}-dependency of PLC activity, estimated from PtdInsP_2 hydrolysis and InsP_3 formation, was studied here in resting and vasopressin-stimulated rat hepatocytes. For this purpose, intact hepatocytes were either loaded with an excess of quin2 or treated with the non-fluorescent Ca^{2+} ionophore ionomycin. Under these conditions Ca^{2+} homoeostasis was not maintained, and rises of up to 50-fold in $[Ca^{2+}]_i$ were observed. These results show that, in intact hepatocytes, the basal PLC activity is dependent on $[Ca^{2+}]_i$ at concentrations between 29 and 160 nM, and is independent of $[Ca^{2+}]_i$ at concentrations above 160 nM. In low-$[Ca^{2+}]_i$ cells, a decrease in InsP_3 and an accumulation of PtdInsP_2 were observed, resulting from the inhibition of PLC and/or activation of PtdIns$P$ kinase. Even at very low $[Ca^{2+}]_i$, PLC was still activated by vasopressin, but to a lesser extent than under normal conditions.

EXPERIMENTAL

Cell isolation and incubation

Isolated hepatocytes from female Wistar rats weighing 200–250 g were prepared as previously described (Mauger *et al.*, 1985). The isolated cells were incubated at about 3×10^6 cells/ml at 37 °C in Eagle's medium containing 116 mM-NaCl, 5.4 mM-KCl, 1.8 mM-$CaCl_2$, 0.81 mM-$MgCl_2$, 0.92 mM-NaH_2PO_4, 25 mM-$NaHCO_3$, glucose (1 g/l), amino acids and vitamins supplemented with 1.5% gelatin (Difco) under O_2/CO_2 (19:1).

$[Ca^{2+}]_i$ determination

$[Ca^{2+}]_i$ was monitored by quin2 fluorescence (see Berthon *et al.*, 1984; Binet *et al.*, 1985). Control cells were incubated at a concentration of 1×10^6–1.5×10^6 cells/ml (condition E, Table 1) in Eagle's medium containing 50 μM-quin2 tetra-acetoxymethyl ester (quin2/AM) for 2.5 min at 37 °C, pH 7.4. To eliminate extracellular quin2/AM, cells were centrifuged once at 50 ***g*** for 1 min and resuspended in Eagle's medium without vitamins, amino acids or gelatin, because the fluorescence of these components impairs Ca^{2+}–quin2 fluorescence emission. When the $[Ca^{2+}]_i$ concentration was lowered to 29 or 35 nM, cells were incubated for either 5 min (condition A, Table 1) or 2.5 min (condition B) in modified Eagle's medium containing 0.12 μM-free Ca^{2+} (i.e. 80 μM-$CaCl_2$/250 μM-EGTA, according to Bartfai, 1979) and 75 μM-quin2/AM. Quin2/AM was eliminated as described above for the controls, cells were resuspended in the same low-Ca^{2+} medium (0.12 μM free Ca^{2+}) and $[Ca^{2+}]_i$ was determined 5 min later. $[Ca^{2+}]_i$ was decreased to 100 nM by loading the hepatocytes with quin2, as described for the controls, and then resuspending them in Eagle's medium containing 0.12 μM free Ca^{2+}. $[Ca^{2+}]_i$ was determined 4 min after cell resuspension (condition C, Table 1). $[Ca^{2+}]_i$ was decreased to 162 nM by loading the cells with quin2 and washing them like the control cells, but resuspending them in Eagle's medium containing 160 μM-Ca^{2+} (condition D). $[Ca^{2+}]_i$ was raised to 1130 nM by loading the cells with quin2 and washing and resuspending them like the control cells. Ionomycin (150 nmol/mg of cell protein) was then added and the external Ca^{2+} concentration was raised to 3 mM. $[Ca^{2+}]_i$ was determined 7.5 min later (condition F).

Phospholipid analysis

Inositol phosphates were measured as described previously (Poggioli *et al.*, 1986). Briefly, hepatocytes labelled with [^{3}H] inositol were resuspended at 4.5×10^6 cells/ml in inositol-free Eagle's medium containing 106 mM-NaCl$_2$ 160 μM-$CaCl_2$ and 10 mM-LiCl. The cell suspension was then preincubated for 10 min with LiCl, after which 0.3 ml samples of this suspension were withdrawn and diluted with 0.3 ml of Eagle's medium in the presence of $CaCl_2$, giving an external Ca^{2+} concentration of 1.8 mM (controls) and incubated for 30 s. Vasopressin or vehicle was then added, and 15 s later 0.5 ml of each sample was transferred to Eppendorf micro-tubes containing 1 ml of ice-cold incubation medium and quickly centrifuged for 5 s at 12000 ***g***. The neutralized extracts were processed by anion-exchange chromatography and sequentially eluted with 20 ml of 150 mM-ammonium formate/5 mM-sodium tetraborate to remove glycerophosphoinositol, with 10 ml of 150 mM-ammonium formate/5 mM-sodium tetraborate to remove Ins$P$, with 10 ml of 400 mM-ammonium formate/ 0.1 M-formic acid to remove InsP_2, and with 6 ml of 1 M-ammonium formate/0.1 M-formic acid to remove InsP_3 (this fraction contains the two isomers of InsP_3 and inositol tetrakisphosphate: Downes & Michell, 1981; Berridge *et al.*, 1983; Batty *et al.*, 1985). For experiments on cells with lower $[Ca^{2+}]_i$, 0.3 ml samples were collected at the end of the 10 min preincubation period, diluted and incubated with 0.3 ml of modified Eagle's medium containing quin2/AM, in order to create the same experimental conditions as those described for $[Ca^{2+}]_i$ measurement as A, B, C and D (Table 1). In these experiments, quin2/AM was not eliminated. The cells were either challenged with vasopressin for 15 s or not so challenged, and then treated like the controls. Cells with high $[Ca^{2+}]_i$ were preincubated, diluted in modified Eagle's medium, and the external Ca^{2+} concentration was raised to 3 mM as described for $[Ca^{2+}]_i$ determination. Ionomycin (150 nmol/mg of cell P) was added 15 s later for 1 min, before the addition of vasopressin or vehicle. Samples were then treated in the same way as controls.

Cell [^{3}H]phospholipid contents were determined under three experimental conditions: in low- and high-$[Ca^{2+}]_i$ cells and in control cells. The experimental procedure was identical with that described for inositol phosphate determination. The reaction was stopped by adding an 0.5 ml portion of the cell suspension to 1 ml of ice-cold 10% (v/v) $HClO_4$. After centrifugation at 1000 ***g*** for 15 min, the [^{3}H]phospholipids were extracted from the $HClO_4$-insoluble pellet. After deacylation, the glycerophosphoryl esters were separated and eluted as described by Creba *et al.* (1983). In some experiments, the total P in the polyphosphoinositides was determined. For this purpose, the cells were labelled by incubation in Eagle's medium with 15 μCi of [^{32}P]P_i for 90 min. Experimental procedures and phospholipid extraction have been described previously. Briefly, phospholipids were separated and assayed as described by Giraud *et al.* (1984). The phospholipids dissolved in chloroform were separated by one t.l.c. run [developed with chloroform/ methanol/4.3 M-NH_3 (9:7:2, by vol.)] on plates treated for 12 h with EGTA (20/1) as described by Steiner & Lester (1972). This procedure was used to separate phosphatidylinositol monophosphate (PtdIns$P$) and PtdInsP_2. Lipids were located on the t.l.c. plates by autoradiography. Radioactive areas were scraped off the

plates for total P measurement as described Rouser *et al.* (1970).

Materials

Collagenase was purchased from Boehringer; [arginine]vasopressin was from Sigma; Dowex 1X8 ion-exchange resin (formate form, 200–400 mesh) was from Bio-Rad; *myo*-[2-^{3}H]inositol was from New England Nuclear; sodium [^{32}P] phosphate was obtained from C.E.A. (France). Quin2/AM was from Lancaster Synthesis (Morecambe, Lancs., U.K.) All other chemicals were of reagent grade. Silica-gel t.l.c. plates were obtained from Schleicher and Schull (Dassel, Germany). X-ray films for autoradiography (X-OMAT AR) were from Kodak (France).

RESULTS

Dependence of basal and vasopressin-stimulated cell InsP_3 contents on $[Ca^{2+}]_i$ in intact isolated hepatocytes

The results in Table 1 clearly confirm that $[Ca^{2+}]_i$ can be decreased in isolated hepatocytes (Berthon *et al.*, 1985; Poggioli *et al.*, 1985) by altering three factors: the Ca^{2+} concentration in the quin2 loading medium, the duration of the quin2 loading and the internal quin2 concentration. A different approach was applied to increase $[Ca^{2+}]_i$: the cells were incubated with the Ca^{2+} ionophore ionomycin in the presence of high external Ca^{2+} concentrations.

Fig. 1(*a*) illustrates the effects of various $[Ca^{2+}]_i$ values on [^{3}H]InsP_3 content in control and vasopressin-treated cells. In non-stimulated control cells, lowering $[Ca^{2+}]_i$ from 193 to 29.5 nM (Fig. 1*b*) induced a statistically significant decrease in InsP_3 content, to $64 \pm 3\%$ ($n = 12$) of that in control cells incubated with 1.8 mM-$CaCl_2$. Raising $[Ca^{2+}]_i$ from 193 to 1130 nM with ionomycin did not modify cell InsP_3 content (Fig. 1*c* and Table 1, condition F versus condition E). The absence of effect of high $[Ca^{2+}]_i$ on InsP_3 was not due to the latter's hydrolysis by Ca^{2+}-activated phosphatases, since neither InsP_2 nor Ins$P$ accumulated under the same experimental conditions (Table 2). Stimulation of hepatocytes with 0.1 μM-vasopressin for 15 s elicited a significant increase in InsP_3 under all the conditions tested. In Fig. 1(*a*), the InsP_3 content determined in cells thus stimulated was plotted as a function of the $[Ca^{2+}]_i$ determined before vasopressin addition. This was of no importance for cells with very low $[Ca^{2+}]_i$ (29.5 or 35.9 nM), since vasopressin did not trigger any rise in $[Ca^{2+}]_i$, as shown in the traces in Fig. 1(*b*) (i.e. under conditions A and B of Table 1). Under the other conditions, the actual $[Ca^{2+}]_i$ was slightly higher than the value plotted, since InsP_3 was measured 15 s after vasopressin addition. Although vasopressin elicited InsP_3 accumulation in low-$[Ca^{2+}]_i$ cells (21.2 ± 2.0 to 46.7 ± 7.0 c.p.m./mg of protein, $n = 12$), this effect was smaller than that observed in the controls [33.5 ± 2.0 c.p.m./mg of protein ($n = 15$) for non-stimulated cells versus 110.0 ± 12.0 c.p.m./mg of protein ($n = 15$) for vasopressin-stimulated cells]. The possibility that the smaller effect of vasopressin on InsP_3 accumulation observed in low-$[Ca^{2+}]_i$ cells might result from a decreased capacity of vasopressin to bind to its receptors was investigated. Hepatocytes were incubated at different external Ca^{2+} concentrations for 10 min, a period short enough not to alter $[Ca^{2+}]_i$ (Binet *et al.*, 1985). When external $[Ca^{2+}]$ was decreased from 3000 to 160 and then to 0.12 μM (conditions F, D and A in Table 1), vasopressin induced the same InsP_3 accumulation

Table 1. Effect of vasopressin on $[Ca^{2+}]_i$ in hepatocytes with $[Ca^{2+}]_i$ fixed to different values before the hormone action

Cells (1.5×10^6–2×10^6 cells/ml) were loaded with 75 μM-quin2/AM for conditions A and B, or 50 μM for conditions C–F, by incubation in Eagle's medium containing a free Ca^{2+} concentration indicated as '$Ca^{2+}{}_1$' for the period designated as '$Time_1$', giving the internal quin2 concentration defined as 'quin2'. Cells were centrifuged once to eliminate external quin2/AM and resuspended in Eagle's medium containing a free Ca^{2+} concentration designated '$Ca^{2+}{}_2$' for the period corresponding to '$Time_2$', and quin2 fluorescence was recorded. Vasopressin (0.1 μM) was then added. After stabilization of the signal, quin2 fluorescence was calibrated in terms of $[Ca^{2+}]_i$ as described by Berthon *et al.* (1984).

	Preliminary incubation conditions					$[Ca^{2+}]_i$ (nM)	
	$Ca^{2+}{}_1$* (μM)	$Time_1$ (min)	quin2 (mM)	$Ca^{2+}{}_2$* (μM)	$Time_2$ (min)	Controls	Vasopressin-treated cells
A	0.12	5	1.5†	0.12	5	29.5 ± 1.6 (30)	33.0 ± 1.8 (90)
B	0.12	2.5	1.5	0.12	5	35.9 ± 2.0 (9)	40.6 ± 4.5 (11)
C	1800	2.5	0.7‡	0.12	4	93.4 ± 4.4 (12)	163.8 ± 15.0 (12)
D	1800	2.5	0.7	160	3	161.7 ± 9.0 (36)	426.1 ± 28.0 (32)
E	1800	2.5	0.7	1800	3	193.0 ± 8.5 (29)	2082 ± 179 (23)
F	1800	2.5	0.7	3000	3	1130 ± 196 (19)	2464 ± 187 (18)

* According to Bartfai (1979).
† from Berthon *et al.* (1985).
‡ from Poggioli *et al.* (1985).

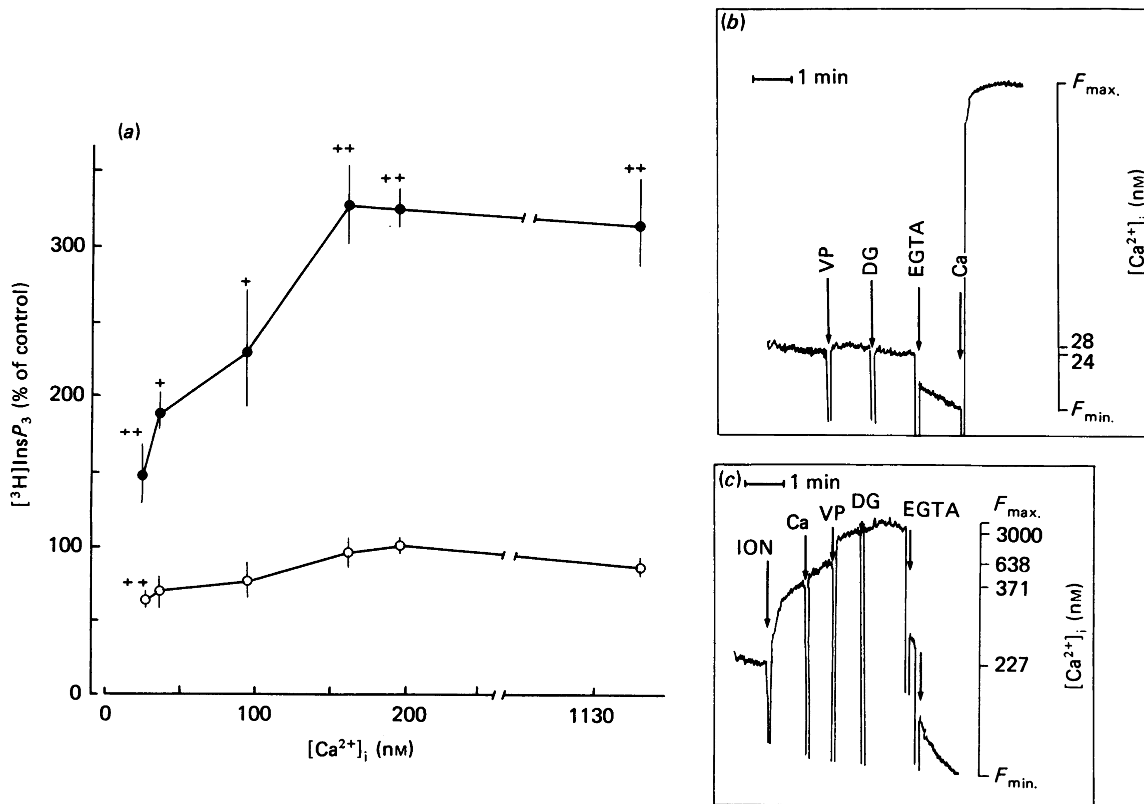

Fig. 1. Relationship between [Ca^{2+}]$_i$ and InsP_3 content in isolated hepatocytes

(*a*) [Ca^{2+}]$_i$ was calculated from quin2 fluorescence as described by Berthon *et al.* (1984) and varied as indicated in the Experimental section; values are means of 9 to 29 determinations on 3 to 13 different cell preparations. For clarity, S.E.M. bars have been omitted; they were generally less than 10% (for details see Table 1). For InsP_3 determinations, cells were labelled by incubation for 90 min in a medium containing 11 μM free inositol and 10 μCi of *myo*-[2-^{3}H]inositol/ml. Cells were washed and preincubated for 10 min in the presence of 10 mM-LiCl. The 0.3 ml samples were diluted with 0.3 ml of Eagle's medium to reproduce the experimental conditions described in Table 1 for [Ca^{2+}]$_i$ determination. When quin2/AM was used, it was not eliminated. It was omitted from the incubation medium of the controls and of the high-[Ca^{2+}]$_i$ cells. The incubations were stopped by adding chloroform/methanol/HCl (100:50:1, by vol.). The water-soluble [^{3}H]inositol phosphates were extracted and separated by anion-exchange chromatography. Cell InsP_3 content was expressed as a percentage of the InsP_3 content of control cells incubated in the presence of 1.8 mM-$CaCl_2$ (33.5 ± 3.0 c.p.m./mg of protein). ○, Non-stimulated cells; ●, cells stimulated for 15 s with 0.1 μM-vasopressin. Each point is the mean ± S.E.M. for 3 to 13 determinations in 3 to 13 cell preparations. (*b*) and (*c*), Cells were loaded with quin2 as indicated in the Experimental section. The quin2 fluorescence was recorded and calibrated in terms of [Ca^{2+}]$_i$ from $F_{max.}$ and $F_{min.}$ determined in the presence of digitonin and EGTA, taking cell autofluorescence into account. Successive additions to the incubation medium were: in (*b*), 0.1 μM-vasopressin (VP), 4 μM-digitonin (DG), 3.2 mM-EGTA and 4 mM-$CaCl_2$ (Ca); in (*c*), ionomycin (ION; 150 nmol/mg of protein), 1.2 mM-$CaCl_2$ (Ca), 0.1 μM-vasopressin (VP), 4 μM-digitonin (DG) and 24 mM-EGTA. Probability of significance according to Student's *t* test: †$P < 0.01$; ††$P < 0.001$.

Table 2. Effect of various [Ca^{2+}]$_i$ concentrations on InsP_2 and Ins$P$ contents in control hepatocytes and in hepatocytes stimulated with vasopressin

[Ca^{2+}]$_i$ and inositol phosphate contents were determined as described in the legend to Fig. 1. The cells were stimulated or not for 15 s with 0.1 μM–vasopressin. InsP_2 and Ins$P$ contents are expressed as percentages of control values determined on cells incubated in the presence of 1.8 mM-Ca^{2+} and in the absence of vasopressin (InsP_2, 70 ± 10 c.p.m./mg of protein; and Ins$P$, 288 ± 68 c.p.m./mg of protein). Each point is the mean ± S.E.M. for *n* determinations on different cell preparations. Probability of significance according to Student's *t* test: *$P < 0.01$; **$P < 0.001$.

[Ca^{2+}]$_i$ (nM)...	29		35		95		162		191		1130	
Vasopressin (0.1 μM)...	0	+	0	+	0	+	0	+	0	+	0	+
InsP_2	77.7 ± 4.0	116.0 ± * 10.0	69.1 ± 9.0	127.0 ± 12.0	93.7 ± 18.0	125.5 ± 10.0	92.0 ± 6.3	323.8 ± ** 41.0	100 ± 12.0	304.7 ± ** 38.0	87.6 ± 4.2	258.4 ± ** 39.0
n	12	12	4	4	7	6	8	8	15	12	9	9
Ins$P$	78.3 ± 5.0	80.6 ± 5.0	80.7 ± 6.0	84.5 ± 6.0	70.7 ± 5.0	96.1 ± 13.0	100.0 ± 9.0	131.5 ± 21.0	100 ± 19	120.2 ± 13.0	89.1 ± 7.0	101.3 ± 15.0
n	12	12	4	4	7	6	9	9	13	11	9	9

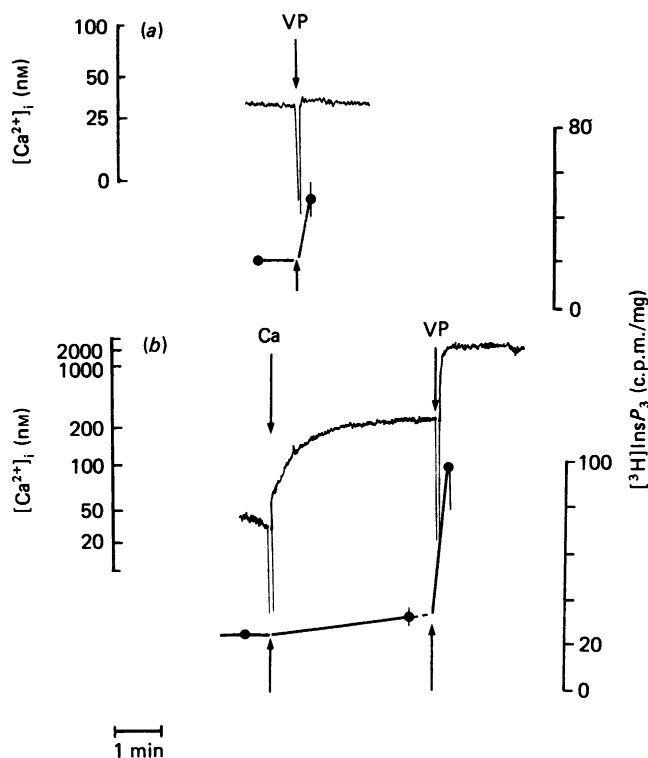

Fig. 2. Reversal by $CaCl_2$ of the effect of quin2 on cell $[Ca^{2+}]_i$ and $InsP_3$ content

(*a*) Cells (1.5×10^6–2×10^6 cells/ml) were loaded for 5 min with 75 μM-quin2/AM in modified Eagle's medium containing 120 nM external free Ca^{2+}, rinsed and re-suspended in the same medium. The quin2 fluorescence was recorded and calibrated in terms of $[Ca^{2+}]_i$, as described in the legend to Fig. 1. $InsP_3$ content was determined in prelabelled hepatocytes (2.3×10^6–3×10^6 cells/ml) loaded with quin2/AM under conditions identical with those used to measure $[Ca^{2+}]_i$, except that quin2/AM was not eliminated. The arrow corresponds to the addition of 0.1 μM-vasopressin (VP). The quin2 fluorescence is shown from one typical experiment out of 36 performed on 12 cell preparations. Each $InsP_3$ value is the mean ± S.E.M of 12 determinations on 12 cell preparations. (*b*) Cells were loaded with quin2/AM as described in (*a*). $CaCl_2$ (Ca) giving an external free Ca^{2+} concentrations of 1.8 mM was added 3 min before 0.1 μM-vasopressin (VP). The quin2 fluorescence is shown from one typical experiment out of 16 performed on three cell preparations. Each $InsP_3$ value is the mean ± S.E.M. for 3 to 12 determinations on 3 to 13 cell preparations.

(results not shown), indicating that the external Ca^{2+} concentration did not change vasopressin binding.

Comparison of the results in Table 1 with those in Fig.s 1(*a*) and 2(*a*) shows that vasopressin elicited $[^3H]InsP_3$ accumulation without triggering any rise in $[Ca^{2+}]_i$. All these observations indicate that, in hepatocytes stimulated with vasopressin, PLC activation occurs at very low $[Ca^{2+}]_i$ values and precedes any movement of Ca^{2+}.

Reversal by $CaCl_2$ of the effect of quin2 on $[Ca^{2+}]_i$ and polyphosphoinositide metabolism

Fig. 2(*a*) shows the effects of vasopressin on $[Ca^{2+}]_i$ and $InsP_3$ content of low-$[Ca^{2+}]_i$ cells. As previously shown, a significant increase in $InsP_3$ was observed without any change in $[Ca^{2+}]_i$. The failure of vasopressin to affect $[Ca^{2+}]_i$ was due both to the absence of external Ca^{2+} and to the depletion of internal Ca^{2+} stores. This was confirmed by the observation that addition of an effective concentration of ionomycin after vasopressin treatment caused no additional change in $[Ca^{2+}]_i$ (results not shown). Consequently, when $CaCl_2$ was added to the incubation medium at a final concentration of 1.8 mM (Fig. 2*b*), 3 min of incubation was long enough to restore completely the basal $[Ca^{2+}]_i$ and $InsP_3$ content in quin2-loaded cells. $[Ca^{2+}]_i$ increased almost 10-fold, from 29.5 to 276 nM, and $InsP_3$ increased from 21.2 ± 2.0 c.p.m./mg ($n = 12$) to 29.2 ± 3.6 c.p.m./mg ($n = 5$). Vasopressin addition induced the expected rise in $[Ca^{2+}]_i$ (from 276 to 1432 nM in that particular experiment) and raised $InsP_3$ content from 29.2 ± 3.6 to 97.2 ± 12.3 c.p.m./mg ($n = 4$). These results strongly support the possibility that in isolated rat hepatocytes Ca^{2+} has a role in PLC activity, since the restoration of external $[Ca^{2+}]$ restored not only the resting $[Ca^{2+}]_i$ and basal content of $InsP_3$ but also their response to vasopressin. As reported by Poggioli *et al.* (1985) and Berthon *et al.* (1985), the above observations rule out the possibility that quin2 loading has a harmful effect on cell metabolism.

Dependence of $InsP_2$ and $InsP$ formation in hepatocytes on $InsP_3$ hydrolysis

As shown in Table 2, $[Ca^{2+}]_i$ was not observed to affect $InsP_2$ and $InsP$ contents in non-stimulated cells. This may be due to hydrolysis of $InsP_3$ by the Mg^{2+}-activated phosphomonoesterase (Storey *et al.*, 1984), since this enzyme is insensitive to $[Ca^{2+}]_i$. In stimulated cells, no significant change was observed in $InsP$ accumulation. $InsP_2$ increased at nearly all the $[Ca^{2+}]_i$ concentrations tested. For the cells with $[Ca^{2+}]_i = 95$ nM, the results were somewhat variable, and no statistically significant change was detected with vasopressin. For $[Ca^{2+}]_i$ 24–95 nM, vasopressin-induced $InsP_2$ accumulation was stimulated by less than 1.5-fold. However, at higher Ca^{2+} concentrations, vasopressin increased cell $InsP_2$ content by 2.5–3-fold. The results might be attributable to the formation of $InsP_2$ in two different ways, depending on the initial $[Ca^{2+}]_i$ value.

Effect of various $[Ca^{2+}]_i$ concentrations on $PtdInsP_2$ content in hepatocytes stimulated by vasopressin and on unstimulated hepatocytes

Since the formation of $InsP_3$ always resulted from its production by the phosphodiesteratic attack on $PtdInsP_2$ by PLC and from its degradation by phosphomonoesterase, the $PtdInsP_2$ content of the cells was measured as a function of increasing $[Ca^{2+}]_i$. Both tritiated and total $PtdInsP_2$ were determined.

Table 3 shows that in non-stimulated cells the $[^3H]PtdInsP_2$ content found in high-$[Ca^{2+}]_i$ cells was not different from that measured in controls. Incubation for 15 s with vasopressin initiated a decrease in $[^3H]PtdInsP_2$ in cells with normal and high $[Ca^{2+}]_i$. Surprisingly, the $[^3H]PtdInsP_2$ content in low-$[Ca^{2+}]_i$ cells was increased, and this accumulation was further enhanced by vasopressin. As these last results were difficult to interpret in the absence of any information about the specific radioactivity of the phospholipids, we decided to

Table 3. PtdInsP_2 content in control hepatocytes and in hepatocytes stimulated with vasopressin

$[Ca^{2+}]_i$ values were calculated from quin2 fluorescence and were changed as indicated in the Experimental section. Cells were labelled and incubated as described in the legend to Fig. 1. Incubations were stopped by adding 0.5 ml of the cell suspension to 1 ml of ice-cold 10% $HClO_4$. The [^{3}H]phosphoinositides were extracted and deacylated. The water-soluble deacylation products were applied to anion-exchange columns and sequentially eluted as described in the Experimental section. The cells were stimulated or not for 15 s with 0.1 μM-vasopressin. Results are expressed as percentages of the [^{3}H]PtdInsP_2 content determined in control cells incubated in the presence of 1.8 mM-Ca^{2+} without vasopressin. Each value is the mean ±S.E.M. for *n* determinations on four cell preparations. The total PtdInsP_2 content of hepatocytes was assayed chemically, as described in the Experimental section. Results are expressed as nmol of P in PtdInsP_2 as a percentage of the nmol of P in total phospholipids. Each value is the mean ±S.E.M. for *n* determinations in two or three cell preparations: N.D., not determined. Probability of significance according to Student's *t* test: *$P < 0.05$; †$P < 0.01$; ††$P < 0.001$.

$[Ca^{2+}]_i$ (nM)...	29		191		1130	
Vasopressin (0.1 μM)...	0	+	0	+	0	+
[^{3}H]PtdInsP_2	121.0±4.9†	171.0±30.0†	100±2.2	61.5±7.9††	86.7±8.0	59.3±5.5††
n	6	7	7	6	7	6
Total PtdInsP_2	0.202±0.008*	0.197±0.019	0.171±0.010	0.134±0.013*	ND	ND
n	5	6	8	8		

determine the total phospholipid concentrations chemically, in normal- and low-$[Ca^{2+}]_i$ cells incubated with or without vasopressin. The results corroborated those obtained with radiolabelled PtdInsP_2 in the controls. Although PtdInsP_2 content rose when $[Ca^{2+}]_i$ was decreased in non-stimulated cells, vasopressin did not elicit any further accumulation of the chemically assayed PtdInsP_2.

Taken together, the present results show that, in low-$[Ca^{2+}]_i$ cells, the synthesis of PtdInsP_2 through PtdIns$P$ kinase activation exceeds its degradation, thus leading to PtdInsP_2 accumulation. The results concerning the effect of vasopressin on PtdInsP_2 content differed, depending on whether this phospholipid was measured chemically or by radiolabelling. This will be considered in the Discussion section.

DISCUSSION

The present results further support the validity of a model for receptor-activated mobilization of intracellular Ca^{2+} in which InsP_3 generation precedes and triggers Ca^{2+} release from endoplasmic reticulum (Michell & Kirk, 1981; Berridge, 1984). We found that in isolated rat liver cells the activation of vasopressin receptors induced both hydrolysis of PtdInsP_2 and generation of InsP_3, as well as a rise in $[Ca^{2+}]_i$, and that the first signal may be observed under conditions in which the second is completely abolished. In agreement with this observation, raising $[Ca^{2+}]_i$ with ionomycin did not trigger any InsP_3 formation. Similar results were reported in platelets (Simon *et al.*, 1984) and human neutrophils (Di Virgilio *et al.*, 1985).

In the present work no attempt was made to separate the two isomers of InsP_3, i.e. Ins(1,4,5)P_3 and Ins-(1,3,4)P_3. In non-stimulated cells, with $[Ca^{2+}]_i$ between 29 and 190 nM, Ins(1,3,4)P_3 is barely detectable (D. Renard, unpublished work) and Ins(1,4,5)P_3 represents about 80% of total InsP_3 (Hansen *et al.*, 1986). This may be explained if in the pathway generating Ins(1,3,4)P_3, the phosphorylation step by InsP_3 kinase, is sensitive to Ca^{2+}, as reported in RINm5F cells (Biden & Wollheim, 1986). In high-$[Ca^{2+}]_i$ cells, although total InsP_3 was not modified, one cannot exclude that it contains a different ratio of the two isomers because of a possible activation of InsP_3 kinase by Ca^{2+}. In vasopressin-stimulated cells, total InsP_3 determinations were performed after 15 s of hormone application, which corresponds to the peak of Ins(1,4,5)P_3 accumulation while the Ins(1,3,4)P_3 concentration remains very low (Burgess *et al.*, 1985; Hansen *et al.*, 1986)

The formation of basal InsP_3 (but not that of InsP_2 or Ins$P$) depends on $[Ca^{2+}]_i$ when the latter's concentrations are between 29 and 100 nM, in keeping with what has been reported regarding the substrate specificity of PLC in liver (Melin *et al.*, 1986), brain (Irvine *et al.*, 1984), sheep seminal vesicles (Wilson *et al.*, 1984) and platelets (Banno *et al.*, 1986). All the studies show that PLC hydrolyses PtdIns$P$ and PtdInsP_2, but that PtdInsP_2 is preferentially degraded in the presence of low $[Ca^{2+}]_i$. The decrease in PLC activity obtained by lowering $[Ca^{2+}]_i$ was reflected by both the lower cell InsP_3 content and the higher PtdInsP_2 content. PtdInsP_2 accumulation in cells with low $[Ca^{2+}]_i$ was also confirmed both when determined by radiolabelling as [^{3}H]PtdInsP_2 and by chemical assay. However, cell PtdInsP_2 content resulted from the balance between its synthesis from PtdIns$P$ by PtdIns$P$ kinase and its degradation by PLC and PtdInsP_2 phosphatase. In this connection, PtdInsP_2 accumulation may be due not only to PLC activity, but also to the inhibition of its degradation and/or acceleration of its synthesis.

In the cells stimulated here by vasopressin, InsP_3 accumulation also depended on $[Ca^{2+}]_i$, but this dependence was not due to any effect on vasopressin binding to its receptors. It might either characterize a PLC-receptor coupling step or result from some direct effect of $[Ca^{2+}]_i$ on PLC activity. It has been proposed that in non-stimulated cells the substrate is unsuitable for hydrolysis by PLC but that binding of the hormones to their receptors makes it accessible (Irvine *et al.*, 1984; Plantavid *et al.*, 1986). This would probably involve the interaction of the activated receptor with a GTP-binding protein (Wallace & Fain, 1985; Uhing *et al.*, 1986). One explanation for the difference between the ranges of Ca^{2+}

concentrations required to activate PLC in intact cells (the present work), in isolated plasma membranes (Wallace & Fain, 1985; Uhing *et al.*, 1986), and as a partially purified enzyme (Nakanishi *et al.*, 1985), might be that all the factors regulating PLC activity are integrated in intact cells, but are necessarily lost in isolated membranes or the purified enzyme. These factors include the ionic strength of the medium, the presence of GTP in the same medium, the physico-chemical form of the substrate and the lipid–protein interaction maintained by the cytoskeleton (Downes & Michell, 1982; Irvine *et al.*, 1984). As regards the effect of GTP, results obtained in leucocytes and GH_3 cells (Smith *et al.*, 1986; Martin *et al.*, 1986) suggested that after hormone treatment a GTP-binding protein stimulates PLC by decreasing the Ca^{2+} requirement for its activity.

Under all the conditions tested here, vasopressin induced $InsP_3$ accumulation. Accordingly, it also elicited a decrease in $PtdInsP_2$ in normal cells and cells with high $[Ca^{2+}]_i$, whether $PtdInsP_2$ was measured by radiolabelling or chemically. Surprisingly, however, measurement of $PtdInsP_2$ by radiolabelling showed that it accumulated in low-$[Ca^{2+}]_i$ cells stimulated by vasopressin. However, when $PtdInsP_2$ was determined chemically, vasopressin still elicited a decline in $PtdInsP_2$ in normal cells, but did not affect it in low-$[Ca^{2+}]_i$ cells, indicating that the rates of $PtdInsP_2$ synthesis and degradation were the same. These results suggest that receptor occupancy accelerates the activation of $PtdInsP$ kinase in addition to that of PLC. Probably because the inositol lipids were not labelled to equilibrium with $[^3H]$inositol, the stimulation of $PtdInsP$ kinase led to an accumulation of $[^3H]PtdInsP_2$.

The accumulation of $InsP_2$ induced by vasopressin can be attributed to $InsP_3$ degradation by a phosphomonoesterase permanently activated by the internal Mg^{2+} concentration (Storey *et al.*, 1984). As regard $PtdInsP$, it was decreased by vasopressin in normal- and high-$[Ca^{2+}]_i$ cells (results not shown), but not in low-$[Ca^{2+}]_i$ cells. This diminution can be attributed to $PtdInsP$ phosphorylation by the $PtdInsP$ kinase located in the plasma membrane (Lundberg *et al.*, 1985), and may occur in order to restore the pool of $PtdInsP_2$, as first suggested for hepatocytes by Michell (1983) and Berridge (1984). Numerous studies on the order of appearance of $InsP_3$, $InsP_2$ and $InsP$ after hormonal stimulation have now shown that $PtdInsP_2$ is the first inositol lipid degraded by PLC (for liver cells, see Charest *et al.*, 1985). Because, in the present work, $InsP_2$ accumulation induced by vasopressin rose sharply at $[Ca^{2+}]_i$ exceeding 100 nM (Fig. 3), we cannot exclude the possibility that, in normal- and high-$[Ca^{2+}]_i$ cells stimulated by a high dose of vasopressin (0.1 μM), $PtdInsP$ might be hydrolysed by PLC too. A similar proposition was formulated for thrombin-activated platelets (Siess & Binder, 1985), fMet-Leu-Phe-stimulated neutrophils (Di Virgilio *et al.*, 1985) and caerulin-stimulated exocrine pancreas (Merritt *et al.*, 1986).

In conclusion, our experiments show that in intact hepatocytes $PtdInsP_2$ hydrolysis by PLC is a process which is only dependent on $[Ca^{2+}]_i$ when the latter's concentrations range from 29 to 160 nM, indicating that, under physiological conditions (i.e. around 200 nM-$Ca^{2+}{}_i$), this process is not greatly affected by the internal Ca^{2+} concentration.

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REFERENCES

Banno, Y., Nakashima, S. & Nozawa, Y. (1986) Biochem. Biophys. Res. Commun. **136**, 713–721

Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. **10**, 219–242

Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) Biochem. J. **232**, 211–215

Berridge, M. J. (1984) Biochem. J. **220**, 345–360

Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) Biochem. J. **212**, 473–482

Berthon, B., Binet, A., Mauger, J.-P. & Claret, M. (1984) FEBS Lett. **167**, 19–24

Berthon, B., Capiod, T. & Claret, M. (1985) Br. J. Pharmacol. **86**, 151–161

Biden, T. J. & Wollheim, C. B. (1986) J. Biol. Chem. **261**, 11931–11934

Binet, A., Berthon, B. & Claret, M. (1985) Biochem. J. **228**, 565–574

Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. & Putney, J. W., Jr. (1984) Biochem. J. **224**, 741–746

Burgess, G. M., McKinney, J. S., Irvine, R. F. & Putney, J. W., Jr. (1985) Biochem. J. **232**, 237–243

Charest, R., Prpic, V., Exton, J. H. & Blackmore, P. F. (1985) Biochem. J. **227**. 79–90

Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) Biochem. J. **212**, 733–747

Di Virgilio, F., Vincentini, L. M., Treves, S., Riz, G. & Pozzan, T. (1985) Biochem. J. **229**, 361–367

Downes, C. P. & Michell, R. H. (1981) Biochem. J. **198**, 133–140

Downes, C. P. & Michell, R. H. (1982) Biochem. J. **202**, 53–58

Giraud, F., M'Zali, H., Chaillet, B. & Mazet, F. (1984) Biochim. Biophys. Acta **778**, 191–200

Hansen, C. A., Mah, S. & Williamson, J. R. (1986) J. Biol. Chem. **261**, 8100–8130

Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. (1984) Biochem. J. **218**, 177–185

Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) J. Biol. Chem. **259**, 3077–3081

Lundberg, G., Jergil, B. & Sundler, R. (1985) Biochim. Biophys. Acta **846**, 379–387

Martin, T. F. J., Lucas, D. O., Bajjalied, S. M. & Kowalchyk, J. A. (1986) J. Biol. Chem. **261**, 2918–2927

Mauger, J. -P., Poggioli, J. & Claret, M. (1985) J. Biol. Chem. **260**, 11635–11642

Melin, P. M., Sundler, R. & Jergil, B. (1986) FEBS Lett. **198**, 85–88

Merritt, J. E., Taylor, C. W., Putney, J. W., Jr. & Rubin, R. P. (1986) Biochem. Soc. Trans. **14**, 605–606

Michell, R. H. (1975) Biochem. Biophys. Acta **415**, 81–147

Michell, R. H. (1983) Trends Biochem. Sci. **8**, 263–265

Michell, R. H. & Kirk, C. J. (1981) Trends Pharmacol. Sci. **2**, 86–89

Nakanishi, H., Nomura, H., Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1985) Biochem. Biophys. Res. Commun. **132**, 582–590

Plantavid, M., Rossignol, L., Chap, H. & Douste-Blazy, L. (1986) Biochim. Biophys. Acta **875**, 147–156

Poggioli, J., Mauger, J. -P., Guesdon, F. & Claret, M. (1985) J. Biol. Chem. **260**, 3289–3294

Poggioli, J., Mauger, J.-P. & Claret, M. (1986) Biochem. J. **235**, 663–669

Putney, J. W., Jr. (1986) Cell Calcium **7**, 1–12

Rhodes, D., Prpic, V., Exton, J. H. & Blackmore, R. F. (1983) J. Biol. Chem. **258**, 2770–2773

Rink, T. J. & Pozzan, T. (1985) Cell Calcium **6**, 133–157

Rouser, G., Fleischer, S. & Yamamoto, A. (1970) Lipids **5**, 494–496

Siess, W. & Binder, H. (1985) FEBS Lett. **180**, 107–112
Simon, M. F., Chap, H. & Douste-Blazy, L. (1984) FEBS Lett. **170**, 43–48
Smith, C. D., Cox, C. C. & Snyderman, R. (1986) Science **232**, 97–100
Steiner, S. & Lester, R. L. (1972) J. Bacteriol. **109**, 81–88
Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) Nature (London) **312**, 371–376
Uhing, R. J., Prpic, V., Jiang, H. & Exton, J. H. (1986) J. Biol. Chem. **261**, 2140–2146
Wallace, M. A. & Fain, J. N. (1985) J. Biol. Chem. **260**, 9527–9530
Williamson, J. R., Cooper, R. H., Joseph, S. K. & Thomas, A. (1985) Am. J. Physiol. **248**, C203–C216
Wilson, D. B., Bross, T. E., Hofmann, S. L. & Majerus, P. W. (1984) J. Biol. Chem. **259**, 11718–11724

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