Stimulated cholesterol-enriched platelets display increased cytosolic Ca^{2+} and phospholipase A activity independent of changes in inositol trisphosphates and agonist/receptor binding

Alexander SORISKY,* Gregory L. KUCERA† and Susan E. RITTENHOUSE†‡

†Department of Biochemistry and *Metabolic Unit, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.

To investigate the mechanism of enhanced responsiveness of cholesterol-enriched human platelets, we compared stimulation by surface-membrane-receptor (thrombin) and post-receptor (AlF_4^-) G-protein-directed pathways. Platelets were labelled with $[^{32}\text{P}]\text{P}_i$ and $[\text{methyl-}^3\text{H}]\text{choline chloride}$, incubated with sonicated lipid dispersions of various ratios of cholesterol and phospholipid, and loaded with the fluorescent Ca^{2+} indicator fura-2. We report the following. (1) Cholesterol enrichment enhances cytosolic Ca^{2+} accumulation and phospholipase A activation in response to both receptor-directed and post-receptor-directed agonists. No enhancement by cholesterol of phospholipase A activity at fixed Ca^{2+} concentrations is observed in lysed platelets, implying that no perturbation by cholesterol of phospholipase A/substrate interaction occurs in our preparations. (2) In both normal and cholesterol-enriched platelets, Ca^{2+} mobilization is promoted by a factor(s) apart from InsP_3 that appear(s) to be modulated by cholesterol. A disproportionate increase in cytosolic Ca^{2+} relative to $[^{32}\text{P}]\text{InsP}_3$ is observed with increasing doses of thrombin in normal, and to a larger extent in cholesterol-enriched, platelets. When AlF_4^- is the agonist, there is no cholesterol-associated enhancement in $[^{32}\text{P}]\text{InsP}_3$ to account for the heightened Ca^{2+} rise seen with cholesterol enrichment. (3) Enhanced phospholipase A activation is not necessarily proportional to cytosolic Ca^{2+} increase. The magnitude of the increase in phospholipase A activity for a given rise in cytosolic Ca^{2+} is greater in cholesterol-enriched platelets that are stimulated by AlF_4^- than in those stimulated by thrombin. We conclude that increased membrane microviscosity associated with cholesterol enrichment may promote G-protein/phospholipase A interaction as well as the Ca^{2+} -release mechanism, without significantly altering G-protein/phospholipase C interaction.

INTRODUCTION

Human platelets enriched with cholesterol *in vitro* display increased reactivity to various agonists. This heightened responsiveness has been ascertained by measurement of various parameters such as aggregation, 5-hydroxytryptamine secretion, and more recently, arachidonate mobilization [1,2]. Interest in this model of cholesterol enrichment stems from observations of increased platelet membrane cholesterol in hypercholesterolaemic subjects [3–5]. Such platelets also demonstrate hyper-reactivity to aggregatory agonists [6], and their role in the increased risk of atherosclerosis in hypercholesterolaemia remains under investigation [7–9]. Although cholesterol incorporation *in vitro* by sonicated lipid dispersions may not mimic the pathophysiological process exactly [10,11], it is a valuable method to examine the mechanism by which alterations in membrane cholesterol modulate platelet activation uncomplicated by variation among donors [12].

It has been suggested that cholesterol enrichment, by increasing platelet membrane microviscosity, may affect stimulus-induced changes in membrane lipids [2]. Alterations could occur at any and/or several levels in the

sequence leading to phospholipase activation and generation of second messengers. One study, based on analysis of binding of agonist by receptors, was interpreted to indicate that cholesterol exerts its effect on thrombin-receptor number rather than on coupling between occupied receptor and the bioresponses of aggregation and 5-hydroxytryptamine secretion [13].

To define more precisely where changes exerted by cholesterol occur, we have analysed the plasma-membrane coupling of activation signal and cellular response by measuring several biochemical sequelae associated with lipid metabolism. In the platelet, as in numerous other cells, such coupling, or signal transduction, involves agonist activation of PLC via a G-protein [14–17]. Subsequent hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by PLC generates $\text{Ins}(1,4,5)\text{P}_3$ and DG [18,19]. $\text{Ins}(1,4,5)\text{P}_3$ has a major role in Ca^{2+} release from microsomes, and DG activates protein kinase C [20]. Liberation of arachidonic acid (the initial step leading to eicosanoid production) may be accomplished by the action of DG and MG lipases on DG [21], or may arise by a direct effect of PLA on membrane phospholipid, such as PtdCho [22]. Previous reports point to the existence of G-protein regulation of PLA in human platelets [23,24].

Abbreviations used: PLA, phospholipase A; PLC, phospholipase C; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concn.; C-N, cholesterol-normal; C-E, cholesterol-enriched; DG, diacylglycerol; MG, monoacylglycerol; G-protein, GTP-binding protein; PtdCho , phosphatidylcholine.

‡ To whom reprint requests should be addressed.

Our approach to elucidate the nature of the effect of cholesterol on the signal-transduction process has been to compare stimulation of platelets by receptor (thrombin) and non-receptor G-protein-directed (AlF_4^-) routes. The data demonstrate an effect of cholesterol on PLA activity and net mobilization of $[\text{Ca}^{2+}]_i$ that is independent of InsP_3 and thrombin-receptor number.

EXPERIMENTAL

Platelet preparation

Human platelet-rich plasma, free from erythrocytes, was prepared as described [25] in the presence of $0.5 \mu\text{M}$ -prostaglandin E_1 (Upjohn Co.) and 1.0 mM -aspirin, and centrifuged at $1600 g$ for 5 min at room temperature. The platelet pellet was resuspended in platelet-poor plasma, pH 7.1, containing 5.0 mM -phosphocreatine and 40 units of creatine kinase/ml.

Preparation of lipid dispersions

Dispersions of cholesterol (99%) and L-3-phosphatidylcholine (dipalmitoyl) (both from Serdary Research Laboratories, Port Huron, MI, U.S.A.) were prepared as described by Shattil *et al.* [1] as modified by Cooper *et al.* [26]. PtdCho (40 mg) and cholesterol [20 mg for cholesterol-normal (C-N) or 80 mg for cholesterol-enriched (C-E)] were sonicated (sonicator model W-375, standard tip, 70 W) in 10 ml of modified Tyrode's buffer (136.9 mM -NaCl, 2.7 mM -KCl, 11.9 mM - NaHCO_3 , 10 mM -Hepes, pH 7.4) for 1 h at 45°C . The sonicated preparations were centrifuged at $27000 g$ for 30 min to sediment any aggregated lipid. The lipid dispersions were stored at 4°C and used within 24 h. Bovine serum albumin (fatty-acid-free; Calbiochem Corp., San Diego, CA, U.S.A.) and glucose were added to final concentrations of 0.35% and 5.5 mM respectively, and the pH was adjusted to that of the anticoagulated plasma before use. T.l.c., elution and subsequent lipid-P analysis of the sonicated lipid dispersions indicated the absence of any lysoPtdCho (detection limit 2% of PtdCho).

Incubation of lipid dispersions with platelets

The platelet suspension sample was mixed with 2 vol. of either C-E or C-N lipid dispersions (control platelets were mixed with Tyrode's buffer only) and incubated for 3 h at 37°C . Platelets were washed twice in buffer (123.2 mM -NaCl, 12.2 mM -sodium citrate, 5.6 mM -dextrose, 2.0 mM -Hepes, 2.8 mM -KCl, 8.9 mM - NaHCO_3 , 0.86 mM - MgCl_2 , 10 mM -Trizma base, 5.0 mM -phosphocreatine and 10 units of creatine kinase/ml, pH 7.3) at room temperature. The magnitude of the cholesterol enrichment (C-E versus C-N platelets) varied from 25 to 53% , which is in agreement with other studies [1,2]. Changes in the lipid P content of C-E versus C-N versus control platelets were minor (34.8 ± 0.4 , 36.4 ± 0.4 and 34.6 ± 0.4 respectively, expressed as $\text{nmol}/10^8$ platelets in duplicate determinations).

Platelet labelling with $[\text{P}^{32}]\text{P}_i$

After preparation from platelet-rich plasma, platelets were incubated for 45 min at 37°C with $[\text{P}^{32}]\text{P}_i$ (Du Pont-New England Nuclear; $0.75 \text{ mCi}/\text{ml}$ of platelet suspension). Incubation with lipid dispersions followed as described above.

Platelet labelling with $[\text{methyl-}^3\text{H}]\text{choline chloride}$

After preparation from platelet-rich plasma, platelets were incubated with $[\text{methyl-}^3\text{H}]\text{choline chloride}$ (Du Pont-New England Nuclear; $20 \mu\text{Ci}/\text{ml}$ of platelet suspension) for 3 h at 37°C simultaneously with the lipid-dispersion incubation. Platelets were washed as described above. Bovine serum albumin (fatty-acid-free) was added to final buffer (see above) at 0.05% .

Aggregometry

After incubation with lipid dispersions, platelets were washed as described but without phosphocreatine or creatine kinase in the final buffer. Samples of 1×10^8 platelets, with or without CaCl_2 (2.8 mM), were equilibrated at 37°C , and then α -thrombin was added (α -thrombin was generously provided by Dr. K. G. Mann, University of Vermont). Aggregation was monitored with a Payton Aggregation Module model 300.

Intracellular Ca^{2+} measurement

Fura-2 (acetoxymethyl ester; Molecular Probes, Eugene, OR, U.S.A.) was used to measure $[\text{Ca}^{2+}]_i$ in C-E and C-N platelets at various concentrations of thrombin or NaF. Appropriate controls of buffer or NaCl were done. No autofluorescence was noted. The fura-2 labelling procedures (carried out after the lipid-dispersion incubation), fluorescence measurements (with a Perkin-Elmer MPF-44A fluorescence spectrophotometer thermostatically maintained at 37°C), and determination of $[\text{Ca}^{2+}]_i$ were as described by Pollock *et al.* [27].

Platelet incubations and $[\text{H}^3]\text{lysoPtdCho}$ measurement

Washed platelets (5×10^8 per sample) were equilibrated at 37°C for 5 min, followed by addition of thrombin (0 – $1.0 \text{ unit}/\text{ml}$) or NaF (0 – 40 mM ; all NaF experiments also contained $10 \mu\text{M}$ - AlCl_3). After specific time intervals, incubations were terminated with 3.75 vol. of chloroform/methanol ($1:2$, v/v). Phases were split with 1 vol. of water and 1 vol. of chloroform. The lower organic phase was chromatographed as described [28], and resolved $[\text{H}^3]\text{lysoPtdCho}$ and $[\text{H}^3]\text{PtdCho}$ were quantified by scintillation spectrophotometry.

Platelet incubations and $[\text{P}^{32}]\text{InsP}_3$ measurement

Washed platelets (1.5×10^9 per sample) were equilibrated at 37°C for 5 min and exposed to agonist as above. Incubations were terminated at 30 s (when thrombin was the agonist) or at 2 min (when AlF_4^- was the agonist) by addition of 3.75 vol. of ice-cold chloroform/methanol/ 1.0 M -HCl ($4:10:1$, by vol.). $[\text{H}^3]\text{Ins}(1,4,5)\text{P}_3$ (courtesy of Dr. Glenn Prestwich, SUNY, Stonybrook, NY U.S.A.; 40000 d.p.m.) was added as tracer. Phases were split by the addition of 0.5 vol. of 1.0 M -HCl and 1 vol. of chloroform. The aqueous upper phases containing labelled inositol phosphates were processed before the h.p.l.c. stages as outlined previously [19].

H.p.l.c. resolution of the inositol phosphates

The Partisphere SAX h.p.l.c. system was used as described by Stephens *et al.* [29]. Radio-label was counted with a Radiomatic Flo-One/Beta in-line scintillation counter.

Measurement of secreted PLA activity

C-N or C-E platelets (1.0×10^9) were equilibrated at 37 °C for 5 min, followed by addition of thrombin at 0.5 unit/ml. After 1 min, the thrombin was inactivated by addition of hirudin (Sigma; 10 units/ml final concn.). The platelets were pelleted at 16000 *g* for 5 min. The supernatant was warmed to 37 °C, and to it were added C-N or C-E platelets (5×10^8 per sample) labelled with [*methyl*- ^3H]choline chloride. After a 2 min incubation, the reaction was terminated by addition of chloroform/methanol (1:2, v/v). Separation of the phases and subsequent measurement of [^3H]lysoPtdCho were as described above.

Studies with frozen/thawed platelets

Once C-N and C-E platelets were labelled with [*methyl*- ^3H]choline chloride, they were washed as above and suspended at 2×10^9 /ml in buffer containing 5.0 mM-EGTA, 0.5 mM-ATP, 2.0 mM-MgCl₂, 40 mM-NaCl, 120 mM-potassium glutamate, 5.0 mM-phosphocreatine and 10 units of creatine kinase/ml, pH 7.3. Platelets were then frozen and thawed twice in a solid-CO₂/ethanol bath and kept on ice. Samples (250 μl) were added to 250 μl of buffer as above containing fixed concentrations of Ca²⁺ [30] that was equilibrated to 37 °C. After 2 min, the incubation was terminated by addition of chloroform/methanol (1:2, v/v). Separation of the phases and subsequent measurement of [^3H]lysoPtdCho were as described above.

Characterization of [^3H]PtdCho and [^3H]lysoPtdCho

Washed platelets were processed as described for [^3H]PtdCho measurement. [^3H]PtdCho and [^3H]lysoPtdCho were resolved by t.l.c., eluted, and hydrolysed under alkaline conditions [31] for cleavage of fatty acid esters, extracted into chloroform, re-chromatographed, and quantified by scintillation spectrophotometry.

Cholesterol analysis

This was performed as described by Rudel & Morris [32].

Lipid-P analysis

Samples for lipid P were ashed [33] and then assayed as described by Chalvardjian & Rudnicki [34].

RESULTS

Thrombin studies

Thrombin-stimulated C-E platelets demonstrated an enhanced aggregatory response compared with C-N platelets or control platelets, measured as % transmission/min $\pm$ range of duplicates (see Fig. 1). In the presence of 2.8 mM-CaCl₂, addition of 0.02 unit of thrombin/ml resulted in values of 28 ± 4.0 (C-E platelets), 5.5 ± 0.5 (C-N platelets) and 4.5 ± 0.5 (control platelets). With no added CaCl₂ and thrombin added at 0.05 unit/ml, the enhancement was maintained, at 19 ± 2.0 (C-E platelets), 13 ± 1.0 (C-N platelets) and 10.5 ± 0.5 (control platelets).

Fig. 2 shows the temporal relationship between the responses of [Ca²⁺]_i and [^3H]lysoPtdCho in thrombin-stimulated platelets. The rise in [Ca²⁺]_i occurs within seconds and then gradually returns to baseline. The accumulation of [^3H]lysoPtdCho appears to increase

Fig. 1. Aggregatory responses of C-E, C-N and control platelets

Washed platelets were prepared as described. Samples (1×10^8 platelets) with CaCl₂ at 2.8 mM were equilibrated at 37 °C and exposed to thrombin at 0.02 unit/ml (arrow). Responses *a*, *b* and *c* are those of C-E, C-N and control platelets respectively. These data are from a single experiment. The results are representative of four similar experiments.

steadily during the time course. Unless otherwise stipulated, in the following studies, for which thrombin was the agonist, [Ca²⁺]_i was calculated as peak minus basal value and [^3H]lysoPtdCho was calculated by subtracting the basal from the 1 min value.

Incorporation of [*methyl*- ^3H]choline chloride into

Fig. 2. Time courses of [Ca²⁺]_i and [^3H]lysoPtdCho accumulation in thrombin-stimulated platelets

Measurements of [Ca²⁺]_i (continuous line is fluorescent scan) and [^3H]lysoPtdCho (dashed line) were as described. C-N platelets (2×10^8 /ml) were exposed to thrombin (0.1 unit/ml) for various time periods at 37 °C. These data, from a single experiment, are expressed as a percentage of the maximum response to the stimulus, and, where indicated, are averages $\pm$ ranges of duplicates. The results are representative of six similar experiments.

Fig. 3. Accumulations of $[Ca^{2+}]_i$ and $[^3H]$ lysoPtdCho in C-E and C-N platelets exposed to thrombin

Human C-N (○, □) and C-E (●, ■) platelets (2×10^8 /ml) labelled with [*methyl*- 3H]choline chloride and loaded with fura-2 as described were incubated with thrombin (0–1.0 unit/ml). $[Ca^{2+}]_i$ represents the peak minus the basal (0.130 μM) values. $[^3H]$ lysoPtdCho represents the 1 min minus basal (1350 d.p.m.) values. These data, from a single experiment, are expressed with respect to varied thrombin concentrations, as averages $\pm$ ranges of duplicates. The results are representative of five similar experiments.

PtdCho did not differ significantly whether or not platelets were incubated under cholesterol-elevating conditions. A 2-fold enhancement of $[^3H]$ lysoPtdCho in C-E versus C-N platelets was observed, however, at thrombin concentrations ≥ 0.25 unit/ml (Fig. 3). At lower concentrations of thrombin, differences were less pronounced and not always noted. Heightened accumulations of $[Ca^{2+}]_i$ in thrombin-stimulated C-E versus C-N platelets were also observed, with a 3-fold difference in $[Ca^{2+}]_i$ obtained at 1.0 unit of thrombin/ml (Fig. 3).

Platelets frozen and thawed twice and then incubated with fixed Ca^{2+} concentrations did not exhibit a cholesterol-dependent increase in PLA activity; C-E and C-N platelets both showed an increase in $[^3H]$ lysoPtdCho of approx. 1000 d.p.m. (10 nM- versus 500 nM- Ca^{2+}). This suggests that the enhanced PLA activity associated with

cholesterol enrichment is not due to a direct effect on PLA–substrate interaction.

A recent report describing secreted PLA₂ from human platelets [35] prompted us to consider whether the PLA activity that we measured could have arisen from hydrolysis of $[^3H]$ PtdCho on the outer membrane by a secreted enzyme fraction. There was no evidence of secreted PLA activity as measured under our assay conditions in the supernatant derived from pelleted thrombin-stimulated platelets (results not shown).

The major species of $[^3H]$ PtdCho in C-E and C-N platelets, at rest and after thrombin stimulation, was diacyl PtdCho (see Table 1). For both C-E and C-N platelets, $[^3H]$ lysoPtdCho was primarily of the acyl form in proportion to PtdCho, under both basal and thrombin-stimulated conditions.

To investigate the mechanism of the increased $[Ca^{2+}]_i$ response, we measured $[^{32}P]InsP_3$ [$Ins(1,4,5)P_3$ + $Ins(1,3,4)P_3$] and $[Ca^{2+}]_i$ in thrombin-stimulated C-E and C-N platelets (Fig. 4a). Incorporation of $[^{32}P]P_i$ into PtdIns P_2 did not differ significantly whether or not platelets were incubated under cholesterol-elevating conditions. Under the reaction conditions used, $InsP_3$ served as a reliable indicator of the Ca^{2+} -mobilizing inositol phosphates (see Fig. 4 legend). At 1.0 unit of thrombin/ml, C-E platelets demonstrated a 3-fold enhancement of $[Ca^{2+}]_i$ compared with C-N platelets, whereas only a modest increase (30–50%) in PLC activity, reflected by $[^{32}P]InsP_3$, was observed. The $[Ca^{2+}]_i$ rose with thrombin dose up to 1.0 unit/ml, whereas $[^{32}P]InsP_3$ virtually levelled off at 0.25 unit/ml. In both C-E and C-N platelets, thrombin disproportionately enhanced $[Ca^{2+}]_i$ with respect to $InsP_3$ (Fig. 4b), although more so in the C-E platelets. Over a dose range of thrombin from 0.1 to 1.0 unit/ml, the stimulated $[Ca^{2+}]_i/[^{32}P]InsP_3$ increased 2-fold for C-N platelets, and 3-fold for C-E platelets. It appears that there is an additional, cholesterol-modulated, factor beyond $InsP_3$ capable of promoting Ca^{2+} mobilization in thrombin-stimulated platelets.

AlF₄[−] studies

It has been proposed that thrombin-receptor number, rather than perturbation of subsequent signal trans-

Table 1. Relative alkyl and acyl species in $[^3H]$ choline-labelled PtdCho and lysoPtdCho of human platelets

$[^3H]$ PtdCho and $[^3H]$ lysoPtdCho of C-N or C-E platelets, with or without thrombin treatment (60s, 1.0 unit/ml) were resolved on t.l.c. plates, eluted, hydrolysed under alkaline conditions for cleavage of fatty acid esters, extracted into chloroform, re-chromatographed, and counted for radioactivity. Results for 'acyl PtdCho' represent the [water-soluble hydrolysate (= 'acyl') $\div$ the sum ('S_p') of water-soluble hydrolysate + lysoPtdCho + PtdCho] $\times 100$. Those for 'alkyl PtdCho' = [(PtdCho + lysoPtdCho) $\div$ S_p] $\times 100$. Results for 'acyl lysoPtdCho' represent the [water-soluble hydrolysate ('acyl') $\div$ the sum ('S_L') of water-soluble hydrolysate + lysoPtdCho] $\times 100$. Those for 'alkyl lysoPtdCho' = (lysoPtdCho $\div$ S_L) $\times 100$. N.D., not detectable. Values are averages $\pm$ ranges of duplicates.

	Species (% of total)							
	C-N				C-E			
	No thrombin		+ thrombin		No thrombin		+ thrombin	
	Alkyl	Acyl	Alkyl	Acyl	Alkyl	Acyl	Alkyl	Acyl
lysoPtdCho	N.D.	100	2.6 $\pm$ 0.2	97.4 $\pm$ 3.6	N.D.	100	3.0 $\pm$ 0.4	97.0 $\pm$ 3.0
PtdCho	2.6 $\pm$ 0.1	97.1 $\pm$ 1.4	2.4 $\pm$ 0.3	97.3 $\pm$ 1.7	2.5 $\pm$ 0.1	97.1 $\pm$ 0.9	2.4 $\pm$ 0.1	97.2 $\pm$ 0.9

Fig. 4. Accumulations of $[Ca^{2+}]_i$ and $[^{32}P]InsP_3$ in C-E and C-N platelets exposed to thrombin

Human C-N (○, △) and C-E (●, ▲) platelets (2×10^8 /ml) were loaded with fura-2 as described and exposed to thrombin (0–1.0 unit/ml). $[Ca^{2+}]_i$ represents the peak minus the basal ($0.130 \mu M$) values. Human C-E and C-N platelets (1.5×10^9 /ml) were labelled with $[^{32}P]P_i$ as described and incubated with thrombin (0–1.0 unit/ml) for 30 s. $[^{32}P]InsP_3$ represents the 30 s minus the basal (1500 d.p.m.) values. $[^{32}P]InsP_3$ consisted primarily of $[^{32}P]Ins(1,3,4)P_3$. However, over the thrombin concentrations examined, $[^{32}P]Ins(1,4,5)P_3$ formed a constant proportion of total $[^{32}P]InsP_3$ for both C-N ($6.3 \pm 0.3\%$) and C-E ($6.5 \pm 0.3\%$) platelets. $[^{32}P]Ins(1,3,4,5)P_4$ also was consistently equivalent to $12 \pm 1\%$ (C-N) and $14 \pm 1\%$ (C-E) of $[^{32}P]InsP_3$. Therefore, measurement of $InsP_3$ serves as a reliable indicator, in short incubation periods, of the Ca^{2+} -mobilizing inositol phosphates. (a) These data, from a single experiment, are expressed with respect to varied thrombin concentrations, as averages $\pm$ ranges of duplicates. The results are representative of three similar experiments. (b) The same data of the experiment described in (a) are displayed to show the relationship between the ratio of thrombin-induced increases in $[Ca^{2+}]_i/[^{32}P]InsP_3$ and thrombin dose.

duction, defines C-E platelet responsiveness [13]. In order to discern effects of cholesterol on post-receptor signal transduction, we have studied a route of stimulation that is independent of plasma-membrane surface receptors. It was not possible to permeabilize platelets with cholesterol-chelating agents (eg. saponin and digitonin) and examine responses to guanosine 5'-[γ -thio]triphosphate. AlF_4^- was therefore employed, as it is a known platelet activator that is thought to by-pass the plasma-membrane receptor by binding at the guanine-nucleotide-occupied site of G_x next to the β -phosphate group of GDP, thus imitating the presence of the γ -phosphate of GTP [36–38].

Fig. 5. Time course of $[Ca^{2+}]_i$ and $[^3H]lysoPtdCho$ accumulation in AlF_4^- -stimulated platelets

Measurements of $[Ca^{2+}]_i$ (continuous line is fluorescent scan) and $[^3H]lysoPtdCho$ (dashed line) were as described. C-N platelets (2×10^8 /ml) were exposed to NaF (40 mM) for various time periods. These data, from a single experiment, are expressed as a percentage of the maximum response to the stimulus, and, where indicated, are averages $\pm$ ranges of duplicates. The results are representative of five similar experiments.

The responses in the levels of $[Ca^{2+}]_i$ and $[^3H]lysoPtdCho$ for platelets activated by AlF_4^- are shown in Fig. 5. As compared with thrombin stimulation, the rise in $[Ca^{2+}]_i$ was much slower, taking approx. 2 min to reach peak value, and no return to baseline was observed. There appeared to be a progressive accumulation of $[^3H]lysoPtdCho$ during the time course. Unless otherwise stated, in subsequent studies with AlF_4^- , $[Ca^{2+}]_i$ was calculated as the 2 min value minus basal and $[^3H]lysoPtdCho$ was calculated as the 5 min value minus basal.

Stimulation of C-E platelets by AlF_4^- (10–40 mM-NaF) resulted in larger accumulations of $[^3H]lysoPtdCho$ than for C-N platelets, indicative of enhanced PLA activity (Fig. 6), as had been observed with thrombin.

Fig. 6. Accumulation of $[Ca^{2+}]_i$ and $[^3H]lysoPtdCho$ in C-E and C-N platelets exposed to AlF_4^-

Human C-N (○, □) and C-E (●, ■) platelets (2×10^8 /ml), labelled with [*methyl*- 3H]choline chloride and loaded with fura-2 as described, were incubated with NaF (0–40 mM). $[Ca^{2+}]_i$ represents the 2 min minus the basal value. $[^3H]lysoPtdCho$ represents the 5 min minus basal value. These data, from a single experiment, are expressed as averages $\pm$ ranges of duplicates. The results are representative of five similar experiments.

Table 2. Accumulation of cytosolic Ca^{2+} and $[^3\text{H}]\text{lysoPtdCho}$ in human C-E platelets exposed to different agonists

Human C-E platelets (2×10^8) labelled with [*methyl*- ^3H]choline chloride and loaded with fura-2 as described were incubated with thrombin (0.1 unit/ml), NaF (40 mM) or buffer. $[\text{Ca}^{2+}]_i$ represents the peak (when thrombin was the agonist) or the 2 min value (when NaF was the agonist) minus the basal value. $[^3\text{H}]\text{LysoPtdCho}$ represents the 5 min minus the basal values. These data, from a single experiment, are expressed as averages $\pm$ ranges of duplicates, and are representative of two experiments.

Agonist	$[\text{Ca}^{2+}]$ (μM)	$[^3\text{H}]\text{LysoPtdCho}$ (d.p.m.)
Thrombin (0.1 unit/ml)	0.200 ± 0.014	1165 ± 106
NaF (40 mM)	0.140 ± 0.014	2713 ± 154

An augmented rise in $[\text{Ca}^{2+}]_i$ in C-E versus C-N platelets also followed exposure to AlF_4^- (Fig. 6). At low concentrations of NaF (10 mM), the enhanced response of $[\text{Ca}^{2+}]_i$ in C-E platelets was variable. Table 2 shows the increases in both $[^3\text{H}]\text{lysoPtdCho}$, calculated as the 5 min increment, and $[\text{Ca}^{2+}]_i$ evoked by thrombin or AlF_4^- in C-E platelets. AlF_4^- stimulation resulted in a larger rise in $[^3\text{H}]\text{lysoPtdCho}$ than that in response to thrombin stimulation, even though the increase in $[\text{Ca}^{2+}]_i$ was smaller than that for thrombin. There was no comparable effect in the C-N platelets, suggesting that cholesterol enrichment promotes PLA activation by AlF_4^- , a G-protein-directed agonist, through a less Ca^{2+} -dependent route.

Cholesterol enrichment did not result in any enhancement of InsP_3 to account for the augmentation in $[\text{Ca}^{2+}]_i$ observed in response to AlF_4^- stimulation (Fig. 7). To examine whether the influx of extracellular Ca^{2+} was

Fig. 7. Accumulation of $[\text{Ca}^{2+}]_i$ and $[^{32}\text{P}]\text{InsP}_3$ in C-E and C-N platelets exposed to AlF_4^-

Human C-N ($\circ$, $\triangle$) and C-E ($\bullet$, $\blacktriangle$) platelets ($2 \times 10^8/\text{ml}$) were loaded with fura-2 as described and exposed to NaF (0–40 mM). $[\text{Ca}^{2+}]_i$ represents the 2 min minus the basal value. Human C-E and C-N platelets ($1.5 \times 10^8/\text{ml}$) were labelled with $[^{32}\text{P}]\text{P}_i$ as described and incubated with NaF (0–40 mM) for 2 min. $[^{32}\text{P}]\text{InsP}_3$ represents the 2 min minus the basal value. These data, from a single experiment, are expressed as averages $\pm$ ranges of duplicates. They are representative of two similar experiments.

selectively altered, we compared AlF_4^- -induced rises in $[\text{Ca}^{2+}]_i$ at buffer Ca^{2+} concentrations with and without 0.1 mM-EGTA. Although the magnitude of the $[\text{Ca}^{2+}]_i$ rise was diminished, the enhancement in the response of the C-E platelets persisted. In the absence of EGTA and in response to 40 mM-NaF, $[\text{Ca}^{2+}]_i$ increased by $0.390 \pm 0.008 \mu\text{M}$ for C-E platelets and $0.306 \pm 0.008 \mu\text{M}$ for C-N platelets. In the presence of EGTA, the increase was $0.162 \pm 0.004 \mu\text{M}$ for C-E platelets and $0.130 \pm 0.007 \mu\text{M}$ for the C-N platelets. It would appear that the cholesterol enrichment affects net mobilization of internal stores as well, rather than specifically perturbing Ca^{2+} influx across the plasma membrane.

DISCUSSION

Our data lead to three major conclusions about signal transduction in human platelets: (1) cholesterol enrichment perturbs Ca^{2+} accumulation and PLA activation at the post-receptor level as well as at the receptor level; (2) in both C-N and C-E platelets Ca^{2+} mobilization is promoted by a factor(s) in addition to InsP_3 ; and (3) enhanced PLA activation is not necessarily proportional to $[\text{Ca}^{2+}]_i$ elevation.

Elevation of platelet-membrane cholesterol is known to increase platelet reactivity to agonists [1], barring any abnormalities in phospholipid or fatty acid composition [39]. An increase in membrane microviscosity resulting from cholesterol enrichment is postulated to affect stimulus-induced changes in membrane lipids [2]. It has not yet been established, however, which of the several steps linking agonist, receptor and second-messenger generation may be altered. One study has concluded, on the basis of analysis of binding of agonist by receptors, that the number of thrombin receptors is increased by cholesterol enrichment [13]. We have analysed several biochemical sequelae, including Ca^{2+} mobilization and PLA activation, that occur beyond surface receptor occupancy, and we provide evidence that cholesterol exerts specific effects at this level of signal transduction as well.

To examine this facet of signal transduction, we needed to establish a route of platelet activation that was not dependent on surface receptors. We explored the possibility of direct G-protein activation in light of the findings by Nakashima *et al.* [23,24], based on the use of non-hydrolysable GTP analogues in saponin-treated human platelets, which pointed to direct regulation of PLA_2 by G-protein(s). Previous studies had indicated that platelet PLC is regulated by G-protein(s) [14–17]. It was not possible to permeabilize platelets with cholesterol-chelating agents in our experiments, given that platelet cholesterol content was the variable of interest. Instead, we have studied intact platelets activated by AlF_4^- , an agonist that appears to bypass the plasma-membrane receptor and mimics the presence of the γ -phosphate of GTP at the GDP-occupied site of G_x [36–38].

The observation that C-E platelets, stimulated by AlF_4^- , exhibit enhanced PLA activation and net Ca^{2+} accumulation as compared with C-N platelets (Fig. 6) indicates an effect of cholesterol at a site distal to the surface membrane receptor, and raises the possibility that this alteration occurs at the level of a G-protein. No enhancement by cholesterol enrichment of PLA activity at fixed Ca^{2+} concentrations is observed in platelets that have been frozen and thawed. This implies that there is

no perturbation by cholesterol at the level of PLA₂/substrate interaction in our preparations. Perhaps, then, G-protein-derived entities (α or $\beta\gamma$) interact more efficiently with PLA and a Ca²⁺-release mechanism in C-E platelets.

Ins(1,4,5)P₃ is established as playing a potentially major role in Ca²⁺ mobilization in platelets [40,41]. We present data pointing to a factor(s), in addition to total InsP₃ (which is a reliable indicator of the Ca²⁺-mobilizing inositol phosphates under our experimental conditions), that influences Ca²⁺ mobilization in both the C-N and C-E platelets. When C-N platelets are exposed to increasing doses of thrombin, the mobilization of [Ca²⁺]_i does not vary as a constant function of InsP₃ accumulation (Fig. 4). This observation indicates that, at higher thrombin doses, a non-InsP₃-dependent signal may influence Ca²⁺ mobilization. C-E platelets, under the same conditions, display an even more disproportionate rise in [Ca²⁺]_i as compared with InsP₃. Since thrombin is the agonist, these results may be attributable to increased numbers of bound receptors, or modulation by cholesterol of the additional signal, or both.

Further data in support of cholesterol modulation of this non-InsP₃ regulator of Ca²⁺ mobilization are shown in Fig. 7. When AlF₄⁻ is the agonist (20–40 mM-NaF), C-E platelets also demonstrate an enhanced rise in [Ca²⁺]_i as compared with C-N platelets in the absence of any such augmentation of InsP₃. Thus cholesterol enrichment appears to cause heightened Ca²⁺ accumulations by a route independent of InsP₃ in platelets stimulated by AlF₄⁻. This augmentation by cholesterol persists in the presence of EGTA, indicating that it includes an effect on net mobilization of Ca²⁺ from internal stores. Additional routes of Ca²⁺ mobilization seem to be triggered by other agonists besides thrombin and AlF₄⁻, since human platelets activated by ADP also undergo a rapid rise in [Ca²⁺]_i without hydrolysis of PtdIns(4,5)P₂ [42–44].

The maximal levels of [Ca²⁺]_i achieved in response to AlF₄⁻ are always much less than those achieved with thrombin. Conceivably, thrombin's role as a serine protease may contribute to these effects. The magnitude of the increase in [³H]lysoPtdCho for a given rise in [Ca²⁺]_i is greater in C-E platelets that are stimulated with AlF₄⁻ than in those stimulated with thrombin (Table 2). The PLA activity in thrombin-stimulated platelets appears to parallel closely changes in [Ca²⁺]_i (Fig. 3). The data in Table 2 would thus suggest another mechanism of PLA activation, perhaps potentiated by a rise in [Ca²⁺]_i, which becomes more evident when AlF₄⁻ is the agonist than when thrombin is the agonist. Several reports have suggested the possibility of PLA activation by Ca²⁺-independent routes in human platelets [27,45,46] as well as by Ca²⁺-dependent routes [28,46,47], and regulation of PLA₂ by a specific G-protein has been reported, as noted above in this Discussion [23,24]. The increased membrane microviscosity induced by cholesterol enrichment may promote G-protein/PLA interaction, resulting in larger accumulations of [³H]lysoPtdCho. Ca²⁺ would not be causal for PLA activation in this mechanism, although it might modulate activity initiated by G-protein(s).

PLC is also a G-protein-regulated enzyme, yet no enhancement of AlF₄⁻-activated PLC is observed in C-E platelets compared with C-N controls. The reason for this apparent discrepancy between PLC and PLA responses to modulation by cholesterol may be due to differences in the types of G-proteins or subunits con-

trolling these two phospholipases. The α -subunit, which binds GTP, is postulated to activate PLC in platelets; however, the $\beta\gamma$ -subunit is capable of stimulating PLA₂ in retinal and heart preparations [48–51]. Therefore the PLA enhancement induced by cholesterol enrichment in human platelets may be due to an effect at the level of either G-protein $\beta\gamma$ -subunit/PLA or unique α -subunit/PLA interactions.

PLA activity is monitored in our studies by the appearance of [³H]lysoPtdCho, a product of [³H]PtdCho hydrolysis. A secreted PLA₂ has been described by Kramer *et al.* [35], but we have found no evidence of this under our experimental conditions. Since the site of the ester bond involved (*sn*-1 or *sn*-2) is not discernible in our assay, we cannot be certain that this PLA activity is responsible for the enhanced release of arachidonic acid from *sn*-2 linkage documented previously in C-E platelets [2]. However, since arachidonic acid can also be liberated via DG lipase and MG lipase action on the product of PLC [21] and PLA₂ action on phosphatidic acid, another indirect PLC product [52], measurement of [³H]lysoPtdCho rather than of labelled arachidonic acid has allowed us to focus more specifically on PLA. Furthermore, characterization of lysoPtdCho from thrombin-activated human platelets has shown that the major fatty acid components are saturated, with a smaller contribution of monounsaturated and only traces of other unsaturated long-chain fatty acids [53]. The absence of arachidonic acid in lysoPtdCho is consistent with hydrolysis by PLA at the *sn*-2 ester bond.

A recent article has indicated that, in rat platelets, diacyl-PtdCho may not be a direct source of arachidonic acid [54]. These authors have suggested that the phospholipid precursors of arachidonic acid might be alkylacyl-PtdCho or diacyl-phosphatidylethanolamine, the hydrolysis of the latter inducing an arachidonate transfer from diacyl-PtdCho. We cannot exclude the contribution of other phospholipid classes to arachidonate release in our study; however, it should be noted that PLA activation in human platelets differs from that in rat platelets [45], and our results (Table 1) indicate that the major lysoPtdCho that accumulates in thrombin-stimulated human platelets is the acyl, rather than the alkyl, form.

The blood-drawing services of the General Clinical Research Center of the Medical Center Hospital of Vermont are gratefully acknowledged. We thank Warren King for skilled assistance in [Ca²⁺]_i measurements. This work was supported by National Institutes of Health Grant HL 38622 (to S.E.R.) and in part by a research fellowship of the Medical Research Council of Canada (to A.S.), and N.I.H. Training Grant T32HL 07594.

REFERENCES

- Shattil, S. J., Anayo-Galindo, R., Bennett, J., Coleman, R. W. & Cooper, R. A. (1975) *J. Clin. Invest.* **55**, 636–643
- Kramer, R. M., Jakubowski, J. A., Vaillancourt, R. & Deykin, D. (1982) *J. Biol. Chem.* **257**, 6844–6849
- Mosconi, C., Colli, S., Tremoli, E. & Galli, C. (1988) *Atherosclerosis* **72**, 129–134
- Shastri, K. M., Carvalho, A. C. A. & Lees, R. (1980) *J. Lipid Res.* **21**, 467–472
- Shattil, S. J., Bennett, J. S., Colman, R. W. & Cooper, R. J. (1977) *J. Lab. Med.* **89**, 341–353
- Carvalho, A. C. A., Colman, R. W. & Lees, R. S. (1974) *N. Engl. J. Med.* **290**, 434–438

7. Ross, R. & Glomset, J. A. (1986) *N. Engl. J. Med.* **314**, 488–500
8. Mendelsohn, M. E. & Loscalzo, J. (1988) *J. Clin. Invest.* **81**, 62–68
9. Takagi, Y., Dyer, C. A. & Curtiss, L. K. (1988) *J. Lipid Res.* **29**, 859–867
10. Harmon, J. T., Tandon, N. N., Hoeg, J. M. & Jamieson, G. A. (1986) *Blood* **68**, 498–505
11. Schick, B. P. & Schick, P. K. (1985) *Biochim. Biophys. Acta.* **833**, 281–290
12. Schick, P. K. (1987) in *Hemostasis and Thrombosis*, 2nd edn. (Colman, R. W., Hirsch, J., Marder, V. J. & Salzman, E. W., eds.), p. 668, J. B. Lippencott Co., Philadelphia
13. Tandon, N., Harmon, J. T., Rodbard, D. & Jamieson, G. A. (1983) *J. Biol. Chem.* **258**, 11840–11845
14. Haslam, R. J. & Davidson, M. L. (1984) *J. Receptor Res.* **4**, 605–629
15. Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) *J. Biol. Chem.* **261**, 16838–16847
16. Banga, H. S., Walker, R. K., Winberry, L. K. & Rittenhouse, S. E. (1988) *Biochem. J.* **252**, 297–300
17. Kucera, G. L. & Rittenhouse, S. E. (1988) *Biochem. Biophys. Res. Commun.* **153**, 417–421
18. Rittenhouse-Simmons, S. E. (1979) *J. Clin. Invest.* **63**, 580–587
19. Tarver, A. P., King, W. G. & Rittenhouse, S. E. (1987) *J. Biol. Chem.* **262**, 17268–17271
20. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
21. Prescott, S. M. & Majerus, P. W. (1983) *J. Biol. Chem.* **258**, 764–769
22. Broekman, M. J., Ward, J. W. & Marcus, A. J. (1980) *J. Clin. Invest.* **66**, 275–283
23. Nakashima, S., Tohmatsu, T., Hattori, H., Suganuma, A. & Nozawa, Y. (1987) *J. Biochem. (Tokyo)* **101**, 1055–1058
24. Nakashima, S., Hattori, H., Shirato, L., Takenata, A. & Nozawa, Y. (1987) *Biochem. Biophys. Res. Commun.* **146**, 820–826
25. Rittenhouse, S. E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5417–5420
26. Cooper, R. A., Leslie, M. H., Fischkoff, S., Shinitsky, M. & Shattil, S. J. (1978) *Biochemistry* **17**, 327–331
27. Pollock, K. W., Rink, T. J. & Levine, R. F. (1986) *Biochem. J.* **235**, 869–877
28. Rittenhouse, S. E. (1984) *Biochem. J.* **222**, 103–110
29. Stephens, L. R., Hawkins, P. T., Barker, C. J. & Downes, C. P. (1988) *Biochem. J.* **253**, 721–733
30. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
31. Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
32. Rudel, L. L. & Morris, M. D. (1973) *J. Lipid Res.* **14**, 364–366
33. Ames, B. N. & Dubin, D. T. (1960) *J. Biol. Chem.* **235**, 769–775
34. Chalvardjian, A. & Rudnicki, E. (1970) *Anal. Biochem.* **36**, 225–226
35. Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R. & Pepinsky, R. B. (1989) *J. Biol. Chem.* **264**, 5768–5775
36. Bigay, J., Deterre, P., Pfister, C. & Chabre, M. (1985) *FEBS Lett.* **191**, 181–185
37. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
38. Chabre, M. (1989) *Biochem. J.* **258**, 931–932
39. Owen, J. S., Hutton, R. A., Day, R. C. Bruckdorfer, K. R. & McIntyre, N. (1981) *J. Lipid Res.* **22**, 423–430
40. Brass, L. F. & Joseph, S. K. (1985) *J. Biol. Chem.* **260**, 15172–15179
41. O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) *J. Biol. Chem.* **260**, 956–962
42. Fisher, G. J., Bakshian, S. & Baldassare, J. J. (1985) *Biochem. Biophys. Res. Commun.* **129**, 958–964
43. Hallam, T. J. & Rink, T. J. (1985) *J. Physiol. (London)* **368**, 131–146
44. Sage, S. O. & Rink, T. J. (1986) *Biochem. Biophys. Res. Commun.* **136**, 1124–1129
45. Nakashima, S., Suganuma, A., Matsui, A., Hattori, H., Sato, M., Takenaka, A. & Nozawa, Y. (1989) *Biochem. J.* **259**, 139–144
46. Ballou, L. R., DeWitt, L. M. & Cheung, W. Y. (1986) *J. Biol. Chem.* **261**, 3107–3111
47. Kramer, R. M., Jakubowski, J. A. & Deykin, D. (1988) *Biochim. Biophys. Acta* **959**, 269–279
48. Jelsema, C. L. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2623–2627
49. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. (1987) *Nature (London)* **325**, 321–326
50. Logothetis, D. E., Kim, D., Northup, J. K., Neer, E. J. & Clapham, D. E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5814–5818
51. Kim, D., Lewis, D. L., Gaziadei, L., Neer, E. J., Bar-Sagi, D. & Clapham, D. E. (1989) *Nature (London)* **337**, 557–560
52. Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399–5403
53. McKean, M. L., Smith, J. B. & Silver, M. J. (1981) *J. Biol. Chem.* **256**, 1522–1524
54. Colard, O., Breton, M., Pepin, D., Chevy, F., Bereziat, G. & Polonovski, J. (1989) *Biochem. J.* **259**, 333–339

Received 1 June 1989/25 September 1989; accepted 10 October 1989