Vol. 155, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1988 Pages 151-159

EARLY EFFECTS OF ESCHERICHIA COLI ENDOTOXIN INFUSION ON VASOPRESSIN-STIMULATED BREAKDOWN AND METABOLISM OF INOSITOL LIPIDS IN RAT HEPATOCYTES

Dr. Elena B. Rodriguez de Turco & Dr. Judy A. Spitzer*

Department of Physiology
Louisiana State University Medical Center
1901 Perdido Street
New Orleans, Louisiana 70112

Received June 10, 1988

The turnover of vasopressin-stimulated ^{32}P-phosphoinositides and ^{32}P-phosphatidic acid and accumulation of [2-^{3}H]-inositol phosphates were examined in hepatocytes from rats infused i.v. with saline and E. coli endotoxin for 3 hrs. Within 60s of VP stimulation the decrease in phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate labeling as well as the increased uptake of ^{32}P into phosphatidic acid were similar in both groups. However, at a later time (300s) the ^{32}P-phosphatidylinositol turnover was greatly decreased concomitantly with a higher labeling of phosphatidic acid. The accumulation of [2-^{3}H]-inositol phosphates in ET-cells was significantly decreased both at 30s and 600s after VP addition. The distribution of [2-^{3}H]-inositol labeling accumulated in the different inositol phosphate fractions over the first 30s of VP stimulation showed a tendency to lower accumulation of inositol trisphosphate, and a signifi- cantly lower accumulation of inositol bisphosphate simultaneously with a higher labeling of the inositol tetrakisphosphate fraction. These observations reflect an early effect of ET-infusion on VP-stimulated inositol lipid turnover and on the subsequent metabolism of the released inositol phosphates.

Several recent studies have shown that during endotoxicosis and sepsis the responsiveness of receptors coupled to inositol lipid metabolism and cytosolic [Ca^{2+}] changes is greatly impaired (1,2). The final consequence is a diminished ability of Ca^{2+}-mobilizing hormones e.g. epinephrine, VP to induce the cellular physiological response in the target organ. Some of the

*To whom correspondence should be addressed.

ABBREVIATIONS

ET, Escherichia coli endotoxin; I, inositol; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; Poly-PI, polyphosphoinositides; PA, phosphatidic acid; VP, vasopressin.

0006-291X/88 $1.50

hemodynamic and metabolic perturbations observed during human gram-negative septicemia have been reproduced in our laboratory by continuously infusing rats with a non-lethal dose of E. coli endotoxin delivered i.v. from a surgically implanted osmotic pump (3). At 30 hs of continuous infusion, at the nadir of the morbid phase, alterations in the hepatocytes' informational cascade initiated by the degradation of Poly-PI included down regulation of α_1-adrenergic and V_1-VP receptors (4), lower hormone-stimulated poly-PI degradation and PI turnover (5,6) and Ca^{2+} mobilization (7), decreased ability of IP_3 to mobilize Ca^{2+} from endoplasmic reticulum stores (8) and therefore a lower hormone-induced activation of glycogen phosphorylase (7). The aim of the present studies was to analyze earlier effects of ET-infusion (over a 3 h period) on VP-stimulated inositol lipid degradation and turnover in rat hepatocytes. Here, we report that VP-stimulated ^{32}P-PI turnover and the accumulation of [2-^{3}H] inositol phosphates were significantly impaired at this time. The kinetics of [^{3}H] labeling of inositol phosphates as a function of time in cells of ET-infused rats was distinctly different from that displayed by cells of matched controls.

MATERIALS AND METHODS

Materials. [2-^{3}H]$_3$myo-inositol was obtained from ARC Chemical Inc. St. Louis, Missouri [2-^{3}H] labeled standards I (1) P, I (1,4) P_2 and I (1,4,5) P_3 were purchased from Amersham (Arlington Heights, IL). ^{32}P- as $H_3{}^{32}PO_4$, carrier free was from ICN Biomedicals, (Irvine, CA). [Arg^8] vasopressin (Pitressin) was from Parke-Davis, (Morris Plains, NJ). Endotoxin (E. coli, 026: B6 prepared by the Boivin method) was obtained from Difco laboratories, (Detroit). Inositol phospholipid standards were purchased from Sigma (St. Louis). Dowex 1-X8 (formate form, 200-400 mesh) was obtained from BioRad lab. (Richmond, CA). All other chemicals used were of the highest purity grade available.

Animal preparation: Male Sprague-Dawley rats (330-360g, Charles River) were anesthetized with ether and arterial and venous catheters were implanted on the day before the experiment. Animals were caged individually, fasted overnight but with free access to water. Between 0800 and 0900 the next morning the experimental group was infused i.v. with E. coli endotoxin (0.25 µg/100g b.w.) over 3h at a rate of 1ml/h. Matched control animals received an equal volume of sterile saline (NaCl 0.9%). Mean arterial blood pressure and heart rate were measured at the beginning and end of the infusion period. A modest hypotension was detected in ET-infused rats.

Hepatocyte isolation and incubation: Hepatocytes were isolated by "in situ" collagenase perfusion as previously described (5). For [2-^{3}H] inositol labeling of lipids, hepatocytes were suspended at a concentration 20 mg dry weight/ml in Krebs-Ringer solution buffered with $NaHCO_3$ (4.2mM) and HEPES (20mM) pH 7.4, containing 1.3 mM $CaCl_2$, 10mM glucose, 2% (w/v) dialyzed bovine serum albumin and myo-inositol (40µCi/ml S.A. 15Ci/mmol) previously passed through a 1cm column of Dowex I-X8 in formate form. Cells were incubated in bulk for 90min at 37°C under oxygen atmosphere, washed twice with unlabeled buffer containing 5mM inositol and resuspended at a concentration 5mg dry weight/ml in Krebs-Ringer-HEPES buffer containing 10mM LiCl. After a preincubation period of 20min, aliquots in triplicates

(0.5ml) were transferred to plastic tubes and incubated in the presence or absence of vasopressin (VP, 10^{-7}M). For the analysis of [2-^{3}H] inositol phosphates, incubations were terminated by the addition of 250µl of cold perchloric acid 12% (w/v) containing 3mM EDTA. Phytic acid hydrolysate (total 25µg phosphorus) was added to the samples to improve the recovery of inositol phosphates as suggested by Wreggett et al (9). After 15 min at ice temperature, the samples were centrifuged at 2,000g for 10min and the supernatant neutralized with 1.8MKOH in 60mM-HEPES. Analysis of [2-^{3}H] inositol lipids was done from parallel incubations terminated with trichloroacetic acid (40%) followed by lipid extraction with an acidified solvent system (5). In those experiments designed to analyze the VP-effect on ^{32}P-PA, PI and poly-PI labeling, cell labeling and lipid extraction were done exactly as described previously (5).

Analysis of the Inositol Phosphates: Neutralized $HClO_4$ extracts were loaded onto 1ml Dowex 1-X8 columns in the formate form and the inositol phosphates eluted following the procedure of Berridge et al (10). Columns were eluted first with 20ml of inositol 5mM to remove inositol; 12ml of 60mM sodium formate - 5mM disodium tetraborate to elute the glycerophosphoinositol; 15ml of 0.2M ammonium formate-0.1M formic acid to elute the IP_1 fraction; 18ml of 0.4M ammonium formate-0.1M formic acid to elute the IP_2 fraction; 18ml of 0.8M ammonium formate-0.1M formic acid to elute the IP_3 fraction and 18ml 1.2M ammonium formate-0.1M formic acid to elute IP_4. Inositol elution was followed by collecting 5ml fractions. In subsequent elution steps, 2.2ml fractions were collected. Aliquots (1ml) of each fraction were mixed with 10ml of ACSII (Amersham Corp.) and radioactivity was determined using a Beckman liquid scintillation spectrometer. Values are expressed as dpm per mg dry weight or as a percent radioactivity of inositol phosphates with respect to basal inositol lipid labeling.

Analysis of phospholipids: For separation of PI and poly-PI labeled with [^{3}-H] inositol, as well as for ^{32}P-poly-PI, the acidic lipid extracts were neutralized with one drop of ammonium hydroxide 7M, taken to dryness, resuspended in a small volume of chloroform/methanol/water (75:25:2, by volume) and applied to K-oxalate precoated high performance thin layer chromatography plates (Silica gel 60, Merk) together with poly-PI standards to aid in the visualization with iodine vapors. After sublimation of the iodine, the spots were scraped into vials containing 0.5ml water and the radioactivity determined using 10ml of Ready Value cocktail (Beckman Instruments, Fullerton, CA) that for [^{3}H] labeled samples contained 20% Triton X 100 (11). Separation of ^{32}P-PA and ^{32}-P-PI was achieved by two dimensional TLC according to Rouser et al (12) on precoated Silica gel H plates (Analtech, Inc., Newark, DE).

RESULTS AND DISCUSSION

Hepatocytes from rats infused for 3 h with ET and Saline (matched controls) were prelabeled with ^{32}P and subsequently exposed to VP (10^{-7}M) for different periods of time (Table 1). Over the first 60 s of hormone stimulation, the decrease in poly-PI (PIP and PIP_2) labeling and the increased ^{32}P uptake into PA were similar for both groups. However 5 min after VP addition, ^{32}P PI turnover in ET-cells was 36% lower than in control cells. At the same time, the uptake of ^{32}P into PA was significantly increased (47% higher than in Sal-cells). Moreover, PI was the only phospholipid showing a lower basal labeling in ET-cells (840 ± 40 vs 1060 ±

Table 1. Changes in ^{32}P labeling of phosphatidic acid and inositol lipids during vasopressin stimulation of hepatocytes

Time sec.	Lipid	Saline	ET
30	PA	+750 ± 190	+805 ± 180
	PI	N.S.	N.S.
	PIP	-215 ± 30	-180 ± 20
	PIP_2	-305 ± 40	-260 ± 25
60	PA	+1,530 ± 150	+1,560 ± 220
	PI	N.S.	N.S.
	PIP	-240 ± 20	-200 ± 20
	PIP_2	-370 ± 40	-450 ± 50
300	PA	2,020 ± 140	+2,960 ± 20[a]
	PI	1,220 ± 180	+780 ± 90[a]
	PIP	-70 ± 30	-100 ± 20
	PIP_2	-345 ± 20	-235 ± 10[a]

Hepatocytes from Sal- and ET- (25µg/100g b.w. over 3h) infused rats, prelabeled with ^{32}P (15µCi per ml), were exposed to vasopressin (10^{-7}M) for different periods of time and lipid labeling analyzed as described in Materials and Methods.
Values are dpm/mg dry weight ± SD from triplicates taken from one cell preparation and represent the difference between VP stimulated and basal values. Similar data were obtained in three independent experiments. N.S., no statistically significant difference with respect to basal values. Statistically significant differences ($p<0.001$, paired t-test) between Saline and ET samples (a).

50 dpm per mg dry weight in ET- and Sal cells respectively). Interestingly, we were able to detect at this early time of ET infusion an impairment in the "PI cycle" when the initial ^{32}P-poly-PI response was not altered. Later on, when rats were in the morbid phase of endotoxemia (by 30 h of ET-infusion), both the early phase of the VP response (i.e. decreased Poly-PI labeling and increased ^{32}P-uptake into PA) and the later stimulated PI turnover were affected (5). Similar results were also obtained in sepsis induced by cecal ligation and puncture (13). It has been previously reported that VP stimulates both degradation and resynthesis of inositol lipids (14,15). Since the steady state level of ^{32}P-Poly-PI attained during 30-60 s of VP stimulation is regulated via phosphodiesterase activity in parallel with phosphatase/kinase mediated interconversion of PIP_2 PIP, and considering that one or more of these metabolic pathways could be altered during ET-infusion, differences in the final poly-PI response to VP, could be masked or underestimated.

Therefore, we analyzed the VP-induced production of inositol phosphates released from hepatocytes prelabeled with [^3H] inositol. Figure 1 shows the elution profiles of [^3H] labeled inositol phosphates by Dowex 1 X-8 (formate form) columns, accumulated in hepatocytes from Saline and ET-infused rats

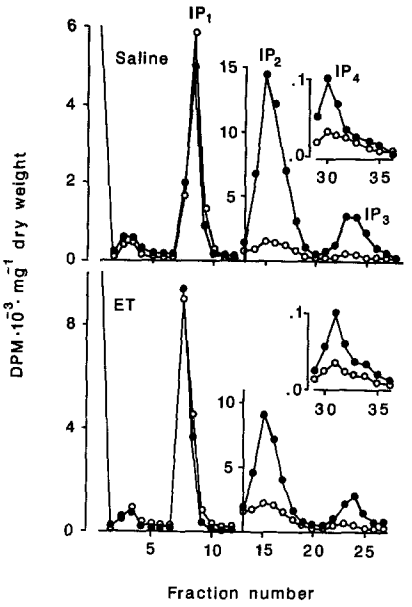

Figure 1
Elution profile of inositol phosphates by anion-exchange chromatography. Hepatocytes from Saline- and ET-infused rats prelabeled with [2-^{3}H] inositol were incubated with (●) or without (○) VP (10^{-7}M) for 30s in the presence of LiCl (10mM). Incubations were terminated with $HCLO_4$ (12%, v/v) and the neutralized acid extracts were applied to Dowex 1-X8 (formate form) columns. Inositol phosphates were eluted as described in the Materials and Methods section.

after 30 s of VP (10^{-7} M) stimulation. By increasing the formate ammonium concentration from 0.2 M to 0.8 M, [^{3}H] labeled IP_1, IP_2 and IP_3 were eluted and identified by [^{3}H] labeled standards. When the strength of the buffer was increased up to 1.2 M, a more polar [^{3}H] inositol-labeled compound was eluted, tentatively ascribed to the [^{3}H] IP_4 fraction. Different isomers of IP_4 (not resolved by Dowex column) can be synthesized in hepatocytes by the action of a 3-kinase on I(1,4,5)P_3 (16) or 6-kinase on I(1,3,4)P_3 (17) and could contribute to the increased labeling of this fraction upon VP stimulation. The profile of eluted water soluble products reflected the high basal labeling of IP_1 fraction in ET-cells unaltered by the presence of VP in Sal and ET-cells and the lower VP-induced accumulation of IP_2 and IP_3 in ET cells. Figure 2 shows the kinetics of accumulation of total [^{3}H] inositol phosphates (IP_2, IP_3 and IP_4) over the first 10-30 s of VP stimulation when no significant accumulation of IP_1 was detected (18 and Fig 1). In cells from saline infused rats, the accumulation of [^{3}H] inositol phosphates (expressed as percentage of basal poly-PI labeling) was very fast, linear with time and by 30 s represented 100% of the initial lipid precursor labeling. This value agrees with the previously reported rate for

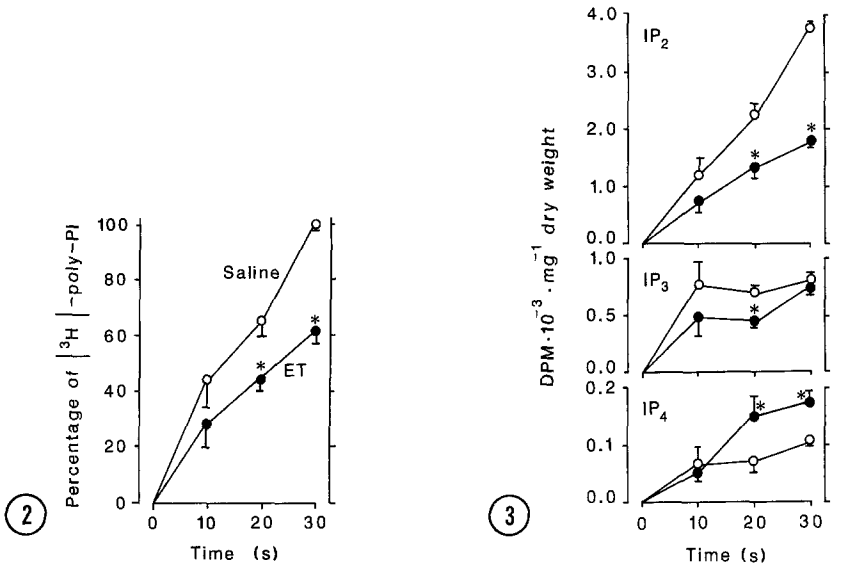

Figure 2
Time course of total [2-^{3}H] inositol polyphosphate accumulation in prelabeled hepatocytes in response to vasopressin. Results shown are percent radioactivity of accumulated IP_2, IP_3 and IP_4 from total radioactivity recovered in Poly-PI (PIP plus PIP_2). The basal labeling (in dpm per mg dry weight) of Poly-PI for Sal- and ET-samples was: 2,810 ± 240 and 2,550 ± 260 (PIP); 1,830 ± 80 and 1,910 ± 240 (PIP_2). For the recovered inositol phosphate fractions the basal labeling was: 342 ± 24 and 417 ± 58 (IP_2); 130 ± 25 and 143 ± 50 (IP_3); 82 ± 4 and 83 ± 6 (IP_4). Data are means ± SD from triplicate incubations. Similar results were obtained in two additional experiments. Asterisk denotes significant differences compared to Saline-samples ($p<0.05$, paired t-test).

Figure 3
Effect of ET-infusion on vasopressin-induced accumulation of inositol polyphosphates. Incubation conditions were the same as in Fig. 1. Asterisk denotes significant difference compared to the saline-sample ($p<0.05$). Data represent means for triplicate incubations ± S.D. The basal labeling of inositol phosphates (given in the legend to Fig. 2) was subtracted from the values attained in the presence of VP. Similar data were obtained in three independent experiments.

poly -PI depletion of 1-3%/s. (18). A significantly lower accumulation was observed in ET-cells (30% and 40% lower than in Sal-cells for 20 and 30 s respectively). This lower phosphodiesterase-mediated release of inositol phosphates occurred concomitantly with a decrease in ^{32}P-Poly-PI labeling similar to that observed in Sal cells (Table 1). This suggests that both Poly-PI degradation and resynthesis were affected to a similar extent, therefore contributing to the observed similarity in the steady-state level of ^{32}P-Poly-PI labeling 30-60 s after VP addition. The kinetics of accumulation of individual inositol phosphates in Sal-Cells showed that while IP_3 and IP_4 labeling reached a plateau within the first 10 s of cell stimulation, IP_2 labeling increased up the last time analyzed (Figure 3). In ET cells, the kinetic profiles of [^{3}H] IP_3 and [^{3}H] IP_2 accumulation were

Table 2. Accumulation of [2-^{3}H] inositol phosphates in vasopressin-stimulated hepatocytes from rats infused with Saline and ET for 3 hrs

Inositol phosphate fraction	Saline		ET	
	30s	600s	30s	600s
IP_1	N.S.	35,625 ± 2660	N.S.	27,915 ± 2,300[b]
IP_2	3,799 ± 136	37,686 ± 1,340	1,800 ± 120[c]	21,330 ± 290[c]
IP_3	825 ± 15	4,460 ± 44	788 ± 90	3,730 ± 70[c]
IP_4	110 ± 11	189 ± 91	172 ± 29[a]	295 ± 15
(%)	1.18 ± 0.02	20.08 ± 0.49	0.83 ± 0.06[c]	15.98 ± 0.58[c]

Values are dpm/mg dry weight ± S.D. from triplicates and represent the difference between VP stimulated and basal values for the different inositol phosphate fractions analyzed. N.S. no statistically significant difference with respect to basal values. The basal labeling of inositol phosphates after 10 min of incubation at 37°C for saline and ET samples was (in dpm/mg of dry weight): 10,055 ± 780 and 12,760 ± 200 (IP_1); 184 ± 20 and 338 ± 11 (IP_2); 99 ± 11 and 140 ± 30 (IP_3); 110 ± 10 and 69 ± 21 (IP_4). The basal labeling of total inositol phospholipids was 391,100 ± 13,565 (Sal) and 328,350 ± 23,800 (ET). (%), percent of radioactivity recovered in the total inositol phosphate fractions from total basal labeling of inositol lipids. Statistically significant difference compared to the saline value: a, $p<0.005$; b, $p<0.025$; c, $p<0.001$.

similar to that of Sal-cells, but showing a significantly lower accumulation of [^{3}H]IP_2 (50% lower than Sal-cells at 30 s). The IP_4 fraction, on the contrary, showed a higher accumulation of ^{3}H-inositol labeling. A similar pattern was observed after 10 min of VP-stimulation (Table 2). At this time, in ET-cells IP_4 was the only fraction that showed a tendency to higher accumulation of labeling while all the other fractions, including IP_1, showed significantly lower values than in Sal-cells.

From these data it can be concluded that the observed impairment in the ^{32}P-PI turnover was due, at least in part, to a lower phosphodiesteratic cleavage of PI that leads to the release of IP_1 and diacylglycerols. To further understand the site(s) of metabolic lesion(s) induced by ET infusion in the "PI-cycle", it will be necessary to individualize the PI derived $I(1)P_1$ from other inositol monophosphate isomers derived from stepwise enzymatic dephosphorylations of inositol poly-phosphates (i.e. $I(4)P_1$ and $I(3)P_1$). Even though 10 mM LiCl was present in the medium to inhibit inositol phosphate monoesterase activity (19), only the dephosphorylation of IP_1 was inhibited 100%, leading to no changes in the [^{3}H] inositol fraction labeling during VP stimulation (data not shown), while the dephosphorylation of IP_3 and IP_2 were only partially inhibited (15,18), therefore contributing to the VP-induced increased labeling of the IP_1 fraction.

In summary, our present study demonstrates that at early stages of ET-infusion the ability of VP to stimulate the phosphodiesteratic cleavage of inositol phospholipids is significantly reduced. Although the amount of

IP_3 released upon VP stimulation induced the same [Ca^{2+}] mobilization in ET cells as in saline cells (Spitzer J.A.and Deaciuc, I.V., unpublished observation), the subsequent [Ca^{2+}] dependent ^{32}P-PI turnover was greatly reduced. This could be due to a) an impairment in the phospholipase C-mediated degradation of PI and/or b) a deficient utilization of PA in the resynthesis of the previously degraded PI as was reflected in a concomitantly high accumulation of ^{32}P into PA. On the other hand, the metabolism of the released inositol phosphates showed a higher capacity of ET-cells to further phosphorylate IP_3. Both the dephosphorylation of I (1,4,5) P_3 by 5-phosphomonoesterase and its phosphorylation by 3-kinase have been involved in the modulation of the IP_3 signal (20), in the latter case with the production of a potential new second messenger: I(1,3,4,5)P_4 (16,21). These early effects of ET-infusion are the first signs of impairment in VP-stimulated inositol lipid turnover and subsequent metabolism of inositol phosphates. This could contribute to the development of diminished responsiveness of the cells to [Ca^{2+}] mobilizing hormones observed later on during the course of chronic endotoxemia.

ACKNOWLEDGMENTS

We would like to thank Ms. Jean Carnal, Ms. Karen duSapin, Mr. Peter O'Malley and Mr. Curtis vande Stouwe for excellent technical assistance.
This work was supported by National Institutes of Health grants. GM-32654 and GM-30312.

REFERENCES

1. Spitzer, J.A., Rodriguez de Turco, E.B., Deaciuc, I.V. and Roth, B.L. (1987). In Progress in Clinical and Biological Research (G. Schlag and H. Redl, Eds.) Vol pp 401-418. Alan R. Liss, Inc., New York.
2. Spitzer, J.A. and Rodriguez de Turco, E.B. (1988). In Molecular and Cellular Mechanisms of Septic Shock. Progress in Clinical Biological Research (B.L. Roth, T. Nielsen and A.E. McKee, Eds.) Alan R. Liss, Inc., New York (in press).
3. Fish, R.E. and Spitzer, J.A. (1984). Circ. Shock 12:135-149.
4. Roth, B.L. and Spitzer, J.A. (1987). Amer. J. Physiol. 252:E699-E702.
5. Rodriguez de Turco, E.B. and Spitzer, J.A. (1987). Metab. Clin. Exp. 36:753-760.
6. Rodriguez de Turco, E.B. and Spitzer, J.A. (1988). Biochem. J. (in press)
7. Deaciuc, I.V. and Spitzer, J.A. Am. J. Physiol. (1986). 251:R984-R995.
8. Spitzer, J.A. and Deaciuc, I.V. (1987). Am. J. Physiol. 253:E130-E134.
9. Wreggett, K.A., Howe, L.R., Moore, J.P. and Irvine, R.F. (1987). Biochem. J. 245:933-934.
10. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983). Biochem. J. 212:473-482.
11. Rodriguez de Turco, E.B. and Aveldano, M.I. (1980). Anal. Biochem. 104:62-69.
12. Rouser, G., Fleischer, S. and Yamamoto, A. (1970). Lipids 5:494-496.

13. Rodriguez de Turco, E.B. and Spitzer, J.A. (1988). Circ. Shock (in press).
14. Litosch, I., Lin, S-H. and Fain, J.N. (1983). J. Biol. Chem. 258:13727-12732.
15. Thomas, A.P., Alexander, J. and Williamson, J.R. (1984). J. Biol. Chem. 259:5574-5584.
16. Hansen, C.A., Mah, S. and Williamson, J.R. (1986). J. Biol. Chem. 261:8100-8103.
17. Shears, S.B., Parry, J.B., Tang, E.K.Y., Irvine, R.F., Michell, R.H. and Kirk, C.J. (1987). Biochem. J. 246:139-147.
18. Palmer, S., Hawkins, P.T., Michell, R.H. and Kirk, C.J. (1986). Biochem. J. 238:491-499.
19. Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982). Biochem. J. 206:587-595.
20. Connolly, T.M., Bansol, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987). J. Biol. Chem. 262:2146-2149.
21. Irvine, R.F. and Moor, R.A. (1986). Biochem. J. 240:917-920.