Stimulation of inositol trisphosphate formation in hepatocytes by vasopressin, adrenaline and angiotensin II and its relationship to changes in cytosolic free Ca^{2+}

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At maximally effective concentrations, vasopressin (10^{-7} M) increased *myo*-inositol trisphosphate (IP_3) in isolated rat hepatocytes by 100% at 3 s and 150% at 6 s, while adrenaline (epinephrine) (10^{-5} M) produced a 17% increase at 3 s and a 30% increase at 6 s. These increases were maintained for at least 10 min. Both agents increased cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) maximally by 5 s. Increases in IP_3 were also observed with angiotensin II and ATP, but not with glucagon or platelet-activating factor. The dose-responses of vasopressin and adrenaline on phosphorylase and $[\text{Ca}^{2+}]_i$ showed a close correspondence, whereas IP_3 accumulation was 20–30-fold less sensitive. However, significant (20%) increases in IP_3 could be observed with 10^{-9} M-vasopressin and 10^{-7} M-adrenaline, which induce near-maximal phosphorylase activation. Vasopressin-induced accumulation of IP_3 was potentiated by 10 mM- Li^+ , after a lag of approx. 1 min. However the rise in $[\text{Ca}^{2+}]_i$ and phosphorylase activation were not potentiated at any time examined. Similar data were obtained with adrenaline as agonist. Lowering the extracellular Ca^{2+} to 30 μM or 250 μM did not affect the initial rise in $[\text{Ca}^{2+}]_i$ with vasopressin but resulted in a rapid decline in $[\text{Ca}^{2+}]_i$. Brief chelation of extracellular Ca^{2+} for times up to 4 min also did not impair the rate or magnitude of the increase in $[\text{Ca}^{2+}]_i$ or phosphorylase *a* induced by vasopressin. The following conclusions are drawn from these studies. 1. IP_3 is increased in rat hepatocytes by vasopressin, adrenaline, angiotensin II and ATP. 2. The temporal relationships of its accumulation to the increases in $[\text{Ca}^{2+}]_i$ and phosphorylase *a* are consistent with it playing a second message role. 3. Influx of extracellular Ca^{2+} is not required for the initial rise in $[\text{Ca}^{2+}]_i$ induced by these agonists, but is required for the maintenance of the elevated $[\text{Ca}^{2+}]_i$.

The idea that changes in phosphoinositide metabolism are integrally involved in the actions of Ca^{2+} -dependent agonists arose out of the pioneering studies of Hokin & Hokin (1953) and

has received vigorous experimental support from the group of Michell and other investigators (for references see Michell, 1975, 1979; Michell & Kirk, 1981; Michell *et al.*, 1981; Berridge, 1984). The initial view was that the primary event following interaction of a Ca^{2+} -dependent agonist with its receptor was the breakdown and resynthesis of PI. However, this was criticized by us as being too slow to explain the physiological responses, at least for vasopressin and adrenaline in liver (Prpić *et al.*, 1982b).

It is now known that, in rat hepatocytes, vasopressin also stimulates the net breakdown of PIP_2 (Michell *et al.*, 1981; Kirk *et al.*, 1981; Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Litosch *et al.*, 1983;

Abbreviations used: PIP_2 , phosphatidylinositol bisphosphate; PIP, phosphatidylinositol monophosphate; GPI, glycerophosphatidylinositol; PI, phosphatidylinositol; IP_3 , *myo*-inositol trisphosphate; IP_2 , *myo*-inositol bisphosphate; IP_1 , *myo*-inositol monophosphate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; Quin-2, 2-[[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxymethyl)-aminoquinoline; Quin-2/AM, Quin-2 tetra-acetoxymethyl ester.

Creba *et al.*, 1983). The effect of vasopressin to decrease PIP_2 occurs more rapidly than the decrease in PI (Michell *et al.*, 1981; Kirk *et al.*, 1981; Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Litosch *et al.*, 1983; Creba *et al.*, 1983), is transient (Rhodes *et al.*, 1983; Litosch *et al.*, 1983), but appears to require higher concentrations of hormone (10^{-8} – 10^{-7} M) than are required for maximum phosphorylase activation and Ca^{2+} mobilization (10^{-9} M) (Michell *et al.*, 1981; Kirk *et al.*, 1981; Creba *et al.*, 1983; Rhodes *et al.*, 1983; Litosch *et al.*, 1983). Because of the latter observation and others, we proposed (Rhodes *et al.*, 1983) that PIP_2 breakdown was not responsible for the initial mobilization of intracellular Ca^{2+} . However, we could not rule out the possibility that a small hormone-sensitive pool of PIP_2 (Rhodes *et al.*, 1983) was being broken down in the plasma membrane. Furthermore, since any PIP_2 broken down could be rapidly resynthesized from PIP and PI, as suggested by Michell *et al.* (1981) and Creba *et al.* (1983), measurements of the level of this phosphoinositide might underestimate the extent of its breakdown.

Recently Berridge (1983) has measured the effects of 5-hydroxytryptamine on the products of phosphoinositide breakdown, namely IP_1 , IP_2 and IP_3 , in blowfly salivary gland. Based on these and other observations, he has proposed that IP_3 functions as a second message for the mobilization of cellular Ca^{2+} by Ca^{2+} -dependent agonists (Berridge, 1984). We decided to investigate the effect of vasopressin and adrenaline on PIP_2 breakdown in rat hepatocytes by measuring IP_3 to see if this would give insight into the role of this inositol phospholipid in the actions of these hormones in liver. The rationale for measuring products of PIP_2 hydrolysis was that if PIP_2 were merely turning over in response to low levels of hormone with little net breakdown, then one could explain the difference in the dose-response curves for PIP_2 breakdown and Ca^{2+} mobilization observed earlier. The present communication describes in detail the effects of hormones on IP_3 formation in hepatocytes and compares the changes with those in phosphorylase *a* and in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) determined by using the fluorescent Ca^{2+} chelator Quin-2 (Tsien *et al.*, 1982; Charest *et al.*, 1983). The findings with vasopressin are similar to those recently reported by Thomas *et al.* (1984). In addition, the possible role of Ca^{2+} influx in the rise in $[\text{Ca}^{2+}]_i$ induced by vasopressin is explored.

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Experimental procedures

Methods

Hepatocytes from male Sprague-Dawley rats (200–250 g body wt.; Harlan Industries) were isolated and incubated as previously described (Hutson *et al.*, 1976). The cell suspensions (approx. 50 mg wet wt./ml) were incubated in Krebs-Henseleit bicarbonate buffer containing 2.5 mM- Ca^{2+} and 1.5% (w/v) gelatin (Difco) and continuously gassed with O_2/CO_2 (19:1).

Hepatocytes were prelabelled for 90 min with 0.1 mM-[2- ^3H]myo-inositol (25 $\mu\text{Ci}/\text{ml}$ of cell suspension). The cells were then washed once and resuspended in medium without [^3H]myo-inositol. After incubation for various periods of time with hormones and agents, the cells plus medium (1 ml, 40–50 mg wet wt./ml) were pipetted into tubes containing 0.2 ml of 80% (w/v) trichloroacetic acid kept on ice. After centrifugation (5000g for 10 min) the supernatant solution was extracted six times with 2 ml portions of diethyl ether to remove trichloroacetic acid. The neutralized extracts were then subjected to ion-exchange chromatography as previously described (Rhodes *et al.*, 1983; Berridge, 1983). The columns used were 5 ml disposable pipette tips for P-5000 Pipetman pipettes (Rainin Instrument Co.), containing approx. 2 ml of Dowex 1 X8 (formate form) ion-exchange resin. Details for the measurement of phosphorylase *a* and $[\text{Ca}^{2+}]_i$ have been described (Blackmore *et al.*, 1978; Charest *et al.*, 1983; Hutson *et al.*, 1976; Morgan *et al.*, 1983a).

Sources of materials

Vasopressin (synthetic [arginine]vasopressin), angiotensin II (grade VII, synthetic), (–)-adrenaline bitartrate, EGTA and myo-inositol were from Sigma. LiCl, diethyl ether, trichloroacetic acid and formic acid were from Fisher. Dowex 1 X8 (formate form) ion-exchange resin, 200–400 mesh, was from Bio-Rad. myo-[2- ^3H (N)]inositol was from New England Nuclear. Before its use the ethanol was removed by lyophilization. Glucagon and A23187 were gifts from Eli Lilly Co. ACS scintillation fluid (Amersham) was used to count column fractions. Quin-2/AM was obtained from Lancaster Synthesis, Morecambe, Lancs., U.K. Platelet activating factor preparations were from Calbiochem.

Results

Levels of myo-inositol phosphates in hepatocytes

Fig. 1 shows the chromatographic separation of the various phosphorylated forms of myo-inositol in cells incubated for 90 min with [^3H]myo-inositol and then for a further 10 min without additions

Fig. 1. Elution profile of water-soluble extract of hepatocytes by anion-exchange chromatography

The water-soluble extract from 30 ml of hepatocytes (30 mg wet wt./ml) labelled with $[2\text{-}^3\text{H}]\text{myo}$ -inositol for 90 min was applied to a column of Dowex 1 (formate form) resin and eluted with a linear gradient of ammonium formate in 0.1 M-formic acid. Fractions (2.0 ml) were collected and 0.5 ml aliquots counted in 15 ml of scintillation fluid (ACS, Amersham). The effect of incubating the cells for 5 min with 10^{-7} M -vasopressin plus 10 mM-LiCl is shown. Note difference in radioactivity scales before and after fraction number 70.

(control) or with 10^{-7} M -vasopressin and 10 mM- Li^+ . Each phosphorylated derivative of *myo*-inositol was well separated, enabling accurate quantification to be obtained. The position of $[^3\text{H}]\text{IP}_1$, $[^3\text{H}]\text{IP}_2$ and $[^3\text{H}]\text{IP}_3$ in the elution profiles was confirmed by chromatographing authentic standards and measuring total phosphate. The standards were kindly supplied by Dr. C. E. Ballou (University of California, Berkeley, CA, U.S.A.) and Dr. F. Eisenberg (National Institutes of Health, Bethesda, MD, U.S.A.). When $[^3\text{H}]\text{IP}_3$ was rechromatographed (after removal of ammonium formate) on formate ion-exchange resin, the ^3H appeared in the same position, as a single symmetrical peak, in the elution profile, with no detectable loss of ^3H . Fig. 1 shows that vasopressin in the presence of Li^+ produced large increases in the levels of IP_1 , IP_2 and IP_3 , but not GPI, with the greatest-fold increase being observed with IP_3 .

As shown in Table 1(a), the labelling of the inositol phosphates in control cells approached equilibrium at 90 min and was unaffected by the pre-

sence of 10 mM- Li^+ . More importantly, Table 1(a) shows that constant ratios between the labelling of IP_3 , IP_2 and IP_1 were observed following 45, 90 or 130 min of incubation with $[^3\text{H}]\text{inositol}$. In addition constant ratios between the labelling of PI, PIP and PIP_2 were also observed at these times (results not shown) with the ratios being the same as those obtained from 24 h labelling experiments *in vivo* (Rhodes *et al.*, 1983). These results support the view that the PI, PIP and PIP_2 pools and IP_1 , IP_2 and IP_3 pools approached isotopic equilibrium at 90 min. Table 1(b) also shows that the effects of vasopressin (10^{-7} M), adrenaline (10^{-5} M) and angiotensin II (10^{-7} M) on IP_3 radioactivity were similar in cells labelled with $[^3\text{H}]\text{myo}$ -inositol for 90 min compared with 130 min. Similar results (not shown) were observed for IP_2 and IP_1 at 90 and 130 min. Assuming isotopic equilibrium of the applied $[2\text{-}^3\text{H}]\text{myo}$ -inositol ($250 \mu\text{Ci}/\mu\text{mol}$) with each phosphorylated *myo*-inositol ester, the basal intracellular concentrations of IP_1 , IP_2 and IP_3 can be calculated from Table 1 to be approx. 4.0, 0.5 and

Table 1. *Formation of inositol phosphates as a function of duration of labelling of hepatocytes with [^3H]myo-inositol* Hepatocytes were incubated with 0.1 mM [^3H]myo-inositol (25 $\mu\text{Ci/ml}$) as described under 'Experimental procedures'. At the times shown, 1 ml aliquots were withdrawn and the content of inositol phosphates was measured after incubation with saline or hormones for 5 min in the presence or absence of 10 mM-LiCl. The values in (a) are the c.p.m. in each inositol phosphate/ml of cell suspension, with the numbers in parentheses being the % of the total c.p.m. in all the inositol phosphates. The values in (b) are given as % increase from relevant control without hormone. The values are the mean (a) or the mean $\pm$ S.E.M. (b) for triplicate incubations from a representative experiment.

Hormone	Fraction	Li ⁺ present	Time of incubation with [³ H]inositol		
			45 min	90 min	130 min
(a)					
None	IP ₃	—	148 (1.9)	193 (1.9)	219 (1.8)
		+	146 (1.9)	192 (1.8)	211 (1.7)
None	IP ₂	—	896 (11.4)	1160 (11.4)	1333 (11.1)
		+	789 (10.1)	1152 (10.8)	1290 (10.6)
None	IP ₁	—	6789 (86.7)	8847 (86.7)	10451 (87.1)
		+	6843 (87.9)	9289 (87.4)	10697 (87.7)
(b)					
Vasopressin (10 ⁻⁷ M)	IP ₃	—	100 ± 7	127 ± 8	126 ± 2
		+	223 ± 4	320 ± 14	360 ± 31
Angiotensin II (10 ⁻⁷ M)	IP ₃	—	31 ± 3	48 ± 3	43 ± 6
		+	167 ± 4	194 ± 22	167
Adrenaline (10 ⁻⁵ M)	IP ₃	—	28 ± 7	22 ± 5	27 ± 5
		+	59 ± 6	68 ± 5	118 ± 4

0.08 μM respectively assuming an intracellular water volume of 0.47 $\mu\text{l/mg}$ wet wt. of cells (Prpić *et al.*, 1982a). However, it must be emphasized that these are only tentative values since it is uncertain that isotopic equilibrium was established for all phosphoinositide pools.

Time courses of vasopressin and adrenaline actions on IP₃, [Ca²⁺]_i and phosphorylase

Fig. 2(a) shows the time courses of maximally effective doses of vasopressin (10⁻⁷ M) and adrenaline (10⁻⁵ M) on the accumulation of IP₃ in hepatocytes. With vasopressin, a 100% increase in IP₃ was detected at 3 s and an increase of 140–170% at 6–15 s, which was maintained for at least 10 min (result not shown). No change in the level of IP₃ was observed in control incubations over a 10 min period (results not shown). For comparison is shown phosphorylase activation, which was significant at 2 s and maximal at 10–15 s (Fig. 2b). Likewise, the increase in [Ca²⁺]_i measured by Quin-2 fluorescence (Fig. 2c), could be detected at 1–2 s and was maximal at 6 s.

No consistent changes in the levels of IP₁ and IP₂ could be observed up to 30 s with 10⁻⁷ M-vasopressin as agonist. However, at 5 min IP₁ and IP₂ had increased by 22 $\pm$ 5% and 23 $\pm$ 4% respectively.

With adrenaline (10⁻⁵ M), a 17% increase in IP₃ was observed at 3 s and a 30% increase at 6 s (Fig. 2a). This was maintained for at least 10 min (result

not shown). This dose of adrenaline produced an increase in [Ca²⁺]_i (Fig. 2c) which was slightly more rapid than that seen with 10⁻⁷ M-vasopressin. Phosphorylase was activated at the same rate as with 10⁻⁷ M-vasopressin (Fig. 2b). Adrenaline caused the same maximum increase in [Ca²⁺]_i (Fig. 2c) and phosphorylase *a* (Fig. 2b) as vasopressin despite the fact it increased IP₃ by only 30% (Fig. 2a).

Concentration-dependence of vasopressin and adrenaline actions

Fig. 3 shows the dose-response of vasopressin on IP₃ accumulation, phosphorylase activation and [Ca²⁺]_i measured at 20 s. There was an approx. 30-fold rightward shift in the curve for IP₃ compared with those for phosphorylase and [Ca²⁺]_i. However, an approx. 20% increase in IP₃ at 20 s could be seen with 10⁻⁹ M-vasopressin, which has near-maximal effects on phosphorylase and [Ca²⁺]_i.

Fig. 4 shows the dose-response of adrenaline on IP₃ accumulation, phosphorylase activation and [Ca²⁺]_i measured at 20 s. The dose of adrenaline producing half-maximal accumulation of IP₃ was approx. 20-fold greater than that required for half-maximal activation of phosphorylase and elevation of [Ca²⁺]_i. Maximal effects on phosphorylase and [Ca²⁺]_i were observed with 10⁻⁷ M-adrenaline and this produced a 20% increase in IP₃ levels at 20 s.

Fig. 2. Time courses of the changes in $[^3H]IP_3$ (a), phosphorylase activation (b) and $[Ca^{2+}]_i$ increases (c) induced by vasopressin ($10^{-7}M$) and adrenaline ($10^{-5}M$)

(a) Hepatocytes (10 ml) labelled with $[2-^3H]myo$ -inositol were incubated at $37^\circ C$. Aliquots (1 ml) were removed at the times indicated into 0.2 ml of 80% trichloroacetic acid at $0^\circ C$. The water soluble products were then chromatographed on Dowex 1 (formate form) resin. The data shown are means $\pm$ S.E.M. from three separate batches of hepatocytes. The radioactivity in IP_3 at 0 s was 123 ± 8 c.p.m./ml of cell suspension. (b) Hepatocytes (5 ml) were incubated with adrenaline ($10^{-5}M$) or vasopressin ($10^{-7}M$). Samples (0.5 ml) were removed for determination of phosphorylase *a* at the times shown. Values shown are means $\pm$ S.E.M. of triplicate incubations from a representative experiment of three. (c) Quin-2-loaded and control hepatocytes were prepared as previously described (Charest *et al.*, 1983). Fluorescence measurements were collected every 0.5 s after the addition, at zero time, of adrenaline ($10^{-5}M$) or vasopressin ($10^{-7}M$). Control incubations ($-Quin-2$) were also run and the change in fluorescence subtracted from that of the Quin-2 loaded cells. The time for addition and mixing of agents in the cuvette, 1.5 s, was not subtracted. The results are presented as the change in Quin-2 fluorescence ($\Delta mV \cdot s$) from zero time, with each curve being the mean of triplicate incubations from a representative experiment of three.

Lack of dependence on extracellular Ca^{2+} of $[Ca^{2+}]_i$ rise with hormones

Although most workers now agree (Blackmore *et al.*, 1978) that mobilization of intracellular Ca^{2+} is responsible for the initial increase in $[Ca^{2+}]_i$ when hepatocytes are stimulated by Ca^{2+} -dependent hormones, some workers still claim that influx of extracellular Ca^{2+} is involved (e.g. Binet & Claret, 1983). We examined this question further utilizing Quin-2-loaded hepatocytes (Charest *et al.*, 1983). Fig. 5(a) shows the effects of vasopressin ($10^{-8}M$) on $[Ca^{2+}]_i$ in hepatocytes suspended in medium in which Ca^{2+} was reduced from 1.0 mM to approx. 30 nM (Bartfai, 1979) by the addition of 2 mM-EGTA for periods from 1 to 10 min. Only after exposure to EGTA for 10 min was the ability of vasopressin to increase $[Ca^{2+}]_i$ slightly altered. At this time, intracellular Ca^{2+} stores would start to be depleted by the chelator (Blackmore *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1977). Fig. 5(b) shows that the rate and extent of phosphorylase activation induced by 10^{-7} , 10^{-9} and $5 \times 10^{-10}M$ -vasopressin were also not affected by the reduction of extracellular Ca^{2+} to 30 nM by EGTA added 30 s prior to agonist. The same results were obtained

with phenylephrine ($10^{-5}M$) (results not shown). When intracellular Ca^{2+} stores are depleted by extensive washing of hepatocytes with EGTA (Assimacopoulos-Jeannet *et al.*, 1977), vasopressin no longer raises $[Ca^{2+}]_i$ (Fig. 5a) or activates phosphorylase (Blackmore *et al.*, 1978).

Fig. 6(a) shows the effects of various extracellular Ca^{2+} concentrations on the ability of $10^{-8}M$ -vasopressin to raise $[Ca^{2+}]_i$ during 9 min. For the first 45 s of vasopressin action, the elevation of $[Ca^{2+}]_i$ was uninfluenced by the extracellular Ca^{2+} concentration. However, beyond 45 s $[Ca^{2+}]_i$ began to fall when the medium Ca^{2+} was 30 μM or 0.25 mM ($P < 0.05$ by paired *t*-test), indicating that at these concentrations, Ca^{2+} influx was no longer able to sustain the elevated $[Ca^{2+}]_i$. Raising the external Ca^{2+} concentrations above 0.5 mM did not alter the time course of vasopressin-induced changes in $[Ca^{2+}]_i$ beyond that seen with 0.5 mM- Ca^{2+} (results not shown).

When phosphorylase was measured a similar pattern was observed, namely, as the extracellular Ca^{2+} was reduced, the phosphorylase activation became more transient (Fig. 6b), showing that Ca^{2+} influx was required to maintain the elevated

Fig. 3. Dose-response of vasopressin on phosphorylase activity, $[Ca^{2+}]_i$ and IP_3 at 20s

For $[Ca^{2+}]_i$ measurements Quin-2-loaded and control hepatocytes were prepared as described by Charest *et al.* (1983). The changes in fluorescence ($\Delta mV \cdot s$) from zero time were determined at 20s after the addition of vasopressin. The values shown are the differences between Quin-2 and control fluorescence changes and are the means $\pm$ S.E.M. of four separate experiments. Phosphorylase activity and IP_3 levels are means $\pm$ S.E.M. from triplicate incubations from a representative experiment of three. The radioactivity in IP_3 in control experiments (no hormone) was 91 ± 2 c.p.m./ml of cell suspension.

$[Ca^{2+}]_i$. These findings confirm earlier observations in perfused livers (Blackmore *et al.*, 1979; Morgan *et al.*, 1982).

Effect of Li^+ on the accumulation of IP_3 , phosphorylase activation and $[Ca^{2+}]_i$

It has been demonstrated that Li^+ potentiates the accumulation of inositol phosphates in salivary gland stimulated by 5-hydroxytryptamine (Berridge *et al.*, 1982) in part because it inhibits the conversion of IP_1 to inositol (Sherman *et al.*, 1981). If IP_3 has a second message role in Ca^{2+} mobilization (Thomas *et al.*, 1983; Berridge, 1983), an increase in its level caused by Li^+ would be expected to potentiate the physiological effects of Ca^{2+} -dependent hormones. Fig. 7(a) shows that when hepatocytes were incubated in the presence of 10mM- Li^+ for 5min the ability of vasopressin to increase IP_3 levels was greatly potentiated. However, Li^+ did not potentiate the ability of 10^{-11} – 10^{-8} M-vasopressin to activate phosphorylase at 4min (Fig. 7b) or earlier times (results not shown), or raise $[Ca^{2+}]_i$ at times up to 10min (results not shown). A similar lack of potentiation of $[Ca^{2+}]_i$ or phosphorylase responses was seen when adrenaline or angiotensin II was the agonist (results not

Fig. 4. Dose-response of adrenaline on phosphorylase activity, $[Ca^{2+}]_i$ and IP_3 at 20s

For experimental details see legend to Fig. 3. The IP_3 values are means $\pm$ S.E.M. from three separate experiments, the values for $[Ca^{2+}]_i$ are from four separate experiments, and phosphorylase values are from a representative experiment performed in triplicate. The radioactivity in IP_3 in control experiments was 108 ± 2 c.p.m./ml of cell suspension.

shown). No effect of Li^+ added alone was observed on $[Ca^{2+}]_i$ or phosphorylase up to 10min (results not shown).

If IP_3 is the second message for intracellular Ca^{2+} mobilization, a possible explanation for the fact that Li^+ does not potentiate the hormone effects on phosphorylase and $[Ca^{2+}]_i$ is that Li^+ is unable to potentiate IP_3 levels at early times after hormone exposure, when Ca^{2+} mobilization is occurring. Fig. 7(a) (insert) shows that there was in fact a delay of 1 min before Li^+ potentiated the increase in IP_3 level with 10^{-9} M-vasopressin. A similar lack of potentiation at early times was observed with 10^{-8} and 10^{-10} M-vasopressin and with adrenaline and angiotensin II (results not shown). Preincubation of the hepatocytes with Li^+ for variable times did not eliminate the lag. As discussed below, the failure of Li^+ to potentiate the phosphorylase response to agonists may also be due to its promoting the accumulation of an IP_3 isomer which is different from the biologically active form.

Effects of other agents on IP_3 levels in hepatocytes

Table 2 shows the effects of a variety of agents on IP_3 formation in normal or Ca^{2+} -depleted hepatocytes. Surprisingly, the divalent cationophore A23187 stimulated the accumulation of IP_3 at 10^{-5} M, but not at 10^{-6} M. The formation of IP_3 by vasopressin, angiotensin II, adrenaline and A23187 was dependent on the presence of intracellular Ca^{2+} , since when hepatocytes were depleted of Ca^{2+} (Assimakopoulos-Jeannet *et al.*,

Fig. 5. Effect of short-term chelation of extracellular Ca²⁺ with EGTA on the ability of vasopressin (10⁻⁸ M) to increase [Ca²⁺]_i (a) or to activate phosphorylase (b)

(a) Quin-2-loaded and control hepatocytes were prepared as described by Charest *et al.* (1983). The level of Ca²⁺ in the incubation medium was 1.0 mM (total) and 2.0 mM-EGTA was added prior to vasopressin addition for the periods indicated. The Ca²⁺-depleted hepatocytes were prepared as described (Assimakopoulos-Jeannet *et al.*, 1977). The values shown are the differences between Quin-2 and control fluorescence changes and are means from three separate incubations. (b) Hepatocytes were incubated in 1.0 mM-Ca²⁺-containing medium. EGTA (2 mM) or saline was added 30 s prior to the addition of vasopressin (10⁻⁷, 10⁻⁹ or 5 × 10⁻¹⁰ M). Phosphorylase values are means ± S.E.M. from triplicate incubations from a representative experiment.

Table 2. Effect of a variety of agents on hepatocyte IP₃ levels

For the '– Calcium' condition, 3.5 mM-EGTA was added to hepatocytes incubated in 2.5 mM-Ca²⁺-containing medium for 15 min prior to addition of hormone. This treatment did not affect basal levels of IP₃ (results not shown). Values shown are means or means ± S.E.M., with the numbers of separate batches of cells shown in parentheses. Abbreviation used: N.D., not determined.

Agent	Concentration (M)	Radioactivity in IP ₃ at 5 min (% change from control)	
		+ Calcium	– Calcium
Vasopressin	10 ⁻⁷	92.8 ± 9.9 (9)	11.2 ± 7.6 (6)
Adrenaline	10 ⁻⁵	47.1 ± 7.3 (9)	6.6 ± 11.8 (5)
Glucagon	10 ⁻⁸	10.8 ± 6.1 (5)	N.D.
A23187*	10 ⁻⁵	38.0 ± 8.3 (9)	1.3 ± 7.6 (6)
A23187*	10 ⁻⁶	5.3 ± 9.7 (4)	N.D.
Angiotensin II	10 ⁻⁸	34.6 ± 10.3 (3)	16.8 ± 6.0 (3)
Platelet-activating factor	10 ⁻⁷	–6.1 ± 0.6 (3)	N.D.
	10 ⁻⁸	–2.3 ± 5.9 (3)	N.D.
	10 ⁻⁹	2.6 ± 15.7 (3)	N.D.
ATP†	10 ⁻⁴	43.0 ± 6.1 (3)	N.D.

* 1.0% (v/v) dimethyl sulfoxide was the control.

† Measured at 15 s.

Fig. 6. Effect of various extracellular Ca^{2+} concentrations on the time courses of the increase in $[Ca^{2+}]_i$ (a) and phosphorylase activation (b) induced by vasopressin (10^{-8} M)

(a) Hepatocytes were loaded with Quin-2 as described (Charest *et al.*, 1983). Control and Quin-2-loaded hepatocytes were washed and resuspended in medium containing either 0.5 mM, 0.25 mM or 30 μM - Ca^{2+} (Bartfai, 1979) for 5 min before fluorescence measurements were made. The values shown are the differences between Quin-2 and control fluorescence changes and are the means from four separate experiments. Vasopressin was added at 1 min. (b) Hepatocytes (6 ml) were incubated for 10 min in medium containing various levels of free Ca^{2+} (Bartfai, 1979). At the indicated times 0.5 ml aliquots were removed for the determination of phosphorylase a activity. Each value is the mean of triplicate determinations; S.E.M. bars have been omitted for the sake of clarity.

1977) the effect of each agent was inhibited. ATP (10^{-4} M) also increased IP_3 (Table 1) in addition to $[Ca^{2+}]_i$ and phosphorylase a (R. Charest, P. F. Blackmore & J. H. Exton, unpublished work). Glucagon (10^{-8} M) was without significant effect

on IP_3 formation although it raised $[Ca^{2+}]_i$ as high as did adrenaline and vasopressin (Charest *et al.*, 1983).

Recently Shukla *et al.* (1983) reported that platelet activating factor (PAF) caused a rapid

Fig. 7. Effect of Li⁺ on the ability of vasopressin to increase IP₃ levels and activate phosphorylase

(a) The dose-response of vasopressin on IP₃ levels in the absence and presence of 10 mM-Li⁺. In the case of the Li⁺-treated cells, it was added 5 min before hormone addition. The insert shows the time course of 10⁻⁹M-vasopressin to increase IP₃ levels in the presence and absence of 10 mM-Li⁺. The results are the mean (insert) or mean $\pm$ S.E.M. for triplicate incubations from a representative experiment of four. The radioactivity in IP₃ in control incubations in the absence or presence of Li⁺ was 279 ± 10 and 285 ± 15 c.p.m./ml of cell suspension, respectively. (b) The dose-response of vasopressin on phosphorylase *a* in the presence and absence of 10 mM-Li⁺. The results are the means $\pm$ S.E.M. for triplicate incubations from three different cell preparations.

(maximum by 15s) and transient breakdown of PIP₂ in isolated hepatocytes and increased glycogenolysis in the perfused rat liver (Shukla *et al.*, 1983; Buxton *et al.*, 1984). The ability of platelet-activating factor (10⁻⁹–10⁻⁷ M) to increase IP₃, [Ca²⁺]_i and phosphorylase in isolated hepatocytes was therefore tested. However, no increases in IP₃ could be detected up to 5 min (Table 1). Likewise, no increases in [Ca²⁺]_i or phosphorylase *a* could be detected (R. Charest, P. F. Blackmore & J. H. Exton, unpublished work). Thus platelet-activating factor does not appear to cause PIP₂ breakdown via phosphodiesterase hydrolysis or cause glycogenolysis via a rise in Ca²⁺ and activation of phosphorylase. Two preparations of factor were used in these studies; both caused platelet aggregation at a final concentration of 10⁻¹⁰–10⁻⁹ M. The findings of Shukla *et al.* (1983) could be due to activation of a phospholipase A type enzyme.

Discussion

PIP₂ breakdown and regulation of cytosolic Ca²⁺

Because chemical methods to detect cellular levels of IP₃ are lacking, changes in the compound are measured isotopically in cells or tissues labelled with [³H]myo-inositol or ³²P_i. In such studies, isotopic equilibrium between PI, PIP and PIP₂ needs to be established to exclude possible artifacts. In contrast to a recent report in which this issue was not addressed (Thomas *et al.*, 1984), isotopic equilibrium

was demonstrated to be approached at the time of hormone addition in the present study (90 min) by measurements of PI, PIP, PIP₂, IP₁, IP₂ and IP₃ radioactivity in hepatocytes labelled for various times. Seyfred & Wells (1984a) have recently also demonstrated isotopic equilibrium between PI, PIP and PIP₂ in the plasma membranes of hepatocytes incubated with ³²P_i for 90 min.

The present data show that vasopressin, adrenaline, angiotensin II and ATP stimulate IP₃ formation in rat hepatocytes. This corresponds to the hydrolysis of PIP₂ previously observed (Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Litosch *et al.*, 1983; Creba *et al.*, 1983). As discussed below, the time course and concentration-dependence of the increase in IP₃ are consistent with it being a second messenger for Ca²⁺ mobilization (Figs. 2–4).

The two means by which cytosolic Ca²⁺ can be increased in cells are by a net influx of Ca²⁺ across the plasma membrane and a net release of Ca²⁺ from intracellular stores. Our findings would appear to rule out the hypothesis that PIP₂ breakdown initially raises [Ca²⁺]_i because it opens 'Ca²⁺ gates' in the plasma membrane (Downes & Michell, 1982). As shown in Fig. 5 the initial rise in [Ca²⁺]_i and the activation of phosphorylase induced by vasopressin are unaffected by a reduction in extracellular Ca²⁺ to approx. 30 nM. This would reverse the gradient for Ca²⁺ across the plasma membrane and, as concluded earlier (Rhodes *et al.*, 1983), render the Ca²⁺ 'gating' hypothesis unten-

able as a mechanism for the effect of vasopressin on $[Ca^{2+}]_i$ at early times. Net influx of Ca^{2+} is only observed in the liver when a hormone stimulus is terminated (Morgan *et al.*, 1982; Reinhart *et al.*, 1984) or when cyclic AMP is elevated simultaneously with the application of a Ca^{2+} -mobilizing hormone (Morgan *et al.*, 1983b).

Evidence for IP_3 being a second messenger

Before IP_3 can be accepted as a physiological intracellular messenger, certain criteria must be met. Firstly, it must increase prior to or simultaneous with the responses it is presumed to mediate. The data in Figs. 2, 3 and 4 show that with adrenaline (10^{-5} M) and vasopressin (10^{-7} M), $[Ca^{2+}]_i$ increases at 1 s and reaches a maximum by 5 s, and phosphorylase activation follows after a slight lag. On the other hand, IP_3 increases by 100% at 3 s with 10^{-7} M-vasopressin and by 17% at 3 s with 10^{-5} M-adrenaline. Thus the IP_3 increase precedes or coincides with the increases in $[Ca^{2+}]_i$ and phosphorylase.

Secondly, the dose-response relationships between agonists and their presumed messenger must be similar to those for the physiological responses unless there are large numbers of spare receptors or there is amplification between the messenger and physiological responses. Figs. 3 and 4 show that an increase in IP_3 with 10^{-9} M-vasopressin or 10^{-7} M-adrenaline of approx. 20% is associated with maximal increases in $[Ca^{2+}]_i$ and phosphorylase *a*. This relatively small increase in IP_3 with maximally effective concentrations of adrenaline and vasopressin indicates that IP_3 would have to be effective in the submicromolar range to be a second messenger (calculated from Fig. 1). It is of interest to note that Thomas *et al.* (1984) have reported a good correlation between the dose-response curves for vasopressin on the initial rates of IP_3 production and increase in $[Ca^{2+}]_i$. However, their calculations do not take into account the large time lags that occur between application of low concentrations of agonists and the increases in IP_3 and $[Ca^{2+}]_i$ (R. Charest, P. F. Blackmore & J. H. Exton, unpublished work).

Thirdly, agents which augment (or abolish) the increase in the second messenger with agonists should potentiate (or eliminate) the physiological responses. This was tested using Li^+ as described previously by Berridge *et al.* (1982). At first sight, the hypothesis that IP_3 is a regulator of intracellular Ca^{2+} appears not to be supported by the data in Fig. 7. The failure of Li^+ to modify the physiological responses to hormones was also observed by Thomas *et al.* (1984) and could be due to the fact that Li^+ took more than 1 min to potentiate IP_3 levels (Fig. 7a, insert). At this time, mobilization of intracellular Ca^{2+} is diminishing (Black-

more *et al.*, 1979) and inhibition of Ca^{2+} efflux (Prpić *et al.*, 1984) appears to be a major determinant of $[Ca^{2+}]_i$ (Fig. 6a). Alternatively, Li^+ could act to promote the accumulation of an IP_3 isomer(s) different from the biologically active form, *myo*-inositol-1,4,5-trisphosphate. This possibility is supported by recent observations by Irvine *et al.* (1984).

Two other criteria for identifying a second messenger are that non-physiological agents which increase the concentration of the messenger should reproduce the effects of physiological agonists, and that addition of the second messenger to cells or subcellular fractions should produce the expected changes. The first of these criteria has not been satisfied for IP_3 as yet. However, the second criterion has recently been met for liver by the studies of Joseph *et al.* (1984), Burgess *et al.* (1984) and Thiyagarajah *et al.* (1985) who demonstrated that IP_3 released Ca^{2+} from permeabilized cells. Additionally, Thiyagarajah *et al.* (1985) showed that IP_3 released Ca^{2+} from 'heavy' particulate fractions enriched in mitochondria and microsomes.

Possible role of diacylglycerol

The other product of PIP_2 hydrolysis is 1,2-diacylglycerol. This is an activator of the Ca^{2+} -phospholipid-dependent protein kinase designated protein kinase C (Kaibuchi *et al.*, 1982). If this enzyme is located in the plasma membrane, its activity may be altered following PIP_2 hydrolysis by phospholipase C. The functions of protein kinase C and its physiological substrates in liver are presently unknown. The addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (10^{-7} – 10^{-5} M), a stimulator of protein kinase C (Kaibuchi *et al.*, 1982), to isolated hepatocytes does not cause phosphorylase activation (Garrison *et al.*, 1984; Roach & Goldman, 1983), Ca^{2+} efflux or Quin-2 fluorescence (R. Charest, P. F. Blackmore & J. H. Exton, unpublished work). Garrison *et al.* (1984) have shown that active phorbol esters increase the phosphorylation of three of the ten cytosolic substrates affected by Ca^{2+} -dependent hormones in hepatocytes.

Role of extracellular Ca^{2+}

Fig. 5 shows that the ability of vasopressin to raise $[Ca^{2+}]_i$ or activate phosphorylase during the initial 30–45 s is independent of the depletion of extracellular Ca^{2+} for times up to 4 min. Furthermore, incubation of hepatocytes with 10 mM-EGTA for 30 s prior to the addition of 10^{-9} M-vasopressin had no effect on the initial Quin-2 response, although after approx. 1 min the $[Ca^{2+}]_i$ began to decline rapidly (R. Charest, P. F. Blackmore & J. H. Exton, unpublished work). Only when intracellular stores of Ca^{2+} are severely de-

pleted (Blackmore *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1977) are hormone effects on $[Ca^{2+}]_i$ and phosphorylase abolished. Although the initial changes in $[Ca^{2+}]_i$ and phosphorylase a induced by vasopressin are unaltered in cells exposed to low- Ca^{2+} media, these parameters decline more rapidly than normal in such cells (Fig. 6). Thus a certain level of extracellular Ca^{2+} , and hence influx of Ca^{2+} , is necessary to maintain an elevated $[Ca^{2+}]_i$ with vasopressin (Fig. 6a).

Possible sources of Ca^{2+} mobilized by PIP_2 turnover

As described more fully elsewhere (Thiyagarajah *et al.*, 1985), IP_3 is capable of mobilizing Ca^{2+} from 'heavy' particulate fractions sedimenting with mitochondria and components of the endoplasmic reticulum. Prentki *et al.* (1984) have also reported that IP_3 releases Ca^{2+} from rat insulinoma microsomes, but were unable to demonstrate Ca^{2+} release from mitochondria isolated from these tumours. The release of Ca^{2+} from these organelles by IP_3 *in vitro* is evident within 1 s (Thiyagarajah *et al.*, 1985). This is consistent with the rapid effects of the hormones on cytosolic Ca^{2+} (Fig. 2c). However, it is possible that the generation of IP_3 by agonist-stimulated breakdown of PIP_2 , which occurs in the plasma membrane (Seyfred & Wells, 1984b), might not be the rate-limiting step in Ca^{2+} mobilization, but rather it might be the diffusion of IP_3 to the intracellular Ca^{2+} stores, particularly since IP_3 appears to be rapidly degraded (Burgess *et al.*, 1984; Thiyagarajah *et al.*, 1985).

Ca^{2+} -dependency of IP_3 accumulation

The fact that a high concentration of A23187 (10^{-5} M) increases the level of IP_3 in a Ca^{2+} -dependent manner (Table 1) raises the question of whether hormones increase the level of this compound merely by raising $[Ca^{2+}]_i$. This seems unlikely since high concentrations (10^{-8} M) of glucagon which raise $[Ca^{2+}]_i$ to as high a level as do vasopressin and adrenaline (Charest *et al.*, 1983) are without effect on IP_3 . Furthermore, IP_3 was unaffected by 10^{-6} M-A23187 which maximally activated phosphorylase; $[Ca^{2+}]_i$ could not be measured due to A23187 fluorescence. Thus it would seem that occupancy of the receptors for the Ca^{2+} -dependent hormones is required for the IP_3 response and that the effect of 10^{-5} M-A23187 may be due to a perturbation of the membrane.

The Ca^{2+} -dependency of the effects of hormones on IP_3 accumulation and PIP_2 hydrolysis (Table 1; Rhodes *et al.*, 1983) could be explained if one or more of the enzymes or proteins involved in coupling the occupied receptor to the elevation of IP_3 were Ca^{2+} -dependent. This would characterize the response as being Ca^{2+} -dependent, but not

mediated by Ca^{2+} , as discussed in detail by Michell *et al.* (1981).

Concluding remarks

The hydrolysis of PIP_2 and accumulation of IP_3 appear to be closely linked to the binding of the Ca^{2+} -mobilizing agents to their receptors, since maximally effective doses of these agents (vasopressin, angiotensin II and adrenaline) produce different magnitudes of PIP_2 (and PI) breakdown, although they are equally effective at mobilizing intracellular Ca^{2+} and activating phosphorylase (Creba *et al.*, 1983; Blackmore *et al.*, 1978; Prpić *et al.*, 1982b). The different extents of hydrolysis of PIP_2 add support to the view that it is not secondary to the rise in $[Ca^{2+}]_i$.

Another possible role for PIP_2 hydrolysis is regulation of the plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase involved in the transfer of Ca^{2+} from the cytosol to the extracellular space (Lin *et al.*, 1983; Prpić *et al.*, 1984). Consistent with this proposal are the data in Fig. 8 which show the relationship between the ability of hormones to inhibit Ca^{2+} transport into liver plasma membrane vesicles (Prpić *et al.*, 1984) and to stimulate IP_3 formation (the present paper). Supraphysiological concentrations were used to ensure maximal effects on phosphoinositide metabolism and Ca^{2+} -pump activity (Prpić *et al.*, 1984). Fig. 8 shows that each hormone

Fig. 8. Correlation between the inhibition of Ca^{2+} pump activity and increases in IP_3 levels elicited by a variety of hormones

Pump activity was measured after 3 min of hormone exposure (Prpić *et al.*, 1984) while the IP_3 levels are taken from Table 1 of the present paper. Maximally effective doses of each hormone were used and were glucagon (10^{-8} M), angiotensin II (10^{-8} M) vasopressin (10^{-7} M) and adrenaline (10^{-5} M).

produces a different effect on both parameters, whereas they are all capable of maximally activating phosphorylase, raising $[Ca^{2+}]_i$, mobilizing mitochondrial Ca^{2+} or promoting Ca^{2+} efflux (Blackmore *et al.*, 1978; Charest *et al.*, 1983; Blackmore *et al.*, 1979). Although the data support the view that phosphoinositides regulate the Ca^{2+} pump (Buckley & Hawthorne, 1972; Penniston, 1983), it should be recognized that they are also consistent with regulation by 1,2-diacylglycerol or inositol phosphates.

Together, the data of the present and other reports (Joseph *et al.*, 1984; Burgess *et al.*, 1984; Thiagarajah *et al.*, 1985) allow the conclusion that IP_3 has the appropriate characteristics for the putative intracellular signal generated in response to activation of α_1 -adrenergic and V_1 -vasopressin receptors in the hepatocyte plasma membrane which induces net calcium release from intracellular organelles.

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