Effect of Chronic Endotoxemia on Concanavalin A-Stimulated Inositol Lipid Metabolism in Rat Splenocytes

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The effect of a chronic, nonlethal infusion of endotoxin (ET) on the phosphatidylinositol (PI) cycle in rat splenocytes was evaluated. Rats were infused intravenously with sterile saline or *Escherichia coli* ET (0.1 mg/100 g body weight/24 hr) for 30 hr via subcutaneously implanted osmotic pumps. The splenocytes were then labeled in vitro for 90 min with \[^{32}P\]P04 or for 3 hr with \[^{3}H\]myoinositol to assess the status of the resynthesis and degradative parts of the PI cycle, respectively. PI cycle activity was depressed in splenocytes of ET-infused rats as evidenced by a 25% reduction in the incorporation of \[^{32}P\]P04 into phosphatidic acid (PA), PI, and the polyphosphoinositides and by a similar decrease in the production of \[^{3}H\]inositol phosphates. Stimulation of splenocytes with concanavalin A (Con A) resulted in dose-dependent increases in the incorporation of \[^{32}P\]P04 into PA and PI and in the production of \[^{3}H\]inositol phosphates, indicating that Con A stimulates the PI cycle in these cells. The Con A-stimulated increase in inositol phosphate production was higher in splenocytes from ET-infused rats. We have previously shown that splenocytes from rats infused for 30 hr with ET exhibit a decreased blastogenic responsiveness to Con A and lipopolysaccharide [Spitzer et al., *Proc. Soc. Exp. Biol. Med.* 186,27, 1987]. The present data do not support the notion that inositol lipid-mediated signalling mechanisms are solely responsible for the expression of the appropriate functional response and suggest that in ET-infused rats there is an uncoupling of the initial response to Con A (i.e., the production of inositol lipid-derived second messengers) and the long-term (i.e., mitogenic) response.

Key words: phosphatidylinositol, phosphatidic acid, polyphosphoinositides

INTRODUCTION

A number of studies have demonstrated that following trauma lymphoid cells are unable to respond to various antigens and mitogens [1,21]. The nature of this posttraumatic immunosuppression remains incompletely defined. Our laboratory has shown that splenocytes from rats infused for 30 hr with *Escherichia coli* endotoxin (ET splenocytes) exhibit depressed in vitro blastogenic responses to lipopolysaccharide (LPS) and concanavalin A (Con A) [29]. A similar 30 hr infusion of endotoxin has also been shown to result in a decrease in basal and epinephrine- and vasopressin-stimulated glycogen phosphorylase activity in rat hepatocytes [6]. Under these conditions there is also an attenuation of the vasopressin-stimulated breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) in rat hepatocytes [24]. Thus work from this laboratory has demonstrated that bacterial endotoxins are capable of disrupting the inositol lipid signalling system in hepatocytes and that this disruption may be responsible for altered physiological responses during chronic endotoxemia. In the inositol lipid signalling system, the binding of an agonist to its receptor activates a phospholipase C, which hydrolyzes PIP2 to inositol trisphosphate (IP3) and diacylglycerol (DAG). Both of these products can act as second messengers. IP3 releases Ca2+ from intracellular stores, whereas DAG activates protein kinase C. Con A is a polyclonal T-lymphocyte mitogen, which stimulates the breakdown of inositol lipids, with concomitant production of inositol phosphates [15,27,30]. The goal of the present study was to determine whether a 30 hr infusion of ET disrupts this signalling pathway in rat splenocytes and may thereby affect the ability of splenocytes to respond to Con A.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were held for at least 1 week prior to being used. Animals (350–400 g) were anesthetized with a mixture of ketamine HCl (10 mg/100 g BW) and Rompun (1 mg/100 g BW). An osmotic minipump (Alzet 2 ML; Alza Corp., Palo Alto, CA) was implanted subcutaneously and either

Received August 15, 1990; accepted November 8, 1990.

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sterile saline or ET was infused at a rate of 10 μl/hr into the right jugular vein. For the ET-infused rats sterile saline was infused for the first 42 hr after pump implantation to allow for postsurgical recovery. After this 42 hr recovery period *E. coli* ET (026:B6; Difco Laboratories, Inc., Detroit, MI) was delivered at a rate of 100 μg/100 g BW/24 hr. The rats were sacrificed 30 hr after the onset of ET infusion (72 hr postsurgery), and the spleens were removed.

**Splenocyte Isolation**

After removal, the spleen wet weight was determined. Cell suspensions were then prepared by passing the spleens through nylon mesh in phosphate-buffered saline (PBS; pH 7.2). The cells were recovered by centrifugation and treated with 0.75% NH₄Cl in 17 mM Tris (pH 7.2) for 10 min at room temperature to lyse the red blood cells [4]. The splenocytes were washed once with PBS and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; pH 7.45).

**32P Experiments**

For experiments with [32P]PO₄, the splenocytes were resuspended in DMEM at 4 × 10⁷ cells/ml. [32P]PO₄ (50 μCi/ml) was added, and the splenocytes were incubated in a shaking water bath for 90 min at 37°C. At the end of the labeling period, 0.25 ml aliquots were transferred to 1) tubes containing 6 ml of CHCl₃/CH₃OH (1:1, v/v) for phospholipid (PL) analysis, 2) tubes containing 1.5 ml of CHCl₃/CH₃OH/HCl (1:2:0.01, v/v/v) for polyphosphoinositide (PPI) analysis, or 3) tubes containing 0.25 ml of buffer or buffer plus Con A. Tubes containing splenocytes and buffer or buffer plus Con A were incubated in a shaking water bath at 37°C for 30 min. Reactions were stopped by adding either 6 ml of CHCl₃/CH₃OH (1:1) or 1.5 ml of CHCl₃/CH₃OH/HCl (1:2:0.01).

**Phospholipid Analysis**

The phospholipid samples were allowed to stand at room temperature (20–25°C) for 2 hr or stored overnight in the refrigerator (10°C). The tubes were centrifuged, and the supernatant was decanted. The pellets were extracted once more with 3 ml of CHCl₃/CH₃OH (2:1), and the supernatants were combined. Phase separation was achieved by adding 3 ml of CHCl₃ and 1.4 ml of 0.05% CaCl₂. The lower phase was washed once with Folch theoretical upper phase (CHCl₃/CH₃OH/0.05% CaCl₂, 3:48:47, v/v/v), to remove excess water-soluble ³²P-labeled compounds. Extracts were dried under N₂, and redissolved in 50 μl of CHCl₃/CH₃OH (2:1) for spotting on thin-layer chromatography plates (silica gel H + 7.5% magnesium acetate; Analtech, Newark, DE). The plates were developed in the first dimension using chloroform/methanol/ammonium (13:5:1, v/v/v), dried thoroughly, and then developed in the second dimension using chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, v/v/v/v/v). The plates were dried and visualized with I₂ vapors, and spots corresponding to phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were marked. After sublimation of the I₂, the spots were scraped into scintillation vials, and the radioactivity determined using 10 ml of Ready Solv. EP or Ready Value scintillation fluid (Beckman Instruments, Fullerton, CA) by liquid scintillation spectrometry.

The PPI samples were placed on ice for 15–30 min and then centrifuged. The supernatant was decanted, and phase separation was achieved by the addition of 1.5 ml of CHCl₃. The lower phase was washed successively with CHCl₃/CH₃OH/0.1 N HCl (3:48:47, v/v/v) and CHCl₃/CH₃OH/0.01 N HCl (3:48:47, v/v/v). The samples were neutralized to pH 7–8 by the addition of one drop of 7 M NH₄OH and evaporated to dryness under N₂. The lipid extracts were redissolved in 50 μl of CHCl₃/CH₃OH/H₂O (75:25:2, v/v/v) and spotted on silica gel H plates (Analtech) that had previously been coated with 1% potassium oxalate. A polyphosphoinositide standard was cospotted with each sample for visualization purposes. The plate was developed using chloroform/methanol/4 M NH₄OH (9:7:2, v/v/v). After development the plate was dried and visualized with I₂ vapors, and spots corresponding to phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP₂) were marked. Spots were then scraped and counted as previously described for the PL samples.

**[³H]inositol Experiments**

The splenocytes were resuspended in DMEM at 8 × 10⁷ cells/ml. [2-³H]myo-inositol (50 μCi/ml) was added, and the cells were incubated at 37°C for 3 hr in a shaking water bath. At the end of the labeling period, the splenocytes were collected by centrifugation, washed once with PBS (pH 7.2) to remove any unincorporated inositol, and resuspended in PBS containing 10 mM LiCl (pH 7.2) at 4 × 10⁷ cells/ml. Aliquots of 0.25 ml were taken for analysis of the incorporation of [2-³H]myoinositol into PI, PIP, and PIP₂. The inositol phospholipids were extracted and chromatographed as described above for the PPI samples, except that the PI and LPI spots were also scraped and the samples were counted in Ready Gel scintillation fluid (Beckman Instruments).

For the analysis of inositol phosphate production, 0.25 ml aliquots were transferred to plastic tubes containing buffer or buffer plus Con A. The tubes were then incubated at 37°C for 30 min in a shaking water bath. Reactions were stopped by adding 0.25 ml of cold perchloric acid (12%, w/v) containing 3 mM (ethylenedinitrilo)-tetracetic acid (EDTA). The tubes were placed on ice for 15–30 min. Phytic acid hydrolysate (25 mg P
per sample) was added, and the tubes were centrifuged at 2,000g for 10 min. The supernatant was decanted and the pellet extracted once with 1.2% perchloric acid (w/v) containing 0.3 mM EDTA. The supernatants were combined and neutralized to pH 7–8 with 1.8 M KOH in 60 mM 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid (HEPES). The neutralized extracts were applied to AG1-X8 (formate form) anion exchange resin (Bio-rad, Richmond, CA), and the inositol phosphates were eluted following the procedure of Berridge et al. [2], as previously described [26].

**Adenosine Triphosphate (ATP)-Specific Activity**

Splenocytes were resuspended in DMEM at 4 × 10⁷ cells/ml. [³²P]P₀₄ (50 μCi/ml) was added, and the cells were incubated in a shaking water bath at 37°C for 90 min. Duplicate 50 μl aliquots were taken for protein determination by the method of Peterson [22]. The cell suspension was then split in half for the determination of the ATP concentration and the amount of [γ-³²P]ATP. The samples were centrifuged at 1,000g for 10 min, and the supernatants were discarded. To the pellets was added 0.1 ml of buffer and 0.5 ml of 10% perchloric acid. The samples were vortexed and placed on ice for 5–15 min. The solutions were centrifuged at 1,000g for 10 min. To 0.5 ml aliquots of the supernatant was added 50 μl of 1 M triethanolamine buffer (pH 7.4). The samples were then neutralized (pH 6.5–7.5) with 20% KOH and centrifuged at 1,000g for 10 min, and the supernatants were stored at −70°C until analysis. The ATP concentration was determined by the enzymatic method of Lamprecht and Trautschold [16]. The amount of [γ-³²P]ATP was determined by the method of Hawkins et al. [11], which involves the cyclic adenosine monophosphate (AMP)-dependent protein kinase phosphorylation of histone H2A.

**Statistical Analyses**

The data presented in this paper are from experiments involving pairs of rats, one infused with saline and the other with endotoxin. The labeling, whether with [³²P]P₀₄ or with [¹³H]myo-inositol, was variable between experiments. Thus it was necessary to analyze the data with either a paired t test, as was done in Tables 1 and 3, or to normalize the data, as was done in Figures 2–4. When the data were normalized, or when a representative experiment is shown (Figs. 1, 5), the data from saline and ET splenocytes were compared using a Student’s t test for independent samples. In all analyses, the results were considered statistically significant at P < 0.05.

**Chemicals**

Ketamine-HCl was from Aveco Co., Inc. (Fort Dodge, IA), and Rompun was purchased from Mobay Corp. (Shawnee, KS). DMEM was bought from GIBCO (Grand Island, NY). Phospholipid standards, Con A, phytoic acid, triethanolamine, histone H2A, cAMP, protein kinase A, and ATP were from Sigma (St. Louis, MO). HEPES, tris (hydroxymethyl) aminomethane (Tris), NADP, glucose-6-phosphate dehydrogenase, and hexose kinase were bought from Boehringer Mannheim Biochemicals (Indianapolis, IN). [³²P]P₀₄ (carrier-free, 285 Ci/m mole) and [γ-³²P]ATP (25 Ci/m mole) were purchased from ICN Radiochemicals (Irvine, CA), and [²-³H]myo-inositol (10–20 Ci/m mole) was from Amersham (Arlington Heights, IL).

**RESULTS**

Splenocytes from both saline and ET-infused rats readily incorporated [³²P]P₀₄ into phospholipids (Fig. 1). Initial incorporation was greatest for the polyphosphoinositides, PIP, and PIP₂. This probably reflects a relatively high turnover of the monoester phosphate groups on the inositol moiety, since these phospholipids are much less abundant than PC, PE, and PI.

![Fig. 1. Incorporation of [³²P]PO₄ into splenocyte phospholipids. Splenocytes were incubated at 4 × 10⁷ cells/ml with 50 μCi/ml of [³²P]PO₄. At the times indicated, 0.25 ml aliquots were taken and the phospholipids were extracted and analyzed as described in Materials and Methods. Values represent averages of triplicate determinations from one of four experiments, which gave similar results. Standard error measurements are not shown because they were generally smaller than the size of the symbols. Significant differences (P < 0.05) between saline (solid circles) and ET (open circles) are indicated by asterisks.](image-url)
Isotopic equilibrium was not attained within the 3 hr labeling period. In fact, in one experiment in which the cells were exposed to \([^{32}P]PO_4\) overnight, isotopic equilibrium was not attained even after 24 hr (data not shown). Since we were interested in determining the effect of an in vivo exposure to ET, it was desirable to use the splenocytes as quickly as possible after sacrificing the rats. Thus, a 90 min labeling time was adopted for all subsequent experiments with \([^{32}P]PO_4\).

Significant differences were observed in the incorporation of \([^{32}P]PO_4\) into splenocyte phospholipids from saline- vs ET-infused rats (Fig. 1, Table 1). The incorporation of label into all of the phospholipids of the PI cycle, i.e., PA, PI, PIP, and PIP₂, was lower in splenocytes from ET-infused animals, whereas the labeling of PC was significantly increased. These differences in \([^{32}P]PO_4\) incorporation were not due to differences in ATP concentration or in the specific activity of the intracellular ATP pool between saline and ET splenocytes (Table 2). There was also no change in the endogenous content of PA, PI, PS, sphingomyelin, PC, or PE following a 30 hr infusion of endotoxin (data not shown).

Splenocytes prelabeled for 90 min with \([^{32}P]PO_4\) and then treated with Con A for 30 min exhibited an increased incorporation of \([^{32}P]PO_4\) into PA and PI but not into PC or PE. This effect was dose-dependent and was observed in cells from both saline- and ET-infused rats (Table 3). The relative degree of stimulation was the same in both groups of splenocytes as evidenced by the fact that the percentage increase in \([^{32}P]PO_4\) incorporation due to Con A was the same. No consistent changes in the labeling of PIP and PIP₂ following stimulation with Con A were observed in rat splenocytes (data not shown).

As with the incorporation of \([^{32}P]PO_4\), the incorporation of [2-³H]myoinositol into splenocyte phospholipids did not reach isotopic equilibrium within three hours. Unlike the \([^{32}P]PO_4\) incorporation, however, no consis-

### Table 3. Effect of Concanavalin A on the Incorporation of \([^{32}P]PO_4\) Into Splenocyte Phospholipids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PL</th>
<th>2</th>
<th>20</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>PA</td>
<td>1.04 ± 0.07</td>
<td>1.43 ± 0.30*</td>
<td>1.78 ± 0.18*</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>1.04 ± 0.07</td>
<td>1.12 ± 0.03*</td>
<td>1.36 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>1.04 ± 0.03</td>
<td>1.06 ± 0.04</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>1.00 ± 0.14</td>
<td>0.99 ± 0.20</td>
<td>1.02 ± 0.18</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>PA</td>
<td>1.11 ± 0.07</td>
<td>1.40 ± 0.29*</td>
<td>1.66 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>1.06 ± 0.05</td>
<td>1.25 ± 0.13*</td>
<td>1.32 ± 0.07*</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>0.99 ± 0.01</td>
<td>1.01 ± 0.04</td>
<td>0.98 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>0.94 ± 0.04</td>
<td>1.02 ± 0.17</td>
<td>1.04 ± 0.06</td>
</tr>
</tbody>
</table>

*Cells were incubated with \([^{32}P]PO_4\) for 90 min and then treated with various concentrations of Con A for 30 min. Data are presented as the ratio of stimulated to unstimulated cells and represent the means ± standard errors of four experiments. The ratio for each individual experiment was determined from the means of triplicate determinations in which the original data were expressed as dpm/10⁷ cells.

*Significant differences (P < 0.05) between stimulated and unstimulated cells.

### Table 2. Endogenous Content and \([γ-^{32}P]ATP\) Specific Activity in Splenocytes From Rats Infused With Saline or Endotoxin

<table>
<thead>
<tr>
<th>ATP (nmoles ATP/ mg protein)</th>
<th>([γ-^{32}P]ATP) (nCi/mg protein)</th>
<th>Specific activity (nCi/nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>11.71 ± 1.62</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>14.74 ± 3.71</td>
<td>0.63 ± 0.20</td>
</tr>
</tbody>
</table>

*Rats were infused for 30 hr with either sterile saline or E. coli endotoxin (0.1 mg/100 g body weight/24 hr). Measurements were made following a 90 min labeling of cells with 50 μCi/ml (4 × 10⁶ cells/ml) of \([^{32}P]PO_4\) and represent the means ± standard deviations of three experiments performed in triplicate.

*Significant differences (P < 0.05) between saline- and ET-infused rats.
tent differences in the [2-3H]myoinositol labeling of splenocyte phosphoinositides from saline- and ET-infused rats were observed (data not shown).

Splenocytes from ET-infused rats exhibited a significantly lower basal production of inositol phosphates than splenocytes from saline-infused animals (Fig. 2). This reflected primarily a decrease in the amount of IP₁, IP₂ was also significantly decreased, whereas there were no significant differences in the levels of IP₃ and IP₄.

Exposure of rat splenocytes to Con A for 30 min resulted in a dose-dependent increase in the production of inositol phosphates (Fig. 3). The increase was greater for splenocytes from ET- as opposed to saline-infused rats. This was particularly true at the highest dose of Con A used, 200 μg/ml. Whereas the production of inositol phosphates increased in an exponential fashion with increasing amounts of Con A in ET splenocytes, the level of inositol phosphates declined slightly between 20 and 200 μg/ml of Con A in saline splenocytes.

The amounts of IP₁, IP₂, IP₃, and IP₄ were all increased following exposure of splenocytes to Con A for 30 min (Fig. 4). The response of IP₁ and IP₃ to increasing concentrations of Con A was similar to that described above for the total inositol phosphates in both saline and ET splenocytes. In saline splenocytes, the increase in IP₂ was independent of the mitogen concentration, whereas, in ET splenocytes, IP₂ reached a plateau between 20 and 200 μg/ml of Con A. The amount of IP₄ increased between 2 and 20 μg/ml of Con A in both sets of splenocytes, and there was no significant change between 20 and 200 μg/ml. At 20 and 200 μg/ml of mitogen, the increases in IP₁ and IP₂ were significantly greater in splenocytes from ET- as opposed to saline-infused rats. There were no significant differences in IP₃ accumulation, whereas the level of IP₄ was significantly higher in ET splenocytes at 200 μg/ml of Con A.

Because no differences in IP₃ accumulation were observed between splenocytes from saline- and ET-infused rats after a 30 min exposure to Con A, shorter time periods were investigated. The results of a representative experiment are shown in Figure 5 for the total inositol phosphates. No significant production of inositol phosphates was detected 1 min after the addition of Con A. Between 5 and 30 min, inositol phosphates accumulated in a linear fashion. The accumulation was significantly greater in the ET as opposed to the saline splenocytes. A similar time course was observed for IP₁.
and IP₂, whereas a 15 min stimulation with Con A was required in order to detect a significant accumulation of IP₃ and IP₄ (data not shown).

**DISCUSSION**

Splenocytes isolated from rats subsequent to a 30 hr nonlethal infusion of ET exhibited a depression of basal PI cycle activity, but an enhanced production of inositol phosphates following stimulation with Con A. The depression of the basal PI cycle is evidenced by both the decreased incorporation of [³²P]PO₄ into the phospholipids of the PI cycle (PA, PI, PIP, and PIP₂) and the lower production of inositol phosphates. In both instances the values were approximately 25% less in splenocytes from ET- as opposed to saline-infused rats. Since there is no difference in the molar amounts of the different phospholipids following a 30 hr infusion of ET, the decreased ³²P labeling indicates that the turnover of the PI cycle lipids is depressed. In contrast, there was no difference in the incorporation of [³²P]PO₄ into the inositol lipids of saline and endotoxin splenocytes. This suggests that the de novo synthesis of PI is not affected by a 30 hr infusion of endotoxin and that the decreased incorporation of [³²P]PO₄ is due to a decreased turnover of the phosphat moieties of the phospholipids.

Although there was a decline in the incorporation of [³²P]PO₄ into the PI cycle lipids, there was an increased labeling of PC in splenocytes from ET-infused rats. Since the molar amount of PC was unchanged, it is likely that the turnover of PC is higher in splenocytes following a 30 hr infusion of endotoxin. Hepatocytes from ET-infused rats also exhibit an increased incorporation of [³²P]PO₄ into PC with no change in PC mass [24,25]. Experiments with [²H]glycerol indicated that de novo PC synthesis was also stimulated in ET hepatocytes [25]. This raises the possibility that small changes in the molar amount of PC cannot be quantitated due to the relatively large cellular mass of this phospholipid and/or that the stimulated de novo synthesis is coupled to a higher turnover of PC with no change in mass. The mechanism by which an in vivo infusion of ET increases the turnover of PC remains to be determined.

Con A caused a stimulation of the PI cycle in splenocytes from both saline- and ET-infused rats. Evidence for this comes both from the increased incorporation of [³²P]PO₄ into PA and PI and from the increased production of inositol phosphates following a 30 min exposure of splenocytes to the mitogen. The increased incorporation of [³²P]PO₄ into PA and PI was dose dependent and occurred in the absence of any changes in the labeling of the more abundant phospholipids PC and PE. This observation is in accord with previous reports [9,18] and is generally taken to indicate a specific stimulation of the PI cycle. No consistent changes in the
labeling of PIP and PIP₂ following exposure of splenocytes to Con A were observed. This probably reflects the relatively high basal turnover of the monoester phosphate groups and the fact that isotopic equilibrium had not been attained after a 90 min labeling period.

The production of inositol phosphates was also increased upon exposure of rat splenocytes to Con A. Of the total inositol phosphates, 80–90% were in the form of IP₁. The present data do not allow us to determine if IP₃ is derived from the hydrolysis of PI or the hydrolysis of PIP₂ followed by a rapid dephosphorylation of IP₃ to IP₂ and IP₁. The assays were performed in the presence of 10 mM LiCl, which primarily blocks the conversion of IP₁ to free inositol. Thus it is possible that most of the IP₃ produced may be converted to IP₁. It has been shown that the stimulation of cloned rat pituitary (GH₃) cells with thyrotropin-releasing hormone (TRH) results in only a transient (1–2 min) hydrolysis of PIP₂ but that PI and PA turnover is enhanced for over 30 min [14]. These data agree with previous observations that TRH stimulation of GH₃ cells results primarily in the production of IP₁, that the production of IP₃ is transient, and that there is a prolonged production of DAG [8]. With rat splenocytes we have not been able to demonstrate an early transient production of IP₃. There is no significant increase in IP₃ levels until 5–15 min after the addition of Con A (data not shown). Thus it is possible that most of the IP₃ produced in the present study comes directly from the hydrolysis of PI. If this is the case, it may also explain our inability to detect changes in [³²P]PO₄ labeling of PIP and PIP₂ following the exposure of splenocytes to Con A. It should be noted, however, that only a fraction of the total splenocyte population is responsive to Con A. Thus it may not be possible to detect small increases in IP₃. Evidence supporting the early production of IP₃ comes from measurements of intracellular Ca²⁺ in which peak responses are attained within 1 min of the addition of Con A (unpublished data).

Whereas a 30 hr infusion of ET resulted in depression of the basal PI cycle, the production of inositol phosphates was higher in Con A-stimulated splenocytes from ET- as compared to saline-infused rats. DAG, one of the phospholipase C-mediated products of phospholipid hydrolysis, activates protein kinase C (PKC) by translocating it from the cytosol to membranes [20]. It has been shown in several different cell types that one of the roles of PKC is to exert feedback control over the receptors that are coupled to inositol phospholipid breakdown such that activation of PKC leads to a decrease in the agonist-induced hydrolysis of inositol phospholipids [20]. In this way, overstimulation of the cells is prevented. LPS, which is a major component of E. coli endotoxin, is capable of inducing PKC translocation [5]. The LPS precursors lipid A and lipid X have been shown to activate PKC in a macrophage cell line [31] and in B lymphocytes [3]. Thus exposure of splenocytes in vivo to LPS might be expected to result in a decrease in inositol phosphate production upon in vitro stimulation with Con A.

An explanation for our results may reside in the effects of long-term activation of PKC. In our study, splenocytes were exposed to LPS for 30 hr. Hepler et al. [12] found that short-term (5–60 min) pretreatment of WB cells with phorbol 12-myristate 13-acetate (PMA) markedly attenuated inositol phosphate accumulation in response to a number of hormones, which is in accordance with the negative feedback role of PKC. Long-term (6–24 hr) exposure to PMA, however, resulted in an enhancement of inositol phosphate production in response to the same hormones. These authors also reported a 90–100% loss of PKC activity in cytosolic and particulate fractions following 18 hr of PMA exposure. A similar down-regulation of PKC was reported for CTL-2 cells following a 48 hr treatment of cells with phorbol 12-13 dibutyrate (PDBu) [23]. Larsen et al. [17] showed that exposure of human peripheral blood T lymphocytes to PMA for 72 hr resulted in down-regulation of PKC and that more than 72 hr was required for the recovery of PKC activation. Thus it is likely that after a 30 hr infusion of endotoxin PKC activity is decreased through down-regulation in rat splenocytes. In fact we have previously shown that the amount of PKC as determined by [³H]PDBu binding is depressed in rat spleens by a 30 hr infusion of ET [13]. Additionally, we have found that splenocytes from ET-infused rats exhibit a significantly lower membrane-associated PKC activity than do splenocytes from saline-infused rats [28]. These data are consistent with a down-regulation of PKC following long-term exposure of splenocytes to PKC activators. Such a down-regulation of PKC by a 30 hr infusion of endotoxin would result in the loss of feedback inhibition upon Con A stimulation, and, therefore, an increased accumulation of inositol phosphates in the presence of LiCl. The increased turnover of PC in splenocytes from ET-infused rats could result in a prolonged activation of PKC through the production of DAG [7]. This would be another mechanism by which ET may cause the down-regulation of PKC.

Down-regulation of PKC may also explain why there was a decline in the production of inositol phosphates between 20 and 200 μg/ml of Con A in saline splenocytes but not in ET splenocytes. High doses of Con A may cause a large initial response, which results in a high level of PKC translocation in splenocytes from saline-infused rats. This overstimulation of PKC activity might then result in an inhibition of PIP₂ hydrolysis, which was manifested 30 min later as a decline in inositol phosphate production. Down-regulation of PKC in ET splenocytes would remove the inhibition and allow higher doses of Con A to cause further increases in inositol phosphate production.
The doses of Con A required to detect increases in $^{32}$P-labeling of PA and PI and in the production of inositol phosphates (i.e., 20 and 200 μg/ml) are supramitogenic, whereas the mitogenic dose (2 μg/ml) did not produce significant changes. Our data are consistent with data reported for rat lymphocytes from mesenteric lymph nodes in which the incorporation of $[^32]$P into phospholipids was dependent on the dose of Con A up to 200 μg/ml [10]. It is likely that the magnitude of the PI response is proportional to the number of Con A receptors occupied and that the inhibitory effect of high concentrations of Con A on mitogenesis occurs subsequent to the “PI response.” McClain and Edelman [19] reported that high doses of Con A cause lymphocytes to become committed to mitogenesis but also result in the generation of a negative growth signal, which blocks cell cycle progression.

It would be expected that an increased breakdown of inositol phospholipids would result in an increased availability of DAG for recycling and hence an increased incorporation of $[^32]$PPO$_4$ into PA and PI. Our data indicate that the degree of stimulation of PA and PI synthesis by Con A was the same in both saline and ET splenocytes. Since neither PA nor PI showed higher rates of $[^32]$P incorporation in ET splenocytes, it appears that DAG was not phosphorylated to PA as quickly in these cells. This may reflect an inhibitory effect of endotoxin on the activity of DAG kinase. DAG has metabolic fates other than conversion to PA. It can be used in the synthesis of PC and PE, acylated to triacylglycerols (TAG), or degraded to free fatty acids (FFA), and monoacylglycerol (MAG). The incorporation of $[^32]$PPO$_4$ into PC and PE was not stimulated by Con A in splenocytes from either saline- or ET-infused rats. Thus it does not appear that this metabolic fate of DAG is enhanced in ET splenocytes. It remains to be determined whether the production of TAG or the degradation of DAG to FFA and MAG is enhanced in rat splenocytes following a 30 hr infusion of endotoxin.

We have previously shown that a 30 hr infusion of E. coli ET results in a decreased blastogenic response to in vitro stimulation of splenocytes with Con A [29]. Our present results suggest that in splenocytes from ET-infused rats the perturbation in the informational cascade leading to the reduced blastogenic response is likely to be placed beyond the early degradation of inositol phospholipids. Moreover, the fact that PKC activity is decreased as a consequence of long-term ET infusion [28] suggests that the failure of Con A to induce a blastogenic response could be due to an uncoupling of inositol lipid-derived second messengers (e.g., DAG) from subsequent activating mechanisms, whose expression is required for the ultimate functional response (i.e., cell proliferation).

ACKNOWLEDGMENTS

This study was supported by National Institute of Health grants GM32654 and HL07098. All experiments were conducted in adherence to the NIH guidelines for the care and use of experimental animals.

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