Kinetics of diacylglycerol accumulation in response to vasopressin stimulation in hepatocytes of continuously endotoxaemic rats

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The content and composition of 1,2-diacyl-*sn*-glycerol (1,2-DAG) was determined in hepatocytes from saline (0.9 % NaCl)- and *Escherichia coli* endotoxin (ET)-infused rats upon continuous vasopressin (VP) (10^{-8} M) stimulation. In both experimental groups the accumulation of 1,2-DAG was detected after a lag period (2–5 min), was sustained up to the last time analysed (10 min), and $C_{18:0}$ - and $C_{20:4}$ -fatty-acid-containing-DAG accumulation preceded that of DAG containing other acyl groups. In hepatocytes from ET-infused rats the VP-induced accumulation of DAG was delayed and was decreased by 50 %, showing a $C_{18:0}/C_{20:4}$ molar ratio of 1.6 as compared with 1.1 for cells from saline-infused rats. A similar lower cellular response to VP stimulation was observed in cells prelabelled with [14 C] $C_{20:4}$ fatty acid. The accumulation of [14 C] $C_{20:4}$ -DAG (lower in ET than in saline-infused rats) was paralleled by a decrease in phosphatidylinositol (PI) labelling, whereas phosphatidic acid showed a transient increase by 5 min in saline- but not in ET-infused rats. The present results demonstrate that the previously reported impairment in the early degradation of poly-PI and later in the 'PI cycle' during VP stimulation [Rodriguez de Turco & Spitzer (1987) *Metab. Clin. Exp.* 36, 753–760] is also reflected at the level of their phosphodiesteratic product, DAG. Moreover, the kinetics of the accumulation of DAG acyl groups is consistent with the idea that the initial release of $C_{18:0}$ - and $C_{20:4}$ -DAG (possibly derived from inositol lipids) could regulate the subsequent enlargement of this pool by stimulating a phospholipase C-mediated degradation of other phospholipids (e.g. phosphatidylcholine).

INTRODUCTION

During endotoxemia and sepsis, that is, in the face of detectable circulating levels of bacterial cell-wall components known as endotoxins, or live microbial organisms and their secretory products, hepatocytes sustained severe perturbations in the transmission of information from extracellular Ca^{2+} -mobilizing glyco-lytic hormones [1]. We have further characterized the interference by endotoxaemia in the regulation of cell metabolism linked to the inositol phospholipid/ Ca^{2+} signalling mechanism using rats continuously infused with a non-lethal dose of *Escherichia coli* endotoxin (ET) [2]. This model has been extensively used in our laboratory [3–5], owing to the fact that it reproduces many of the haemodynamic and metabolic perturbations observed during human 'Gram-negative' septicaemia [6,7]. Previous studies conducted 30 h after continuous ET-infusion (the morbid phase of the process) indicated a perturbation in Ca^{2+} homeostasis and phosphorylase activation when hepatocytes were challenged with vasopressin (VP) or adrenaline (epinephrine) [8,9]. These metabolic perturbations occurred concomitantly with 'structural' and 'functional' changes, that is, a decrease in the number of α_1 -adrenergic and V_1 -vasopressin receptors [10–11] and a significant attenuation of V_1 function as measured by VP-stimulated poly[32 P]PI (poly[32 P]phosphoinositol) degradation and [32 P]PI turnover [1]. Additionally, Spitzer & Deaciuc [12] observed

that the ability of *myo*-inositol 1,4,5-trisphosphate (IP_3) to release Ca^{2+} from endoplasmic-reticulum stores in permeabilized hepatocytes from ET-infused rats was greatly diminished.

It is well known that the early event triggered by Ca^{2+} -mobilizing agonists in a variety of tissues is the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol (1,2-DAG) and IP_3 (for reviews see [13–17]). The subsequent increase in PI turnover has been suggested as the main source of agonist-stimulated production of $C_{20:4}$ -DAG [17]. The co-ordinate and possibly different temporal roles of the two second messengers released from inositol lipids involve the early Ca^{2+} mobilization by IP_3 from the endoplasmic reticulum [14,18–20] and the stimulation of a Ca^{2+} - and phospholipid-dependent protein kinase C by DAG [21,22].

In our present paper we report that the time course of the DAG signal generation through hepatocyte V_1 -vasopressin receptor stimulation involved the primary release of $C_{18:0}$ - and $C_{20:4}$ -DAG. This was followed by a sustained enlargement of the DAG pool by other fatty acids (i.e. $C_{16:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{22:6}$). Endotoxaemia greatly diminished the magnitude of the DAG signal, both at early and later times of VP stimulation. This could result in an altered transmembrane control of protein kinase C-mediated protein phosphorylation, contributing to the observed impairment in the regulation of cell metabolism.

Abbreviations used: DAG, 1,2-diacyl-*sn*-glycerol; ET, *Escherichia coli* endotoxin; NEFA, non-esterified fatty acids; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; TAG, triacylglycerol; VP, vasopressin. $C_{x:y}$ -DAG, $C_{x:y}$ -fatty-acid containing diacylglycerol; BSA, bovine serum albumin.

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EXPERIMENTAL

Materials

Collagenase (CLS II, containing 170 units/mg) was purchased from Cooper Biomedical (Irvine, CA, U.S.A.), endotoxin (*E. coli*, 026: B6, prepared by the Boivin method) was from Difco Laboratories (Detroit, MI, U.S.A.). [Arg⁸]Vasopressin (VP) (Pitressin) was from Parke-Davis (Morris Plains, NJ, U.S.A.). Precoated silica-gel H plates and silica-gel GHL plates were from Analtech (Newark, NJ, U.S.A.). Lipid standards and 14% (w/v) BF₃ in methanol were from Sigma (St. Louis, MO, U.S.A.). [1-¹⁴C]Arachidonic acid (sp. radioactivity 58 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Ready-Solv EP was from Beckman Instruments (Fullerton, CA, U.S.A.). All solvents used were h.p.l.c. grade from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.).

Animals

Male Sprague-Dawley rats (350–400 g) were used throughout and were given standard rat chow (Purina) and water *ad libitum* until the day of surgery. At this time an osmotic minipump (Alzet 2ML 1) was implanted subcutaneously over the scapulae under diethyl ether anaesthesia [2]. Pumps were set up to deliver sterile saline (0.9% NaCl) at a rate of 10 μ l/h into the right jugular vein. After a postsurgical recovery period of 42 h, ET (0.1 mg/24 h per 100 g body wt.) ('ET-pump rats') or saline ('saline-pump rats') was infused continuously and animals were killed 30 h later. At this time ET-infused rats showed some sustained haemodynamic and metabolic perturbations, which were reversed thereafter as tolerance to endotoxin evolved [2,23]. Experiments were also done in control rats (fed *ad libitum*) and in pair-fed rats (normal rats whose food intake was decreased to match that voluntarily consumed by ET-pump rats).

Preparation of hepatocytes

Isolated liver cells were prepared using modifications of the procedure of Berry & Friend [24] as previously described [1]. The perfusion medium was a Ca²⁺-free haemoglobin-free Hanks bicarbonate buffer constantly gassed with O₂/CO₂ (19:1). This was followed by recycling the same oxygenated buffer containing CaCl₂ (2.5 mM) and collagenase (4250 units, corresponding to 25 mg of enzyme) for 13 min. The isolated liver cells were washed three times by centrifugation (60 g, 2 min) in a modified Krebs-Ringer bicarbonate buffer containing (mmol/l): NaCl, 120; KCl, 5.4; CaCl₂, 1.8; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 4.2; Hepes, 20; glucose, 11; and dialysed bovine serum albumin, 2% (w/v), at pH 7.45. Cells were resuspended in the same medium, adjusted to 40 mg wet wt./ml and kept at room temperature under O₂ until incubations were started. Viability was routinely tested. Cell preparations were considered suitable when more than 90% excluded Trypan Blue. In this procedure a few drops of an aqueous solution of 0.4% Trypan Blue dye are added to a suspension of cells and the cells are then observed under an optical microscope. Exclusion of the dye from the interior of the cell is a commonly accepted criterion for cell membrane integrity and thus viability.

Incubation of cells and extraction of lipids

Aliquots (1 ml) of cell suspensions (40 mg wet wt./ml) were transferred to plastic tubes and preincubated at

37 °C in a shaking incubator for 3 min before addition of hormone. Incubations were continued for the period specified in the presence or absence of VP (10⁻⁸ M), and were terminated by the addition of 7 ml of chloroform/methanol (1:1, v/v). After at least 2 h at room temperature, the extracts were centrifuged and the residue extracted once more with 3 ml of chloroform/methanol (2:1, v/v). The combined supernatant was washed [25], evaporated to dryness under N₂ and redissolved in chloroform/methanol (2:1, v/v) before storage at -70 °C under N₂. To avoid peroxidation of unsaturated fatty acids, the solvents used for lipid extraction and t.l.c. contained 2,6-di-*t*-butyl-*p*-cresol (50 mg/l) as an antioxidant.

An aliquot of the sample was spotted on to silica-gel GHL plates, and 1,2-DAG was separated by one-dimensional t.l.c., with chloroform/acetone (24:1, v/v) as a solvent system. Under these conditions, 1,3- and 1,2-DAG were clearly separated from other lipids and identified by a standard co-chromatographed with the samples. The plates were sprayed with 2',7'-dichlorofluorescein in methanol, and the spots were revealed under u.v. light, scraped into tubes and then derivatized to methyl esters of fatty acids with 14% BF₃ in methanol [26]. Blanks containing 1 ml of buffer were subjected to the same methodological steps as the sample.

Fatty acid analysis by g.l.c.

The methylated fatty acids were analysed in a Hewlett-Packard 790 series chromatograph equipped with a terminal computer integrator system and a flame-ionization detector. A capillary column (15 m long $\times$ 0.2 mm inside diameter) packed with 10% SP 2330 on 100–200 Chromosorb WAW (Supelco, Bellefonte, PA, U.S.A.) and He carrier (flow rate 1 ml/min) were used. The oven was programmed from 170 to 220 °C at 4 °C/min. The fatty acid peaks were identified by comparison of their retention time with those of standards run under identical conditions, and the mass distribution was determined in the presence of an internal standard of methyl nonadecanoate. When 1,2-dinonadecanoyl-DAG was added to the samples before lipid extraction, the final recovery was 95 $\pm$ 1%. This correction factor was not applied to the present results.

Incorporation of [1-¹⁴C]arachidonic acid into glycerolipids

Hepatocytes (50 mg wet wt./ml) were incubated in the presence of [1-¹⁴C]arachidonic acid (1 μ Ci/ml, sp. radioactivity 58 mCi/mmol) in a shaking incubator at 37 °C under O₂ for 30 min. The cells were then washed twice with buffer containing 2% bovine serum albumin (BSA; fatty-acid-free) to remove excess [¹⁴C]C_{20:4} fatty acid, and then resuspended in Krebs-Ringer bicarbonate buffer without BSA at 40 mg wet wt./ml. Aliquots (0.3 ml) of the cell suspensions were transferred to plastic tubes and the incubation continued for 60–300 s and 600 s in the presence or absence of VP (10⁻⁸ M). Incubation was terminated by adding 3.5 ml of chloroform/methanol (1:1, v/v). After lipid extracts were prepared and purified [25], a portion of the samples was spotted under N₂ on precoated silica-gel-H plates (Analtech) of 0.25 mm thickness, and phospholipids were isolated by two-dimensional t.l.c. as described by Rouser *et al.* [27]. For neutral lipids, portions were applied on a silica-gel-GHL plate and resolved by a one-

dimensional system using hexane/diethyl ether/acetic acid (13:7:1, v/v). Poly-PI were extracted with an acidified solvent system and isolated by t.l.c. on potassium oxalate-impregnated plates [1]. The plates were developed with I_2 vapour, the spots revealed being scraped into vials and the silica gel dispersed in 0.5 ml of water. Radioactivity was counted in a Beckman liquid-scintillation spectrometer using 10 ml of Ready Solv EP scintillation fluid (Beckman) containing 20% (v/v) Triton X-100 [28]. Blanks were prepared to enable the non-specific adsorption of the precursor to be subtracted.

Expression of results

Results are presented as mean values $\pm$ S.D. for the numbers of incubations specified for each experiment. For analysis of the DAG pool, values from cells incubated in duplicate from two different experiments are presented. By using another batch of the same collagenase (CLS II) in two other experiments, higher basal values for DAG were observed. Although these pools are 'artefactually' increased during cell isolation, differences between saline and ET-infused cells in VP-induced DAG accumulation were reproducible. Student's *t* test was used for statistical analysis of the data. Results were considered statistically significant when *P* was less than 0.05.

RESULTS

Accumulation of DAG and its fatty acid content during VP stimulation

Changes in 1,2-DAG content of hepatocytes from saline- and ET-infused ('sal-cells' and 'ET-cells' respectively) rats during incubation in the presence or absence of VP (10^{-8} M) are shown in Fig. 1. After 2 min of

Fig. 1. Time course of diacylglycerol accumulation in response to VP stimulation

Hepatocyte suspensions (1 ml, 40 mg wet wt/ml) prepared from saline- ('Saline pump') and ET-('ET pump')infused rats were incubated in the presence (●) or absence (○) of VP (10^{-8} M) for different periods of time. The incubation was terminated by adding 7 ml of chloroform/methanol (1:1, v/v). Separation of DAG, derivatization to fatty acid methyl esters and their quantification by g.l.c. were performed as described in the Experimental section. Each point is the mean value $\pm$ S.D. for four incubations from two different cell preparations. The values for 2 min of VP stimulation in ET-cells were the only ones not statistically significantly different (*P* > 0.05) from their respective basal values.

Fig. 2. Kinetics of DAG-acyl-group accumulation during VP stimulation of hepatocytes from saline-infused rats

After 2 min of VP stimulation, only the contents of $C_{18:0}$ and $C_{20:4}$ fatty acids were significantly increased. Values for $C_{18:0}$ and $C_{20:4}$ content (nmol/100 mg dry wt.) in the absence and presence of VP for 2 min were: 18.8 ± 1.6 versus 25.2 ± 1.2 (*P* < 0.001) and 11.5 ± 1.2 versus 20.4 ± 1.8 (*P* < 0.001) respectively. Other details were as for Fig. 1.

Fig. 3. Kinetics of VP-induced DAG-acyl-group accumulation in hepatocytes from ET-infused rats

Asterisks denote statistically significant differences from their respective basal values (*P* < 0.05). Other details are as for Fig. 1.

incubation at 37 °C (plus 3 min of preincubation) the amount of 1,2-DAG was 142 ± 4 and 96 ± 2 nmol of fatty acid/100 mg of dry weight for saline- and ET-infused rats respectively. ET-cells accumulated, as a function of time, less 1,2-DAG than did sal-cells, both under basal conditions and during VP stimulation. Moreover, addition of VP provoked a 14% and 79% increase in the 1,2-DAG pool within 2 and 5 min respectively in sal-cells, whereas in ET-cells a sustained delay was observed, the increase becoming significant only at 5 min (36%).

Table 1. Stearic acid ($C_{18:0}$) and arachidonic acid ($C_{20:4,n-6}$) contents of PI and DAG accumulated during VP stimulation of hepatocytes

Values are means $\pm$ S.D. for the numbers (n) of individual samples shown. Hepatocytes were obtained from control (normal rats fed *ad libitum*), pair-fed (rats whose food intake was reduced to match that voluntarily consumed by ET-infused rats), saline-infused and ET-infused rats. DAGs released were calculated as the difference between the concentration attained when the cells were incubated for 10 min in the presence of VP (10^{-8} M) and that in its absence. Values given in square brackets are percentages of $C_{18:0}$ - and $C_{20:4}$ -DAG released with respect to the content of these fatty acids esterified into PI. Total values represent total (nmol) fatty acids in PI and DAG released/mg dry wt. Significance of differences between the $C_{18:0}$ - and the $C_{20:4}$ -fatty acid content in PI and DAG released from ET-pump cells: * $P < 0.025$; ** $P < 0.005$. Other details are as in Fig. 1.

Experimental conditions	Lipid	n	Content (nmol/mg dry wt.)			Ratio $C_{18:0}/C_{20:4}$
			$C_{18:0}$	$C_{20:4,n-6}$	Total	
Control	PI*	6	10.50 ± 0.70	9.60 ± 0.90	23.80 ± 2.00	1.09
	DAG	4	0.41 ± 0.09 [3.9]	0.40 ± 0.02 [4.2]	2.60 ± 0.15 [6.9]	1.03
Pair-fed	PI	3	9.40 ± 1.70	8.60 ± 0.60	18.80 ± 2.40	1.09
	DAG	4	0.56 ± 0.12 [6.0]	0.55 ± 0.12 [6.4]	1.82 ± 0.29 [6.9]	1.02
Saline pump	PI	5	10.20 ± 0.80	9.00 ± 1.2	22.10 ± 2.10	1.13
	DAG	4	0.49 ± 0.04 [4.8]	0.46 ± 0.03 [5.1]	1.63 ± 0.06 [7.4]	1.07
ET pump	PI	6	9.70 ± 1.40	$7.20 \pm 0.90^{**}$	19.90 ± 3.00	1.35
	DAG	4	0.27 ± 0.05 [2.8]	$0.17 \pm 0.03^{*}$ [2.4]	0.81 ± 0.06 [4.1]	1.59

* Recalculated from [38].

Under basal conditions all acyl groups contributed to a similar extent to the increase in DAG acyl groups in sal-cells, whereas, during VP stimulation, $C_{18:0}$ - and $C_{20:4}$ -DAG showed the earliest and greatest detectable changes (Fig. 2). Within 2 min of VP stimulation, $C_{18:0}$ and $C_{20:4}$ accounted for the total observed DAG accumulation, and by 10 min they contributed 58% of the total DAG acyl groups accumulated. In ET-cells the observed low accumulation of DAG during VP stimulation is reflected to a similar extent at the level of all individual acyl groups (Fig. 3). As previously observed for sal-cells, the increase in $C_{18:0}$ - and $C_{20:4}$ -DAG was detected before other DAG acyl groups. By 5 min, $C_{18:0}$ and $C_{20:4}$ fatty acids were the only ones significantly increased over basal values, and by 10 min they accounted for 57% of the total DAG acyl groups accumulated.

The magnitude and the fatty acid profile of the 1,2-DAG accumulated within 10 min of VP stimulation were similar in control cells (from normal rats fed *ad libitum*), in cells from pair-fed rats (normal rats whose food intake was reduced matching that voluntarily eaten by ET-infused rats) and sal-cells (Table 1). Only ET-cells showed a 50% decrease in DAG accumulation, suggesting that neither surgical trauma and/or pump implantation nor the nutritional state of the rats, but rather ET infusion, was the triggering factor involved. Moreover, the $C_{18:0}/C_{20:4}$ molar ratio in the DAG released in cells stimulated with VP was about 1.0, except for ET-cells, which showed a value significantly higher (1.59) (Table 1). This was paralleled by a similar trend of differences in the $C_{18:0}/C_{20:4}$ molar ratio esterified in PI (1.13 and 1.35 for sal- and ET-cells respectively.)

Effect of VP stimulation on [14 C]arachidonoyl-containing-phospholipid metabolism

When hepatocytes were prelabelled with [14 C] $C_{20:4}$ fatty acid, no significant changes in lipid labelling were

Fig. 4. Effect of VP on the accumulation of labelled PA and DAG in hepatocytes prelabelled with [14 C]arachidonic acid ($C_{20:4}$)

Hepatocytes were prelabelled with [14 C] $C_{20:4}$ ($1 \mu\text{Ci/ml}$) for 30 min. The plotted points represent mean values ($\pm$ S.D.) for triplicates of the difference between the lipid labelling attained in the presence and that obtained in the absence of VP (10^{-8} M) for the different time points analysed. No changes were observed in PA labelling on VP stimulation of ET-cells. Decreases in PI labelling after 10 min of VP stimulation (d.p.m./mg dry wt.) were: for control cells, -970 ± 160 ; pair-fed, -500 ± 240 ; saline pump, -700 ± 340 ; ET pump, -380 ± 100 . (a) Open symbols, control; closed symbols, pair-fed; $\circ$, $\bullet$, DAG; $\square$, $\blacksquare$, PA; (b) Open symbols, saline-pump; closed symbols, ET pump; $\circ$, $\bullet$, DAG; $\square$, PA.

observed within the first 60 s of VP stimulation (Fig. 4). By 5 and 10 min the labelling of DAG was significantly increased, concomitantly with a proportional decrease in

PI labelling (see the legend to Fig. 4). From the four experimental groups of cells analysed, namely control, pair-fed, saline- and ET-infused rats, only the last showed a significantly lower accumulation of $[1-^{14}\text{C}]C_{20:4}$ -DAG. Also, in cells from control and saline-infused rats the labelling of PA was increased by 5 min, returning to basal values thereafter. In pair-fed animals, a sustained increase in PA labelling was observed, whereas no significant change could be detected in ET-cells.

Similarly to the observed VP-induced $C_{20:4}$ -DAG accumulation (Table 1), the amount of $[1-^{14}\text{C}]C_{20:4}$ -DAG accumulated showed, in ET-cells, the lowest percentage value with respect to basal $[1-^{14}\text{C}]C_{20:4}$ -PI labelling (Table 2). By 10 min of VP stimulation the accumulation of $[1-^{14}\text{C}]C_{20:4}$ -DAG in ET-cells represented 2.7% of the PI basal labelling as compared with 6.4% in sal-cells, values close to that obtained for the content of $C_{20:4}$ -DAG accumulated (2.4 and 5.1% respectively; Table 1). In pair-fed rats, on the other hand, both the masses of $C_{20:4}$ -DAG and $[1-^{14}\text{C}]C_{20:4}$ -DAG accumulated showed higher percentage values

(6.4 and 7.5% respectively) than those in control cells (4.2 and 5.9% respectively).

Although we succeeded in labelling the poly-PI pool, the basal uptake of $[1-^{14}\text{C}]C_{20:4}$ was very low, representing 2–3% of PI labelling (Table 3). In ET-cells this value was doubled (6%), mainly due, as previously observed in ^{32}P -labelled cells, to a very high labelling of phosphatidylinositol 4-phosphate (PIP) [1]. No significant change in their labelling could be detected within the first 60 s of VP stimulation (results not shown).

DISCUSSION

We previously reported that chronic non-lethal endotoxaemia resulted in remarkable impairment in VP-stimulated poly $[^{32}\text{P}]$ PI degradation and resynthesis and $[^{32}\text{P}]$ PI turnover [1]. The present results provided further evidence that, as a consequence of ET infusion, the mechanism of DAG signal generation initiated by the breakdown of inositol lipids upon V_1 receptor stimulation was delayed and the magnitude of the response greatly diminished.

To our knowledge this is the first time that the kinetics of individual DAG acyl groups accumulation during continuous VP stimulation of hepatocytes has been reported. Several aspects of the VP-induced DAG changes were features common to both sal- and ET-cells. (1) Detectable accumulation of DAG occurred after a lag period. We never succeeded in observing any significant change in the DAG pool at a time shorter than 2 min of VP stimulation, in either control or sal- and ET-cells (E. B. Rodriguez de Turco & J. A. Spitzer, unpublished work). (2) The accumulation of total DAG was sustained up to the last time analysed (10 min), a finding similar to that previously obtained for control cells [29–32] and at variance with the transient increase reported by others [33,34]. (3) The release of $C_{18:0}$ - and $C_{20:4}$ -DAG was detected before other DAG acyl groups. In addition, the kinetics of DAG-acyl-group accumulation showed a higher rate of $C_{18:0}$ - and $C_{20:4}$ -fatty acid accumulation within the first 5 min of VP stimulation, whereas the release of other DAG acyl groups, i.e. $C_{22:6}$ -DAG, was sustained up to the last time analysed (10 min). (4) The fatty acid composition of the DAG pool accumulated after 10 min of VP stimulation reflected the fact that, as previously suggested [31,33,35], other phospholipids in addition to inositol phospholipids contributed to the sustained enlargement of this pool. Although $C_{18:0}$ and $C_{20:4}$ fatty acids made up only 55% of the total DAG acyl groups released, these two fatty acids accounted for 87% of the total PI acyl groups (Table 1). The amount of $C_{18:0}$ - and $C_{20:4}$ -DAG accumulated in sal-cells represented about 5% of the total content of these fatty acids esterified in PI (Table 2). Similar values were obtained for cells from control (4%) and pair-fed (6%) rats. This value was close to that reported by Kirk & Michell [36] for PI depletion (6%) when hepatocytes were challenged with VP (10^{-8} M) for 15 min. Moreover, in cells prelabelled with $[1-^{14}\text{C}]C_{20:4}$ fatty acid, the VP-induced accumulation of labelled DAG generally paralleled the data from $C_{20:4}$ -DAG mass accumulation in the four experimental groups analysed. Whether the previously reported decrease of PI during VP stimulation of hepatocytes mainly reflected its utilization for the replenishment of the hormone-induced depletion of the poly-PI pool [37] or, at least in part, was also due to its

Table 2. $[^{14}\text{C}]$ Arachidonic acid labelling of PI and DAG released during VP stimulation

Hepatocytes (40 mg wet wt./ml) were prelabelled with arachidonic acid ($[^{14}\text{C}]C_{20:4}$ ($1 \mu\text{Ci/ml}$)). Triplicate samples were subsequently incubated without or with VP (10^{-8} M) for 10 min.

Conditions	Labelling (d.p.m./mg dry wt.)		
	PI	DAG released	(%)*
Control	13 720 $\pm$ 220	+ 810 $\pm$ 75	(5.90)
Pair-fed	10 175 $\pm$ 60	+ 760 $\pm$ 125	(7.47)
Saline pump	17 300 $\pm$ 145	+ 1100 $\pm$ 70	(6.36)
ET pump	10 480 $\pm$ 70	+ 285 $\pm$ 55	(2.72)

* Percentage of $[^{14}\text{C}]C_{20:4}$ -DAG released with respect to the basal labelling of PI.

Table 3. $[^{14}\text{C}]$ Arachidonic acid ($C_{20:4}$) labelling of hepatocyte poly-PIs

Values are means $\pm$ S.D. for at least four individual samples. Other details are as given in Table 1.

Conditions	Labelling (d.p.m./100 mg dry wt.)		(%)*
	PIP	PIP ₂	
Control	240 $\pm$ 20	130 $\pm$ 10	(2.7)
Pair-fed	200 $\pm$ 20	100 $\pm$ 12	(3.1)
Saline pump	196 $\pm$ 10	160 $\pm$ 10	(2.1)
ET pump	460 $\pm$ 32	190 $\pm$ 26	(6.3)

* Percentage of poly-PI labelling with respect to the $[1-^{14}\text{C}]C_{20:4}$ -PI basal labelling; $[1-^{14}\text{C}]$ PI (d.p.m./mg dry wt.): control, 13 570 $\pm$ 450; pair-fed, 9740 $\pm$ 100; saline-pump: 16 740 $\pm$ 550; ET-pump, 10 320 $\pm$ 110.

conversion into DAG at a time when PI turnover was greatly increased remains to be determined. In the present study we observed a close relationship between PI and its phosphodiesteratic product, DAG. The molar ratio of $C_{18:0}$ to $C_{20:4}$ fatty acids in PI and in the DAG pool accumulated during VP stimulation was close to 1.0, except for ET cells, which showed significantly higher values for both lipids. Although we have not yet analysed the fatty acid composition of poly-PI, one of our previous studies showed that PI was the only phospholipid whose endogenous content of $C_{20:4}$ fatty acid was decreased during chronic endotoxaemia [38]. Moreover, $[1-^{14}C]C_{20:4}$ -PI was the only phospholipid whose labelling was decreased during VP stimulation concomitantly with the accumulation of labelled DAG. These observations give support to the idea that a hormone-sensitive PI pool [39,40] of very high $C_{20:4}$ -fatty acid turnover is likely to be the precursor of a fraction of the DAG pool enriched in $C_{18:0}$ and $C_{20:4}$ fatty acids. However, the contribution of other tetraenoic species of phospholipids to this pool cannot be excluded at present. Because of the characteristic fatty acid composition of individual phospholipids [38], the accumulation of DAG containing $C_{16:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{22:6}$ fatty acid could be derived mainly from PC.

The contribution of PC breakdown to the enlargement of the DAG pool was supported by the reported occurrence of a phospholipase C that selectively degraded this phospholipid [35,41–43] and whose activity can be regulated via protein kinase C activation [35,42–44]. Moreover, this could also be one of the mechanisms by which Ca^{2+} -mobilizing hormones, such as VP, stimulated DAG release from PC [35,43]. Our present results are in good agreement with these previous suggestions. The observed early accumulation of $C_{18:0}$ - and $C_{20:4}$ -DAG upon VP stimulation (derived mainly from inositol phospholipids) could result, via protein kinase C stimulation, in the subsequent stimulated release of DAG from other phospholipids (e.g. PC). To underscore the significance of the time course of these events, in ET-infused rats not only the accumulation of $C_{18:0}$ - and $C_{20:4}$ -DAG was inhibited by 50%, but also, in a similar proportion, the later accumulation of all other DAG acyl groups. This observation is consistent with the notion that the DAG released from VP-stimulated inositol lipid turnover could efficiently *regulate* the increased availability of the DAG signal and the degree of protein kinase C activation [35,43]. The differences observed in the fatty acid composition of DAG accumulated over the time course of the VP response may serve as a possible mechanism for the attenuation of the DAG second-messenger function. Previous studies demonstrated that DAGs of different-chain-length fatty acids are not uniform in their effectiveness in activating protein kinase C in platelets and in a variety of biological systems [45].

The different accumulation kinetics of individual DAG-acyl groups possibly released from different lipid substrates and involving various subcellular levels, the regulation of their release and subsequent metabolism as well as the physiological implications of these signals in the time course of VP stimulation comprise an exciting area growing in complexity and deserving of further study. Briefly, the initial DAG signal generated when Ca^{2+} -mobilizing glycogenolytic hormones interact with their receptors involves poly-PI breakdown [37,46–49].

The magnitude of this primary DAG signal could not be detected either in mass over the relatively high basal levels of DAG in the cells or in cells prelabelled with $[1-^{14}C]C_{20:4}$, owing to the very low labelling of poly-PI as compared with the basal DAG labelling. In addition, DAG is efficiently removed by phosphorylation to PA [1,39], whose increase in mass has been detected prior to any change at the level of the DAG pool [32]. However, the transient accumulation of DAG derived from poly-PI at the plasma-membrane level could efficiently result in the observed early changes (within 5–30 s) in protein phosphorylation through protein kinase C stimulation [50,51]. Later on the overstimulation of PI turnover may contribute to a detectable and sustained accumulation of both $C_{18:0}$ - and $C_{20:4}$ -DAG [29–32; the present study]. The DAG accumulated at the onset of stimulation could play a *key role* in the subsequent enlargement of the DAG pool from other phospholipids, e.g. PC. The function of this latter and sustained accumulation of DAG when protein phosphorylation has already occurred remains to be determined. However, the release of $C_{20:4,n-6}$ and $C_{22:6,n-3}$ fatty acids from the previously accumulated DAG could be coupled to their efficient oxidation at the microsomal level [52–54]. Although the biological function of these oxidized metabolites is not known at present, they could be involved in modulating the cells' physiological response to hormonal stimulation.

In summary, our present study demonstrated that the reported impairment in the VP-induced poly $[^{32}P]$ PI degradation and $[^{32}P]$ PI turnover in hepatocytes from chronically endotoxaemic rats [1] is also reflected in a significantly lower DAG signal. This could in turn attenuate the magnitude of protein kinase C activation that contributes to the appropriate cellular physiological response.

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