A transient increase in diacylglycerols is associated with the action of vasopressin on hepatocytes

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1. Vasopressin induced a transient increase of 50% in the total concentration of diacylglycerols (determined by g.l.c.) in isolated hepatocytes. The increase was maximal at 0.25 min, and the concentration of diacylglycerols in cells treated with vasopressin had returned to the basal value by 4 min. No change in the concentration of diacylglycerols was observed after the treatment of cells with glucagon. 2. The dependency of this effect on the concentration of vasopressin was similar to that of the effect of the hormone on $^{45}\text{Ca}^{2+}$ efflux measured at 0.1 mM extracellular Ca^{2+} . 3. Vasopressin increased the proportion of arachidonic acid and stearic acid and decreased the proportion of oleic acid present in the diacylglycerols. 4. In hepatocytes prelabelled with [^{14}C]arachidonic acid, vasopressin increased the amount of [^{14}C]diacylglycerol. 5. The effects of vasopressin on the total concentration of diacylglycerols and [^{14}C]diacylglycerol were mimicked by an exogenous phospholipid phosphodiesterase (phospholipase C) from *Clostridium perfringens*. 6. The results are consistent with the conclusion that the transient increase in diacylglycerols induced by vasopressin is caused by the rapid hydrolysis of both the phosphoinositides and one or more other phospholipids.

It is now generally accepted that the stimulatory effects of α -adrenergic agonists, vasopressin and angiotensin on glycogen breakdown in rat liver requires an increase in the cytoplasmic Ca^{2+} concentration (reviewed by Exton, 1981; Williamson *et al.*, 1981). Further, a body of evidence suggests that a major source of this Ca^{2+} is the mitochondria (Blackmore *et al.*, 1979; Babcock *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981; Berthon *et al.*, 1981; Kimura *et al.*, 1982; Reinhart *et al.*, 1982). The proposal has developed that, after binding of α -adrenergic agonists or vasopressin to their receptors, a signal or intracellular mediator is generated which leads to the release of Ca^{2+} from the mitochondria (reviewed by Exton, 1981; Williamson *et al.*, 1981). Several workers have postulated that one or more of the products of the breakdown of the phosphoinositides may be candi-

dates for the putative intracellular mediator (Billah & Michell, 1979; Barritt, 1981; Thomas *et al.*, 1983).

Vasopressin has been shown to cause a rapid decrease in the concentrations of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (Kirk *et al.*, 1981; Michell *et al.*, 1981; Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Creba *et al.*, 1983). These data are consistent with the proposal that α -adrenergic agents and vasopressin stimulate the activity of phosphoinositide phosphodiesterase (phospholipase C), which *in vivo* converts $\text{PtdIns}(4,5)\text{P}_2$ into diacylglycerol and L-*myo*-inositol 1,4,5-trisphosphate (Berridge, 1983). Indeed it has been shown that the addition of an exogenous phospholipid phosphodiesterase mimics the effect of phenylephrine on Ca^{2+} transport in hepatocytes (Barritt & Whiting, 1983).

In order to test further the involvement of phospholipid phosphodiesterase, we have investigated the effect of vasopressin on the immediate product of the reaction catalysed by this enzyme, namely diacylglycerol. The effects of vasopressin have been compared with those of an exogenous phospholipid phosphodiesterase. There has been

Abbreviations used: PtdIns , 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol; $\text{PtdIns}4\text{P}$, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; $\text{PtdIns}(4,5)\text{P}_2$, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate.

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considerable interest in diacylglycerol owing to the characterization of a diacylglycerol-activated protein kinase (protein kinase C) and its postulated role in platelet activation by thrombin (Takai *et al.*, 1979; Kikkawa *et al.*, 1982; Kaibuchi *et al.*, 1983). It has been proposed that *in vivo* this protein kinase may be specifically activated by diacylglycerol, the concentrations of which may be increased by the breakdown of phosphoinositides when cells are stimulated by a wide variety of agents, including α -adrenergic agents (Kaibuchi *et al.*, 1983).

Experimental

Isolation and incubation of hepatocytes

The isolation of hepatocytes from fed male rats and measurement of $^{45}\text{Ca}^{2+}$ release at 0.1 mM- Ca^{2+} were performed as described previously (Barritt *et al.*, 1981). In experiments involving the measurement of diacylglycerol concentrations, the conditions for hepatocyte incubation were identical with those described previously (Barritt & Whiting, 1983). The incubation medium contained, in a final volume of 7.6 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-2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulphonic acid/KOH, pH 7.4, 0.1 mM- CaCl_2 , 30 mg wet wt. of cells/ml and other additions as indicated. A pre-equilibration time of 15 min was allowed before any additions were made. Vasopressin was dissolved in 0.1 M-acetic acid as a stock solution of 0.13 mM and kept at -20°C . It was diluted before use at least 100-fold in 0.9% (w/v) NaCl. Control incubations received an equal volume of 0.1 M-acetic acid diluted accordingly in 0.9% NaCl. Phospholipase C from *Clostridium perfringens* was used as previously described (Barritt & Whiting, 1983).

Extraction of lipids, isolation of diacylglycerols, transmethylation of fatty acids and g.l.c.

The procedures were based on established methods (Banschbach *et al.*, 1981). All organic solvent mixtures contained 0.05% (w/v) of the antioxidant butylated hydroxytoluene. At the desired time, 7.0 ml samples of the cell incubations were placed into 15 ml of ice-cold methanol/chloroform (2:1, v/v), mixed vigorously and stored on ice until the end of all incubations. The lipids were extracted by shaking in 50 ml glass separating funnels after the addition of 5 ml each of chloroform and 2 M-KCl 5 mM- Na_2EDTA , to each sample. The chloroform layer was evaporated to dryness with a rotary evaporator. The lipid extracts were transferred in 0.5 ml of chloroform into glass vials with Teflon screw caps and evaporated to approx. 0.1 ml under N_2 .

Diacylglycerols were separated from other lipids

by t.l.c. on silica-gel-pre-coated plastic-backed plates (silica gel 60 F254; E. Merck, Darmstadt, Germany), activated at 110°C for 1 h immediately before use. The solvent system was light petroleum (b.p. $40\text{--}60^\circ\text{C}$)/diethyl ether/acetic acid (80:20:1, by vol.). At the conclusion of the chromatography the plates were sprayed with 0.01% (w/v) 2,7-dichlorofluorescein in 90% (v/v) ethanol and observed under u.v. light.

The spots containing both the 1,3- and 1,2-isomers of diacylglycerols were scraped off, combined, and transferred into screw-capped glass test tubes, and 5 ml of methanol containing 1% (v/v) conc. H_2SO_4 was added. An internal standard of 5 μl of 4 mm-heptadecanoic acid was added to each tube. Transmethylation was performed by heating at 70°C for 3 h. The methylated fatty acids were extracted with 2 ml of light petroleum (b.p. $40\text{--}60^\circ\text{C}$) and the extracts evaporated to dryness under N_2 .

For g.l.c. 0.1 ml of chloroform was added to each sample and 1 μl was injected on to a silicone-treated glass column (internal diameter 1 mm $\times$ length 1.80 m) packed with 10% DEGS-PS on 80/100 Supelcoport (Supelco, Bellefonte, PA, U.S.A.), incorporated into a 5830A gas chromatograph equipped with a 18850A Terminal/Integrator (Hewlett Packard, Avondale, PA, U.S.A.). The column was run at a N_2 flow rate of about 35 ml/min, and the oven, injector and flame-ionization-detector temperatures were 175, 220 and 220°C respectively.

Samples (5–60 μl) of a standard solution of approx. 1 mM each of palmitic acid, stearic acid, oleic acid, linoleic acid and arachidonic acid in chloroform were methylated as described above. In the cell extracts only these acids were found to be present in measurable quantities. Diacylglycerol concentrations in the cell extracts were calculated from the amounts of the component fatty acids. Blank extractions in which 7.0 ml samples of cell incubation medium were taken through the entire procedure were always conducted. The values obtained for the component fatty acids were subtracted from the values obtained for the cell extracts.

The recovery of diacylglycerols was estimated by the inclusion of additional samples which contained 7.0 ml of cell-incubation buffer and 625 nmol of 1,2-diolein, 1,2-dipalmitoylglycerol or 1,3-dipalmitoylglycerol. These were taken through the entire procedure and served as markers for the t.l.c. step. The recovery of the added diacylglycerols was $74.4 \pm 2.5\%$ ($n = 10$).

Incorporation of [^{14}C]arachidonic acid into diacylglycerol

Isolated hepatocytes (30 mg wet wt./ml) were incubated for 30 min at 37°C in incubation medium

(30 ml total volume) in the presence of 15 mM-glucose, 0.1 mM- CaCl_2 and tracer [^{14}C]arachidonic acid [0.5 $\mu\text{Ci/ml}$, dissolved in dimethyl sulphoxide (final concn. 1%, v/v). Bovine serum albumin (1 mg/ml) was then added in order to bind unincorporated [^{14}C]arachidonic acid, and the cells were washed once in incubation medium which contained 0.1 mM- CaCl_2 but no [^{14}C]arachidonic acid. Cells (30 mg wet wt./ml) labelled with [^{14}C]arachidonic acid were incubated for 5 min at 37°C in incubation medium (0.1 mM- CaCl_2) before the addition of vasopressin or vehicle (control incubations). Samples were removed at 0, 1 and 5 min for measurement of [^{14}C]diacylglycerol. The extraction of lipids and isolation of diacylglycerols were performed as described by Billah *et al.* (1980). The areas of the plate corresponding to both the 1,2- and 1,3-isomers of diacylglycerol were scraped off, and the total amount of [^{14}C]diacylglycerols present was determined by liquid-scintillation counting in ACS (aqueous counting scintillant) II (Amersham Australia Pty., Sydney, N.S.W., Australia). The amount of radioactivity present in the diacylglycerols at 0 min after addition of vasopressin (or vehicle) was 1342 ± 106 c.p.m. (mean $\pm$ S.E.M., $n = 7$) per spot, which represented one-quarter of the lipids extracted from 45 mg wet wt. of cells.

Expression of results

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 for paired samples. Values of $P > 0.05$ were considered to be not significant.

Materials

[Arginine]vasopressin, phospholipid phosphodiesterase (phospholipase C) from *Clostridium perfringens*, butylated hydroxytoluene, 2,7-dichlorofluorescein, fatty acids and diacylglycerol standards were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The solvents methanol, chloroform and diethyl ether were from E. Merck. Light petroleum (b.p. 40–60°C) was from Ajax Chemicals, Sydney, N.S.W., Australia. $^{45}\text{CaCl}_2$ and [^{14}C]arachidonic acid (57.6 Ci/mol) were obtained from Amersham Australia. All other reagents were of the highest grade available.

Results

Exposure of hepatocytes to a maximally effective concentration of vasopressin (10 nM) led to a transient increase in the total concentration of diacylglycerols (Fig. 1). The maximal effect was observed at 0.25 min after addition of vasopressin (the shortest time period measured). The concen-

Fig. 1. Time course of the effect of vasopressin on the concentration of diacylglycerols present in isolated hepatocytes

The concentration of diacylglycerols in the presence and absence of 10 nM-vasopressin was determined as described in the Experimental section. The concentration of diacylglycerols present in vasopressin-challenged hepatocytes at a given time is expressed as a percentage of the corresponding value in control cells. Each point is the mean $\pm$ S.E.M. for four separate cell preparations, each of which was incubated in triplicate or quadruplicate. The mean control diacylglycerol content was 1.48 ± 0.25 nmol/mg wet wt. of cells. The degrees of significance are $P < 0.05$ for the 0.25 and 2 min values, and $P < 0.025$ for the 1 min value.

tration of diacylglycerols returned to the basal value by 4 min. The maximal effect of vasopressin was obtained with a concentration of 1 nM, and a half-maximal effect with approx. 0.1 nM-vasopressin (Fig. 2). A similar concentration-dependency was observed for the effect of vasopressin on $^{45}\text{Ca}^{2+}$ efflux measured at 0.1 mM extracellular Ca^{2+} (Fig. 2). No change in the concentration of diacylglycerols was observed after treatment of hepatocytes with 10 nM-glucagon. At 1 and 5 min after addition of the hormone, the concentrations of diacylglycerols were 105 ± 12 and 92 ± 6 ($n = 3$) % of the control value at these time periods.

Measurement of the fatty acid composition of the diacylglycerols indicated that at 1 min exposure to vasopressin there is an increase of 41 mol% and a decrease of 30 mol% in arachidonic acid and oleic acid present respectively (Table 1). At 0.25, 2 and 4 min exposure to vasopressin the increases in arachidonic acid present in diacylglycerols isolated from cells treated with vasopressin were 17, 40 and 63 mol% respectively, and the changes in the proportion of oleic acid were +24, -17 and -25 mol% respectively ($n = 4$). No significant

Fig. 2. Effect of increasing concentrations of vasopressin on the concentration of diacylglycerols present in isolated hepatocytes

The concentration of diacylglycerols (●) was measured at 1 min after the addition of vasopressin as described in the Experimental section. The values are the means $\pm$ S.E.M. for eight incubations conducted using two separate preparations of cells. The loss of $^{45}\text{Ca}^{2+}$ from isolated hepatocytes induced by vasopressin (○) was measured at 0.1 mM extracellular Ca^{2+} as described in the Experimental section. The values are the means of experiments conducted with two separate preparations of cells.

changes were observed in the mole per cent of the other fatty acids present at any of the times tested (results not shown). Analysis of the fatty acid composition of the diacylglycerols formed as a result of vasopressin action indicated that these species are enriched in stearic acid and arachidonic acid and have low amounts of oleic acid compared with diacylglycerols isolated from control cells (Table 1).

Vasopressin increased the amount of [^{14}C]diacylglycerol present in isolated hepatocytes pre-

labelled with [^{14}C]arachidonic acid. At 1 and 5 min after the addition of 10 nM vasopressin, the amount of [^{14}C]diacylglycerol was 101 ± 1.5 and 112 ± 4.0 ($n = 6$, $P < 0.05$)% of the value at zero time, respectively. The values for control cells were 97 ± 2 and 98 ± 4 %, respectively, of the value at zero time.

In hepatocytes exposed to exogenous phospholipid phosphodiesterase from *Clostridium perfringens* (1.5 units/ml) for periods of 2 and 4 and 10 min, the concentration of diacylglycerols was increased to 310, 292 and 347%, respectively, of the control value (means of two or three experiments). The proportion of fatty acids present in the diacylglycerols formed as a result of the action of exogenous phospholipid phosphodiesterase (4 min) was palmitic acid 29, stearic acid 18, oleic acid 17, linoleic acid 18 and arachidonic acid 18 mol%. Exposure of hepatocytes pre-labelled with [^{14}C]arachidonic acid to exogenous phospholipid phosphodiesterase (1 unit/ml) for 10 min increased the amount of [^{14}C]diacylglycerol from 0.8 ± 0.3 to 11.7 ± 0.4 % of the total radioactivity in the lipid extract ($n = 3$). The amount of [^{14}C]phosphatidylcholine decreased from 32 ± 1 to 26 ± 2 % of the total radioactivity in the lipid extract after 10 min exposure to the phospholipid phosphodiesterase.

Discussion

The present results indicate that vasopressin causes a large transient increase in total diacylglycerols in the liver cell. This change, which is rapid in onset, is associated with a sustained increase in the proportion of arachidonic acid in the diacylglycerols. Vasopressin has been shown to cause a decrease in the concentrations of $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}4\text{P}$ and PtdIns in hepatocytes (Kirk *et al.*, 1981; Michell *et al.*, 1981; Prpić *et al.*, 1982; Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Creba *et al.*, 1983; Litosch *et al.*, 1983). The onset of the effect on $\text{PtdIns}(4,5)\text{P}_2$ is observed within about 5 s (Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Creba *et al.*, 1983; Litosch *et al.*, 1983). It is concluded that the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ as well as of $\text{PtdIns}4\text{P}$ and PtdIns [through conversion into $\text{PtdIns}(4,5)\text{P}_2$ (Creba *et al.*, 1983)] accounts for some of the diacylglycerols formed in the presence of vasopressin. This conclusion is based on (a) the observation that in cells labelled with [^{14}C]arachidonic acid vasopressin increases the amount of [^{14}C]diacylglycerol, and (b) comparison of the fatty acid compositions of the diacylglycerols formed in the presence of vasopressin or exogenous phospholipid phosphodiesterase, which caused a decrease chiefly in [^{14}C]phosphatidylcholine, with that of the phosphoinositides (Holub *et al.*, 1970; Holub & Kuksis, 1971; Allan & Cockcroft, 1983) and phosphatidylcholine (sum-

Table 1. *Effect of vasopressin on the fatty acid composition of diacylglycerols isolated from hepatocytes*

The isolation of diacylglycerols from suspensions of hepatocytes incubated for 1 min in the presence or absence of 10 nM-vasopressin, determination of the fatty acid composition and calculation of the proportion of fatty acids present were performed as described in the Experimental section. The values are the means $\pm$ S.E.M. of the results obtained with four separate cell preparations, for each of which triplicate or quadruplicate determinations were made. The degrees of significance are: * $P < 0.025$. The fatty acid composition of the diacylglycerols formed as a result of vasopressin action were determined as follows. For a given fatty acid, the total amount derived from diacylglycerols in control cells (nmol/g wet wt.) was subtracted from that derived from diacylglycerols in vasopressin-treated cells. This was expressed as a percentage of the sum of the amounts of all fatty acids present in the diacylglycerols formed as a result of vasopressin action [the sum of all fatty acids (nmol/g wet wt.) in diacylglycerols in control cells subtracted from the sum of all fatty acids in diacylglycerols in vasopressin-treated cells].

Fatty acid	Proportion of fatty acid in diacylglycerols (mol%)		
	Control cells	Cells treated with vasopressin	Diacylglycerols formed as a result of vasopressin action
Palmitic (C _{16:0})	27.1 $\pm$ 1.8	22.8 $\pm$ 1.2	17 $\pm$ 4
Stearic (C _{18:0})	18.6 $\pm$ 4.0	24.2 $\pm$ 1.9	32 $\pm$ 7
Oleic (C _{18:1})	18.0 $\pm$ 3.3	10.9 $\pm$ 1.9*	1 $\pm$ 7
Linoleic (C _{18:2})	21.1 $\pm$ 3.3	20.6 $\pm$ 2.5	20 $\pm$ 8
Arachidonic (C _{20:4})	15.2 $\pm$ 1.9	21.4 $\pm$ 0.9*	30 $\pm$ 4

marized by Holub & Kuksis, 1978). Takenawa *et al.* (1982) and Thomas *et al.* (1983) have also reported an increase in the amount of [¹⁴C]diacylglycerol in hepatocytes labelled with [¹⁴C]arachidonic acid and subsequently treated with vasopressin.

Assuming that the rates of PtdIns(4,5)P₂ resynthesis and metabolism of diacylglycerol are small during the first 1 min of vasopressin action, the amount of diacylglycerol formed by vasopressin-induced hydrolysis of phosphoinositides, estimated from the data of Creba *et al.* (1983), is about 30 nmol/g wet wt. Higher or lower values are obtained by using data from other laboratories (Rhodes *et al.*, 1983; Thomas *et al.*, 1983; Litosch *et al.*, 1983). These calculations indicate that at least half of the observed increase in diacylglycerol (0.7 μ mol/g wet wt.) is likely to be derived from sources other than the phosphoinositides.

Triacylglycerols, phosphatidic acid or other phospholipids could also be precursors of diacylglycerol. Evidence that vasopressin or Ca²⁺ affect some of the reactions involved in the conversion of these lipids into diacylglycerol has been obtained (Allan & Michell, 1977; Williamson *et al.*, 1980; Sugden & Williamson, 1981; Alemany *et al.*, 1982; Pollard & Brindley, 1984). Comparison of the fatty acid composition of the diacylglycerols formed in the presence of vasopressin with that of triacylglycerols (Åkesson, 1969), the observations that vasopressin increases the rate of fatty acid esterification (Williamson *et al.*, 1980) and the amount of [³²P]phosphatidic acid in cells prelabelled with [³²P]P_i (Thomas *et al.*, 1983; Litosch *et al.*, 1983), and the failure of glucagon [which has been shown to alter hepatic triacylglycerol metab-

olism (reviewed by Saggerson & Bates, 1981; Pelech *et al.*, 1983)] to induce a transient change in diacylglycerols suggest that stimulation by vasopressin of the net conversion of triacylglycerol or phosphatidic acid into diacylglycerol is unlikely. However, the possibility that diacylglycerols are formed by stimulation of the hydrolysis of other phospholipids has not been excluded. Moreover, the present results have demonstrated that this can be achieved by exogenous phospholipid phosphodiesterase. Since the amounts of phosphatidylcholine and phosphatidylethanolamine present in the liver cell plasma membrane are much higher than the phosphoinositides (White, 1973), hydrolysis of only about 3% of one of these lipids would yield the observed increase in diacylglycerols.

The time course for changes in total diacylglycerols observed in the present experiments is similar to that for the effects of thrombin and other agents on the concentration of diacylglycerols in blood platelets (Bell *et al.*, 1979; Rittenhouse-Simmons, 1979, 1981) and of thyrotropin-releasing hormone on [³H]diacylglycerol in pituitary cells (Rebecchi *et al.*, 1983). For blood platelets, evidence consistent with a role for diacylglycerol in the physiological activation of protein kinase C has been presented (Kaibuchi *et al.*, 1983). The present results are consistent with a role for the activation of protein kinase C by diacylglycerol in the mechanism by which vasopressin induces physiological responses in the liver cell (Cooper *et al.*, 1984; Garrison *et al.*, 1984).

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