Phosphatidylinositol turnover in mitogen-activated lymphocytes

Suppression by low-density lipoproteins

David Y. HUI and Judith A. K. HARMONY*

Department of Chemistry, Indiana University, Bloomington, IN 47405, U.S.A.

(Received 29 February 1980/Accepted 21 April 1980)

Low-density (LD) lipoproteins inhibit phytohaemagglutinin-enhanced turnover of phosphatidylinositol in human peripheral lymphocytes. Turnover was assessed by ^{32}P incorporation into phospholipids and by loss of ^{32}P from [^{32}P]phosphatidylinositol. Inhibition of lipid turnover by LD lipoproteins is not the result of a change in the amount of phytohaemagglutinin required for maximum cellular response. Neither phytohaemagglutinin nor LD lipoproteins influence ^{32}P incorporation into phosphatidylethanolamine and phosphatidylcholine during the first 60 min after mitogenic challenge. The extent of inhibition of phosphatidylinositol turnover by LD lipoproteins depends on the concentration of LD lipoproteins present in the incubation medium: 50% of maximum inhibition occurs at a low-density-lipoprotein protein concentration of $33\text{ }\mu\text{g/ml}$ and maximum inhibition occurs at low-density-lipoprotein protein concentrations above $100\text{ }\mu\text{g/ml}$. Phytohaemagglutinin stimulates ^{32}P incorporation into phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. However, LD lipoproteins abolish ^{32}P incorporation into phosphatidylinositol without affecting incorporation into phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. The ability of LD lipoproteins to inhibit phytohaemagglutinin-induced phosphatidylinositol turnover is mimicked by EGTA. Furthermore, inhibition of LD lipoproteins by phytohaemagglutinin-induced ^{32}P incorporation into phosphatidylinositol correlates directly with inhibition by LD lipoproteins of Ca^{2+} accumulation. These results suggest that Ca^{2+} accumulation and turnover of phosphatidylinositol are coupled responses in lymphocytes challenged by mitogens. The step in phosphatidylinositol metabolism that is sensitive to LD lipoproteins and, by inference, that is coupled to Ca^{2+} accumulation is release of [^{32}P]phosphoinositol from phosphatidylinositol.

Phytohaemagglutinin-induced lymphocyte proliferation is inhibited by plasma lipoproteins (Curtiss & Edgington, 1976; Chisari, 1977; Morse *et al.*, 1977). The most potent immunosuppressive lipoproteins are those of the low-density classes which have hydrated densities less than 1.063 g/cm^3 (very-low-density lipoproteins, $d < 1.006$; intermediate-density lipoproteins, $d = 1.006\text{--}1.019$; low-density lipoproteins, $d = 1.019\text{--}1.063$). The target cells for

the inhibitory lipoproteins appear to be resting lymphocytes (Curtiss & Edgington, 1977). Inhibition by LD lipoproteins of lymphocyte response to phytohaemagglutinin is not the result of inhibition of mitogen–cell interaction, or of alteration in the amount of phytohaemagglutinin required to elicit maximum cellular response (Hui *et al.*, 1979). Moreover, the immunosuppressive activity of LD lipoproteins appears to be distinct from the ability of the lipoproteins to down-regulate cholesterol biosynthesis in lymphocytes. Cholesterol-depleted LD lipoproteins are as active as native LD lipoproteins in preventing lymphocyte activation (Hui *et al.*, 1979). LD lipoproteins also inhibit activation of lymphocytes obtained from patients with familial hypercholesterolaemia (Curtiss & Edgington, 1978; Hui *et al.*, 1979); the cholesterol-synthesizing

Abbreviations used: VLD lipoproteins, very-low-density lipoproteins ($d < 1.006$); IDL, intermediate-density lipoproteins ($d = 1.006\text{--}1.019$); LDL, low-density lipoproteins ($d = 1.019\text{--}1.063$).

* To whom reprint requests should be sent at the following present address: Department of Biological Chemistry, University of Cincinnati College of Medicine, Cincinnati, OH 45267.

machinery of lymphocytes isolated from familial-hypercholesterolaemia donors is not subject to regulation by LD lipoproteins (Ho *et al.*, 1976). Suppression of phytohaemagglutinin-elicited lymphocyte activation appears to be a direct consequence of lipoproteins binding to the cell membrane since removal of LD lipoproteins from the cell surface with heparin reverses inhibition (Hui *et al.*, 1979). Immunosuppression by LD lipoproteins may thus be the direct result of an alteration of the lymphocyte plasma membrane, which renders the cell non-responsive to the mitogenic signal.

Interaction of LD lipoproteins with lymphocytes inhibits early events elicited by phytohaemagglutinin which are required for induction of cell proliferation. Specifically, LD lipoproteins inhibit phytohaemagglutinin-enhanced accumulation of Ca^{2+} (Hui *et al.*, 1979) and cyclic GMP (Hui & Harmony, 1979a) by lymphocytes; inhibition correlates directly with inhibition by LD lipoproteins of DNA synthesis. An additional lymphocyte-membrane-associated phenomenon that occurs immediately after addition of phytohaemagglutinin is an increased rate of phospholipid metabolism. During the first hours after stimulation, phytohaemagglutinin enhances the incorporation of [^{32}P]P_i into phosphatidylinositol without affecting [^{32}P]P_i incorporation into other major phospholipids (Fisher & Mueller, 1968; Maino *et al.*, 1975). This increase in [^{32}P]phosphatidylinositol is due to the enhanced turnover of the phosphoinositol group of phosphatidylinositol and not to increased biosynthesis of the phospholipid subsequent to stimulation. Since an increased rate of phosphatidylinositol turnover has been implicated as the initial event in the coupling of ligand-receptor interactions and Ca^{2+} transport (Allan & Michell, 1977), the influence of LD lipoproteins on phytohaemagglutinin-enhanced phosphatidylinositol turnover was investigated. The data summarized herein demonstrate that LD lipoproteins inhibit turnover of phosphatidylinositol in mitogen-challenged lymphocytes. Metabolism of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate is not suppressed when lymphocytes are incubated with LD lipoproteins. Interestingly, the extent of inhibition of phosphatidylinositol turnover by LD lipoproteins and of inhibition of Ca^{2+} accumulation by LD lipoproteins coincide at all LD lipoprotein concentrations.

Materials and methods

Lipoproteins were isolated from freshly collected plasma of normolipemic fasted human volunteers by sequential ultracentrifugal flotation in KBr (Havel *et al.*, 1955). Low-density lipoproteins were isolated between $d = 1.019$ and 1.063 by centri-

fugation for 18 h at 50000 rev./min in a Beckman type 50.2 titanium rotor, and were stored at 4°C under N_2 in KBr for no longer than 2 weeks. The lipoproteins were dialysed overnight at 4°C in 150 mM-NaCl before each experiment. The purity of each lipoprotein fraction was assessed by electrophoresis on agarose (1%, pH 8.6) and by immunochemical analysis with antibodies raised against the purified lipoproteins, selected apoproteins (AI, AII, B, E) and against human serum albumin. The lipid composition of LD lipoproteins was within the normal range (Nelson, 1972) and similar to that reported previously (Hui & Harmony, 1979b). Human peripheral blood lymphocytes, obtained by centrifugation of the blood through Ficoll-Paque (Boyum, 1968), were washed twice with 150 mM-NaCl. Lymphocytes were further purified from contaminating adherent cells as described by Hui *et al.* (1979). Accumulation of $^{45}\text{Ca}^{2+}$ by lymphocytes was determined by the method of Hui *et al.* (1979).

The influence of LD lipoproteins and phytohaemagglutinin on phospholipid metabolism in lymphocytes was assessed by determining the amount of ^{32}P incorporated into the lipids. In a typical experiment, 1×10^6 cells suspended in 1 ml of phosphate-free Hanks' balanced salt solution (pH adjusted to 7.0) were incubated with or without added lipoproteins at 37°C in an atmosphere of CO_2/air (1:19). After 1 h, [^{32}P]phosphoric acid ($100 \mu\text{Ci}$; New England Nuclear) was added to the suspension followed immediately by the addition of phytohaemagglutinin (Sigma Chemical Co.). The phytohaemagglutinin used had optimum mitogenic activity at the concentration of $3 \mu\text{g}/\text{ml}$ (Hui *et al.*, 1979). Incubation at 37°C was continued for one additional hour. To assess phospholipid breakdown independently, the phospholipids were labelled with ^{32}P before addition of lipoproteins. Lymphocytes were preincubated with [^{32}P]P_i for 2 h in the presence of phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$). The cells were washed in Hanks' balanced salt solution containing phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$) to remove unbound [^{32}P]P_i, and resuspended in Hanks' balanced salt solution (0.78 mM -[^{31}P]phosphate) and phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$).

All reactions were terminated by centrifugation to pellet the lymphocytes. The sedimented cells were washed twice in 150 mM-NaCl, lysed in 0.5 M -HCl, and the lipids were extracted by addition of an equal volume of methanol/chloroform (1:2, v/v). Individual phospholipids were separated by t.l.c. by published methods (Brewster *et al.*, 1978); identification was achieved by comparison with known standards. Phosphatidylinositol phosphate and phosphatidylinositol bisphosphate were separated on silica gel developed in propanol/4 M-ammonia (2:1, v/v) (Gonzalez-Sastre & Folch-Pi, 1968). After

detection of the chromatogram with I_2 , phospholipids were scraped from the plate and the radioactivity associated with each spot was determined by liquid-scintillation spectrometry in 10 ml of Aquasol (New England Nuclear).

Protein concentrations were determined by a modified method of Lowry *et al.* (1951), with 1% sodium dodecyl sulphate to solubilize the samples and bovine serum albumin as standard. Total phospholipid was measured as phosphorus by the method of Bartlett (1959). Cholesterol and cholesteryl esters were determined by the method of Roeschlau *et al.* (1974) by using the Cholesterol Test Combination Kit (Boehringer-Mannheim Biochemical Co.). Total glycerol was determined enzymically by using the Triglycerides Test Combination Kit (Boehringer-Mannheim Biochemical Co.).

All numbers reported in the present paper represent means obtained from five different experiments. Errors bars in all Figures indicate the range of values obtained in these experiments.

Results

Phytohemagglutinin stimulates ^{32}P incorporation into phosphatidylinositol in lymphocytes (Fisher & Mueller, 1971). As shown in Fig. 1, the amount of ^{32}P in phosphatidylinositol increases linearly with time after mitogen addition, and after a 60 min incubation, the c.p.m. values of [^{32}P]phosphatidylinositol in phytohaemagglutinin-challenged cells is 9.5-times that in unstimulated cells. Preincubation of lymphocytes with LD lipoproteins (150 μg of protein/ml) before the addition of phytohaemagglutinin inhibits the amount of ^{32}P incorporated into phosphatidylinositol. The magnitude of the phosphatidylinositol response of lymphocytes to phytohaemagglutinin depends on the amount of mitogen in the incubation medium; maximum incorporation of ^{32}P occurs at the optimal mitogenic concentration of 3 $\mu\text{g}/\text{ml}$ as shown in Fig. 2. Although the actual phytohaemagglutinin-induced increase in [^{32}P]phosphatidylinositol varies with lymphocyte donor, the mitogen-dependence of the response is invariant. The results presented in Fig. 2 illustrate two additional points. First, LD lipoproteins do not alter the dose-dependence of the phosphatidylinositol response to phytohaemagglutinin. In the presence of a low-density lipoprotein concentration insufficient to afford complete inhibition (30 μg of protein/ml), the maximum amount of [^{32}P]phosphatidylinositol is formed at a phytohaemagglutinin concentration of 3 $\mu\text{g}/\text{ml}$. Secondly, neither phytohaemagglutinin nor LD lipoproteins (150 μg of protein/ml) significantly perturb ^{32}P incorporation into phosphatidylethanolamine. Incorporation of ^{32}P into phosphatidylcholine is similarly uninfluenced by

Fig. 1. The effect of LD lipoproteins on the mitogen-stimulated incorporation of ^{32}P into phosphatidylinositol

Human lymphocytes (1×10^6 cells/ml) in Hanks' salt solution were preincubated with [^{32}P]phosphoric acid with (●) or without LD lipoproteins (▲) (150 μg of protein/ml) for 1 h at 37°C in an atmosphere of CO_2/air (1:19). Phytohaemagglutinin (3 $\mu\text{g}/\text{ml}$ final concentration) was added as indicated by the arrow, and the incubation was continued for one additional hour. The broken line represents the amount of ^{32}P incorporated by unstimulated cells incubated in the absence of phytohaemagglutinin. The methods for cell isolation and for determination of [^{32}P]phosphatidylinositol are described in the Materials and methods section.

phytohaemagglutinin or LD lipoproteins (results not shown).

Incubation of lymphocytes with phytohaemagglutinin also increases the rate of ^{32}P incorporation into phosphatidylinositol phosphate and phosphatidylinositol bisphosphate (Fig. 3). Although [^{32}P]phosphatidylinositol increases linearly with time (Fig. 1), the rate of increase in [^{32}P]phosphatidylinositol phosphate and [^{32}P]phosphatidylinositol bisphosphate is greater at 40–60 min after phytohaemagglutinin addition than it is during the first 40 min. In these experiments lymphocytes were exposed to [^{32}P]P_i 60 min before phytohaemagglutinin addition to establish the rate of ^{32}P incorporation into each phosphoinositide in unstimulated cells (indicated by the broken line in Figs. 1 and 3). In contrast with the influence of LD lipoproteins on phosphatidylinositol turnover, preincubation of lymphocytes with LD lipoproteins has no influence on the ability of phytohaemagglutinin to stimulate ^{32}P incorporation into phosphatidylinositol phosphate or phosphatidylinositol bisphosphate.

The extent of inhibition by LD lipoproteins of ^{32}P incorporation into phosphatidylinositol in phyto-

Fig. 2. The effect of phytohaemagglutinin on the incorporation of ^{32}P into phospholipids by phytohaemagglutinin-stimulated lymphocytes

Human lymphocytes (1×10^6 cells/ml) in Hanks' salt solution were preincubated for 1 h at 37°C in an atmosphere of CO_2/air (1:19) in the presence of $150 \mu\text{g}$ of low-density-lipoprotein protein/ml (● and ○), $30 \mu\text{g}$ of low-density-lipoprotein protein/ml (×), or in the absence of lipoproteins (▲ and Δ) before the addition of [^{32}P]phosphoric acid ($100 \mu\text{Ci}$) and phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$). The incubations were continued for 1 h and the reactions were terminated by centrifugation to pellet the lymphocytes. The cells were washed twice in 150 mM -NaCl and lysed in 0.5 M -HCl; the lipids were extracted in methanol/chloroform (1:2, v/v). Phospholipids were identified by t.l.c. as described in the Materials and methods section. Radioactivity associated with phosphatidylinositol (▲, × and ●) and phosphatidylethanolamine (Δ and ○) was determined by liquid-scintillation spectrometry in Aquasol.

haemagglutinin-stimulated lymphocytes depends on the concentration of LD lipoproteins present in the incubation medium. As shown in Fig. 4, 50% of maximum inhibition occurs at a low-density-lipoprotein protein concentration of about $33 \mu\text{g}/\text{ml}$, maximum inhibition of phytohaemagglutinin-induced phosphatidylinositol turnover occurs at low-density-lipoprotein protein concentrations greater than $100 \mu\text{g}/\text{ml}$. LD lipoproteins at concentrations as high as $200 \mu\text{g}$ of protein/ml have no effect on ^{32}P incorporation into phosphatidylinositol in the absence of phytohaemagglutinin.

The concentrations of LD lipoproteins that inhibit ^{32}P incorporation into phosphatidylinositol by 50 and 100% of maximum (Fig. 4) respectively are quite similar to the concentrations, 22 and $100 \mu\text{g}/\text{ml}$ (Hui *et al.*, 1979), that prevent phytohaemagglutinin-enhanced $^{45}\text{Ca}^{2+}$ accumulation to a similar extent, implying that inhibition of phosphatidylinositol metabolism by LD lipoproteins may be related to the inhibition of Ca^{2+} accumulation by LD lipoproteins. The results presented in Fig. 4 demon-

Fig. 3. The effect of LD lipoproteins on the incorporation of ^{32}P into phosphatidylinositol phosphate (a) and phosphatidylinositol bisphosphate (b)

Human lymphocytes (1×10^6 cells/ml) in Hanks' salt solution were preincubated with [^{32}P]phosphoric acid with (●) or without LD lipoproteins (▲) ($150 \mu\text{g}$ of protein/ml) for 1 h at 37°C in an atmosphere of CO_2/air (1:19). Phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$ final concentration) was added as indicated by the arrow, and the incubation was continued for one additional hour. The broken line represents the amount of ^{32}P incorporated by unstimulated cells incubated in the absence of phytohaemagglutinin. The methods for cell isolation and determination of [^{32}P]phosphatidylinositol phosphate and [^{32}P]phosphatidylinositol bisphosphate are described in the Materials and methods section.

strate that such a correlation indeed exists: inhibition by LD lipoproteins of ^{32}P incorporation into phosphatidylinositol correlates directly with inhibition by LD lipoproteins of $^{45}\text{Ca}^{2+}$ accumulation by activated lymphocytes. With lymphocytes isolated on the same day from the same donor, low-density-lipoprotein concentrations that inhibit $^{45}\text{Ca}^{2+}$ accumulation and incorporation of ^{32}P into phosphatidylinositol by 50% are about 28 and $33 \mu\text{g}$ of protein/ml respectively. Although the response of lymphocytes to phytohaemagglutinin differs among different lymphocyte donors, the extent of inhibition

Fig. 4. The influence of LD lipoproteins on the incorporation of ^{32}P into phosphatidylinositol and on $^{45}\text{Ca}^{2+}$ accumulation by phytohaemagglutinin-activated lymphocytes

Human lymphocytes (1×10^6 cells/ml) in Hanks' salt solution were preincubated at 37°C in an atmosphere of CO_2/air (1 : 19) with LD lipoproteins at the indicated concentrations. After 1 h, $^{45}\text{Ca}^{2+}$ ($1 \mu\text{Ci}$) or [^{32}P]phosphoric acid ($100 \mu\text{Ci}$) was added, followed immediately by phytohaemagglutinin ($3 \mu\text{g}/\text{ml}$). The incubation was continued for 1 h. For determination of $^{45}\text{Ca}^{2+}$ (●) the cell suspension was centrifuged through a water-impermeable layer of dibutyl phthalate into 0.5 ml of 90% formic acid; the amount of cell-associated $^{45}\text{Ca}^{2+}$ dissolved in formic acid was determined by liquid-scintillation spectrometry. Incorporation of ^{32}P into phosphatidylinositol (O) was determined as described in the Materials and methods section.

by LD lipoproteins varies little (five experiments, $P = 0.10$).

The obvious question that arises is: do LD lipoproteins inhibit degradation or synthesis of phosphatidylinositol? To attempt to provide an answer, lymphocyte phosphatidylinositol was prelabelled with ^{32}P . The radiolabel is lost from phosphatidylinositol if the cells are subsequently challenged with phytohaemagglutinin in the absence of LD lipoproteins (Table 1a). This result, which indicates a continuous breakdown of phosphatidylinositol when lymphocytes are incubated in the presence of phytohaemagglutinin, agrees with the report that phytohaemagglutinin stimulates the breakdown of phosphatidylinositol (Fisher & Mueller, 1968). When stimulated lymphocytes are exposed to LD lipoproteins under conditions in which LD lipoproteins activate $^{45}\text{Ca}^{2+}$ efflux from the cells, no loss of radioactivity from phosphatidylinositol occurs during a 5 h incubation period. A similar result is obtained when mitogen-treated cells are incubated with EGTA. Thus LD lipoproteins and EGTA prevent cleavage of [^{32}P]phosphoinositol from phosphatidylinositol. Like LD lipoproteins, EGTA does not influence turnover of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in the presence or absence of mitogens (results not shown). To ascertain whether LD lipoproteins and EGTA inhibit breakdown of [^{32}P]phosphatidylinositol by similar or different mechanisms, the suppressor substances, each present at a

Table 1. Effect of LD lipoproteins and EGTA on release of ^{32}P from phosphatidylinositol in phytohaemagglutinin-activated lymphocytes

The reaction conditions are described in the Materials and methods section. The concentration of phytohaemagglutinin was $3 \mu\text{g}/\text{ml}$. The incubation time was 5 h in (a) and 1 h in (b).

Addition	^{32}P in phosphatidylinositol (c.p.m.)	^{32}P released (%)
(a)		
None	6449	—
Phytohaemagglutinin	694	100
Phytohaemagglutinin + LD lipoproteins ($33 \mu\text{g}$ of protein/ml)	2497	61
Phytohaemagglutinin + LD lipoproteins ($150 \mu\text{g}$ of protein/ml)	6446	0
Phytohaemagglutinin + EGTA (0.5 mM)	3708	43
Phytohaemagglutinin + EGTA (1.25 mM)	5445	14
Phytohaemagglutinin + LD lipoproteins ($33 \mu\text{g}$ of protein/ml) + EGTA (0.5 mM)	6705	0
(b)		
None	9928	—
Phytohaemagglutinin	6053	39
Phytohaemagglutinin + LD lipoproteins ($150 \mu\text{g}$ of protein/ml)	9724	2
Phytohaemagglutinin + dibutyl cyclic AMP (0.1 mM)	6852	31
Phytohaemagglutinin + dibutyl cyclic AMP (10 mM)	8906	11
Phytohaemagglutinin + dibutyl cyclic GMP (10 mM)	6739	32
N-Acetylgalactosamine (1 mM)	7925	20

concentration insufficient to achieve maximum inhibition alone, were combined as outlined in Table 1(a). LD lipoproteins at 33 μg of protein/ml and EGTA at 0.5 mM inhibit release of ^{32}P from [^{32}P]phosphatidylinositol by 39 and 57% respectively. The LD lipoproteins/EGTA combination elicits 100% inhibition, indicating that the effects of LD lipoproteins and EGTA are additive. Dibutyryl cyclic AMP (0.1–10 mM) and *N*-acetylgalactosamine (1 mM), which inhibit lymphocyte proliferation (Lindahl-Kiessling, 1972; Diamanstein & Ulmer, 1975) and dibutyryl cyclic GMP (10 mM), which potentiates lymphocyte activation (Diamanstein & Ulmer, 1975), are relatively ineffective in suppressing phytohaemagglutinin-induced breakdown of phosphatidylinositol (Table 1b).

Discussion

Much of the inositol in eukaryotic cells is present as phosphatidylinositol in cell membranes. In response to extracellular stimuli, enhanced turnover of the phosphoinositol group of phosphatidylinositol occurs by what appears to be a closed cycle of reactions (Michell, 1975). That is, subsequent to stimulation of a number of cell types including lymphocytes, there is an increase in incorporation into phosphatidylinositol of [^{32}P]phosphate and radiolabelled *myo*-inositol without a concomitant increase in the concentration of the phospholipid. The function of this response has yet to be established. An understanding of the process of phosphatidylinositol turnover and of its role in cell activation will be greatly facilitated if inhibitors of phosphatidylinositol breakdown, which block the response elicited by a variety of stimuli, are identified. The data reported herein establish the potential of the plasma lipoproteins as such inhibitors.

Low-density lipoproteins influence phosphatidylinositol turnover in phytohaemagglutinin-stimulated lymphocytes. Specifically, LD lipoproteins suppress phytohaemagglutinin-enhanced incorporation of ^{32}P into phosphatidylinositol. LD lipoproteins, at suboptimal concentrations, do not induce a change in the amount of phytohaemagglutinin required to elicit maximum phosphatidylinositol turnover: lymphocytes incubated with and without LD lipoproteins incorporate ^{32}P into phosphatidylinositol most effectively if the phytohaemagglutinin dose is 3 $\mu\text{g}/\text{ml}$, the optimum mitogenic dose as determined by enhanced DNA synthesis. Inhibition by LD lipoproteins of phosphatidylinositol turnover is selectively suppressed by LD lipoproteins; phytohaemagglutinin-enhanced turnover of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate as detected by ^{32}P incorporation is not perturbed by LD lipoproteins. The significant result

is that inhibition of phosphatidylinositol turnover by LD lipoproteins correlates directly with inhibition by LD lipoproteins of phosphatidylinositol-enhanced $^{45}\text{Ca}^{2+}$ accumulation. This is the first clear demonstration that phosphatidylinositol breakdown and increased cellular Ca^{2+} are coupled events in lymphocyte activation.

A relationship between Ca^{2+} accumulation and phosphatidylinositol metabolism in phytohaemagglutinin-activated lymphocytes is, however, well documented (Allan & Michell, 1974, 1977, 1978; Michell, 1975). The Ca^{2+} ionophore A23187 also stimulates ^{32}P incorporation into phosphatidylinositol (Greene *et al.*, 1976; Allan & Michell, 1977), and inclusion of EGTA in the incubation medium inhibits phosphatidylinositol-stimulated ^{32}P incorporation (Allan & Michell, 1977). However, since at identical EGTA concentrations phytohaemagglutinin-enhanced $^{45}\text{Ca}^{2+}$ accumulation is abolished completely and ^{32}P incorporation into phosphatidylinositol is decreased by 35% only, Allan & Michell (1977) conclude that Ca^{2+} is not the primary factor that controls phosphatidylinositol turnover. These investigators further suggest that the ionophore-triggered Ca^{2+} accumulation in the cell cytoplasm may increase ^{32}P incorporation by increasing the availability of diacylglycerol for the synthesis of phosphatidylinositol, and phytohaemagglutinin may stimulate phosphatidylinositol turnover *per se* resulting in enhanced accumulation of $^{45}\text{Ca}^{2+}$ (Allan & Michell, 1977, 1978). Ca^{2+} is a putative second messenger in lymphocyte activation.

Under the conditions of the experiments reported in the present paper, both EGTA and LD lipoproteins inhibit the release of ^{32}P from [^{32}P]phosphatidylinositol. However, the effects of suboptimal concentrations of LD lipoproteins and EGTA are additive, suggesting that both inhibitors suppress the same step in the phosphatidylinositol cycle, though perhaps by distinct mechanisms. It is likely that EGTA inhibits phosphatidylinositol breakdown by depleting lymphocytes of cellular Ca^{2+} , thus inhibiting the phosphatidylinositol-specific phospholipase C in lymphocytes (Allan & Michell, 1974). This enzyme, when assayed in soluble lymphocyte extracts, is exquisitely dependent on Ca^{2+} for activity (Allan & Michell, 1974). Although it is tempting to propose that LD lipoproteins inhibit phytohaemagglutinin-enhanced $^{45}\text{Ca}^{2+}$ accumulation, which in turn inhibits phosphatidylinositol turnover by preventing the degradation step, inhibition of phosphatidylinositol turnover by LD lipoproteins may be the result of an alteration in the responsiveness of the lymphocyte membrane to mitogenic stimulation which is unrelated to Ca^{2+} transport. The data do not distinguish between the two possibilities. The second possibility is consistent with the phosphatidylinositol $\rightarrow \text{Ca}^{2+}$ sequence sug-

gested by Allan & Michell (1977, 1978), the first is not. In any case, the fact that LD lipoproteins inhibit phosphatidylinositol turnover and $^{45}\text{Ca}^{2+}$ accumulation by phytohaemagglutinin-challenged lymphocytes is important.

The mechanism by which LD lipoproteins inhibit the events of lymphocyte activation that occur immediately after mitogenic challenge is not known. Three possibilities exist: (1) lipoproteins exert a suppressive influence simply by binding to the lymphocyte surface and, as a consequence of this lipoprotein-membrane interaction, alterations in membrane functions due to mitogenic activation are suppressed; (2) inhibition by LD lipoproteins requires the internalization and degradation of the lipoproteins, processes involved for regulation of cellular cholesterol biosynthesis by LD lipoproteins; or (3) inhibition is the result of lipoprotein-cell-surface-lipid transfer. The experimental evidence supports mechanism (1). First, both cellular accumulation of $^{45}\text{Ca}^{2+}$ and phosphatidylinositol turnover are believed to be the result of alterations in the plasma membrane of the cell (Michell, 1975; Greene *et al.*, 1976). Secondly, inhibition by LD lipoproteins of lymphocyte activation is reversed by the addition of heparin and reversal of inhibition correlates with heparin-induced removal of ^{125}I -labelled LD lipoproteins from the cell surface (Hui *et al.*, 1979). Thirdly, addition of LD lipoproteins 5 h after phytohaemagglutinin stimulation of lymphocytes results in rapid and almost complete release of $^{45}\text{Ca}^{2+}$ from the cells without concomitant dissociation of phytohaemagglutinin from the cell membrane. Fourthly, cholesterol-depleted LD lipoproteins, prepared by the method of Gustafson (1965), are more potent than native LD lipoproteins in preventing phytohaemagglutinin activation of lymphocytes (Harmony & Hui, 1980). Cholesterol-depleted LD lipoproteins do not regulate cholesterol biosynthesis in cultured cells (Steinberg *et al.*, 1978), indicating that the immunosuppressive activity of LD lipoproteins is independent of suppression by LD lipoproteins of cholesterol biosynthesis. Finally, LD lipoproteins covalently linked to Sepharose beads are as effective as native LD lipoproteins in the inhibition of lymphocyte stimulation induced by phytohaemagglutinin (Hui & Harmony, 1980). Sepharose-immobilized LD lipoproteins are not internalized by the cells (Steinberg *et al.*, 1978), providing additional support for the conclusion that LD lipoproteins inhibit lymphocyte activation by binding to the cell membrane.

The physiological significance of the immunoregulatory properties of plasma lipoproteins may be that excess LD lipoproteins increase the susceptibility of the host to atherosclerosis. This latter hypothesis is based on the suggestion that viral-induced changes in the smooth muscle of blood

vessels may have a role in the atherogenic process (Benditt & Benditt, 1973; Burch, 1974). If this hypothesis proves to be correct, patients in whom the immune process within the intravascular compartment is suppressed by lipoproteins will be more susceptible to viral-induced changes in the arterial wall. Moreover, the results presented herein support our hypothesis that specific plasma lipoproteins regulate cell physiology via the cell membrane (Hui & Harmony, 1979b,c; Hui *et al.*, 1979). In addition to suppression of $^{45}\text{Ca}^{2+}$ accumulation and phosphatidylinositol turnover in activated lymphocytes, LD lipoproteins promote agglutination of erythrocytes by concanavalin A (B. Shore & V. Shore, 1975), activate Mg^{2+} -dependent ATPase in erythrocyte membranes (V. Shore & B. Shore, 1975), increase [cyclic AMP] in thrombocytes (Taylor *et al.*, 1979), and activate adipocyte-membrane adenylate cyclase (Pairault *et al.*, 1977). The hypothesis provides a working model for studying the role of lipoproteins in normal and in pathological states.

This research was supported by United States Public Health Service Grant HL 20882 from the National Institutes of Health and by Grant 79-1072 from the American Heart Association. D. Y. H. is the recipient of a United States Public Health Service Traineeship T32 GM 07227-04 in Molecular and Cellular Biology. Excellent technical assistance was provided by Mr. Ronald Osborne. We also wish to acknowledge the valuable contribution of Ms. Peggy Gore, who typed the manuscript, and Mr. Roger Purcell, who prepared the Figures. Anti-apoB and anti-apoE were generously provided by Dr. Richard L. Jackson and Dr. Richard J. Havel.

References

- Allan, D. & Michell, R. H. (1974) *Biochem. J.* **142**, 599-604
- Allan, D. & Michell, R. H. (1977) *Biochem. J.* **164**, 389-397
- Allan, D. & Michell, R. H. (1978) in *Cyclitols and Phosphoinositides* (Wells, W. W. & Eisenberg, F., eds.), pp. 325-336, Academic Press, New York
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-468
- Benditt, E. P. & Benditt, J. M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1753-1756
- Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, Suppl. 97, 77-89
- Brewster, M. E., Ihm, J., Brainard, J. R. & Harmony, J. A. K. (1978) *Biochim. Biophys. Acta* **529**, 147-159
- Burch, G. E. (1974) *Am. Heart J.* **87**, 407-412
- Chisari, F. V. (1977) *J. Immunol.* **119**, 2129-2136
- Curtiss, L. K. & Edgington, T. S. (1976) *J. Immunol.* **116**, 1452-1458
- Curtiss, L. K. & Edgington, T. S. (1977) *J. Immunol.* **118**, 1966-1970
- Curtiss, L. K. & Edgington, T. S. (1978) *J. Clin. Invest.* **61**, 1298-1308
- Diamanstein, T. & Ulmer, A. (1975) *Immunology* **28**, 113-119

- Fisher, D. B. & Mueller, G. C. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1396–1402
- Fisher, D. B. & Mueller, G. C. (1971) *Biochim. Biophys. Acta* **248**, 434–448
- Gonzalez-Sastre, F. & Folch-Pi, J. (1968) *J. Lipid Res.* **9**, 532–533
- Greene, W. C., Parker, C. M. & Parker, C. W. (1976) *Cell. Immunol.* **25**, 74–89
- Gustafson, A. (1965) *J. Lipid Res.* **6**, 512–517
- Harmony, J. A. K. & Hui, D. Y. (1980) *Cancer Res.* in the press
- Havel, R. J., Eder, H. S. & Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
- Ho, Y. K., Brown, M. S., Bilheimer, D. W. & Goldstein, J. L. (1976) *J. Clin. Invest.* **58**, 1465–1474
- Hui, D. Y. & Harmony, J. A. K. (1979a) *J. Supramol. Struct.* **10**, Suppl. 3, 264 (abstr.)
- Hui, D. Y. & Harmony, J. A. K. (1979b) *Biochim. Biophys. Acta* **550**, 407–424
- Hui, D. Y. & Harmony, J. A. K. (1979c) *Biochim. Biophys. Acta* **550**, 425–434
- Hui, D. Y. & Harmony, J. A. K. (1980) *Cancer Res.* in the press
- Hui, D. Y., Berebitsky, G. L. & Harmony, J. A. K. (1979) *J. Biol. Chem.* **254**, 4666–4673
- Lindahl-Kiessling, K. (1972) *Exp. Cell Res.* **70**, 17–26
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maino, V. C., Hayman, M. J. & Crumpton, M. J. (1975) *Biochem. J.* **146**, 247–252
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–148
- Morse, J. H., Witte, L. D. & Goodman, D. S. (1977) *J. Exp. Med.* **146**, 1791–1803
- Nelson, G. J. (1972) *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, Wiley-Interscience, New York
- Pairault, J., Levillier, J. & Chapman, M. J. (1977) *Nature (London)* **269**, 607–609
- Roeschlau, P., Bernt, E. & Gurber, W. (1974) *Z. Klin. Chem. Klin. Biochem.* **12**, 403–407
- Shore, B. & Shore, V. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 476 (abstr.)
- Shore, V. & Shore, B. (1975) *Biochem. Biophys. Res. Commun.* **65**, 1250–1256
- Steinberg, D., Nestel, P. J., Weinstein, D. B., Remaut-Desmeth, M. & Chang, C. M. (1978) *Biochim. Biophys. Acta* **528**, 199–212
- Taylor, R. G., Lewis, J. C., Rudel, L. L. & Biddulph, D. M. (1979) *Thromb. Res.* **14**, 799–803