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Molecular link between membrane cholesterol and Na^+/H^+ exchange within human platelets

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Incubation of human platelets with cholesterol-poor, cholesterol-normal and cholesterol-rich liposomes revealed that: (i) acquisition or depletion of platelet membrane cholesterol was highly selective; (ii) variation in membrane cholesterol was highly selective. Variation in membrane cholesterol content (cholesterol-to-phospholipid molar ratio from 0.15-1.2) with respect to values found in unmodified normal platelets, was paralleled by the observed changes in amiloride-sensitive cytoplasmic pH, as well as phospholipase A_2 activity. However, a decrease in cytoplasmic pH was accompanied by an increase in phospholipase A_2 activity; (iii) membrane cholesterol-modulated changes in intra-platelet pH, as well as phospholipase A_2 activity, was completely inhibited when platelets were pretreated with quinacrine (a specific phospholipase A_2 inhibitor) before exposure to various types of liposomes. Although exposure of platelets (pretreated with amiloride) with various types of liposomes resulted in the inhibition of Na^+/H^+ exchange it had no noticeable effect upon the observed phospholipase A_2 activity. Based upon these results we suggest that membrane cholesterol-modulated phospholipase A_2 activity may be the basic mechanism responsible for the nature of Na^+/H^+ exchanger activity observed in cholesterol-enriched platelets, leading these platelets to a hypersensitized state.

Cholesterol; Na^+/H^+ exchange; Phospholipase A_2; Platelet

1. INTRODUCTION

It is now widely recognised that platelets provide an excellent human cellular model for the understanding of cellular activity modulated by second messengers. Interest in platelets has also been generated because of their increased sensitivity to aggregating agents in hypercholesterolemic patients [1,2]. Consequently, a series of in vitro studies were sought to establish the basis of this hypersensitivity: when the cholesterol-to-phospholipid (C/P) molar ratio was elevated by exposure to cholesterol-rich liposomes, the platelets were found to have increased membrane microviscosity [3], increased sensitivity to epinephrine, ADP and thrombin [4] and increased or decreased basal activity of adenyl cyclase [5]. Further, stimulation of Na^+/H^+ exchange has been suggested to play a role in the activation of platelets by thrombin and other agonists [6–10] and it has been concluded that Na^+/H^+ exchange may selectively modulate arachidonic acid mobilization in response to weak agonists (epinephrine, ADP and thrombin at low concentrations), to effect vigorous platelet aggregation and dense granule secretion [8]. A role in cytoplasmic pH regulation has also been proposed [10]. Consequently, keeping in view the complex role of membrane cholesterol, as well as Na^+/H^+ exchange, in the activity of human platelets, the present study was addressed to understand two specific issues: firstly, whether or not variation in platelet membrane cholesterol content has a direct effect upon the cytoplasmic pH and secondly, if so, whether or not membrane cholesterol-modulated cytoplasmic pH is mediated through phospholipase A_2 activation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cholesterol, lecithin, amiloride, 6-carboxyfluorescein diacetate and quinacrine were purchased from Sigma. All other reagents were of the highest quality available.

2.2. *Liposome preparation*

'Cholesterol-poor', 'cholesterol-normal' and 'cholesterol-rich' liposomes, having C/P ratios equal to 0, 1.0 and 2.2, respectively, were made in Tyrode's solution by employing standard methods [4].

2.3. *Platelet incubation experiments*

All blood donors had fasted for 12 h, had abstained from medication for 10 days before blood donation and had normal serum lipoprotein profiles according to standard criteria. Within 45 min of collection, platelet-rich plasma was adjusted to a platelet count of $3-4 \times 10^5$ per μl with platelet-poor plasma, and this mixture was incubated with an equal volume of cholesterol-poor, cholesterol-normal or cholesterol-rich liposomes in tyrode's solution at 37°C for up to 5 h. Samples were removed at various times up to 5 h for analysis of protein [11], phospholipid [12] and cholesterol [13] content of platelets. No platelet aggregation was observed with treatment of liposomes with platelets in up to 5 h of incubation, which is in agreement with results reported by various workers [4]. From these experiments 5 types of platelet preparations (characterized by their membrane C/P ratio, ranging from 0.15–1.2) were used for the analysis of intraplatelet pH, as well as phospholipase A_2 activity within platelets.

2.4. *ATP release measurement*

Since the ATP release by platelets in response to various agonists has been shown to be directly proportional to the extent of aggregation, the platelets enriched with cholesterol for 5 h (C/P ratio = 1.2) were incubated with ADP (0–15 μM), epinephrine (0–15 μM) and collagen (0–5 μg/ml). Subsequently, the ATP release was measured using luciferin–luciferase assay [14].

2.5. *Phospholipase A_2 activity*

Platelet preparations (C/P ratio 0.15–1.2), with or without pretreatment with quinacrine (150 μM), EGTA (final concentration 3 mM) or amiloride (200 mM), were processed for phospholipase A_2 activity according to the method of Wong et al. [15]. Phospholipase A_2 activity in cholesterol-enriched platelets was also studied in the presence of varying concentrations of quinacrine (0–200 μM) or EGTA (0–3 mM).

2.6. *Na^+/H^+ exchange determination*

The activity of Na^+/H^+ exchangers in platelets was measured by monitoring intracellular pH through pH-sensitive probes generated in situ [16]. Washed platelets in HEPES buffer, pH 7.35, were incubated with 6-carboxyfluorescein diacetate (25 μM of dye, final concentration, in dimethyl sulfoxide 0.1% final concentration) at 37°C for 15 min and subsequently gel filtered on a Sepharose 2B column equilibrated with HEPES buffer, pH 7.35, to remove non-internalized probe. These gel-filtered platelets were resuspended in platelet-poor plasma and pretreated without or with either amiloride (200 μM final concentration; a specific blocker of Na^+/H^+ exchange [17]) or quinacrine (150 μM final concentration; a specific blocker of phospholipase A_2 [18]) for 15 min before exposure to either cholesterol-poor, cholesterol-normal or cholesterol-rich liposomes at 37°C for up to 5 h. From these experiments, five types of platelet preparations (characterized by their membrane C/P ratio, ranging from 0.15–1.2) were selected and subsequently gel-filtered again on a Sepharose 2B column equilibrated with HEPES buffer, pH 7.35, and fluorescence was measured at an emission wavelength of 518 nm corresponding to maximal 6-carboxy-fluorescein pH sensitivity [19]. An aliquot of cells was taken immediately prior to stimulation, the platelets centrifuged, and the fluorescence of the supernatant measured and subtracted from the cell suspension to correct for the presence of extracellular dye. Intracellular pH was determined from the calibration curve obtained with and without platelets.

3. RESULTS

Enrichment or depletion experiments employing liposome technology revealed that acquisition or depletion of cholesterol from platelets was highly selective and that there was no significant change in the phospholipid or protein content per platelet (Fig. 1) Further, when the cholesterol-enriched platelets were exposed to ADP (0–15 μM), epinephrine (0–15 μM) and collagen (0–5 μg/ml), there was a dose-dependent increase in ATP secretion by these platelets indicating hypersensitivity to these aggregating agents (Fig. 2) These results are in agreement with those reported by other investigators regarding the sensitivity of cholesterol-enriched platelets [4]. Based upon these results, five types of platelet preparations, characterized by their membrane C/P ratio (ranging from 0.15–1.2 M; (Fig. 1) were employed

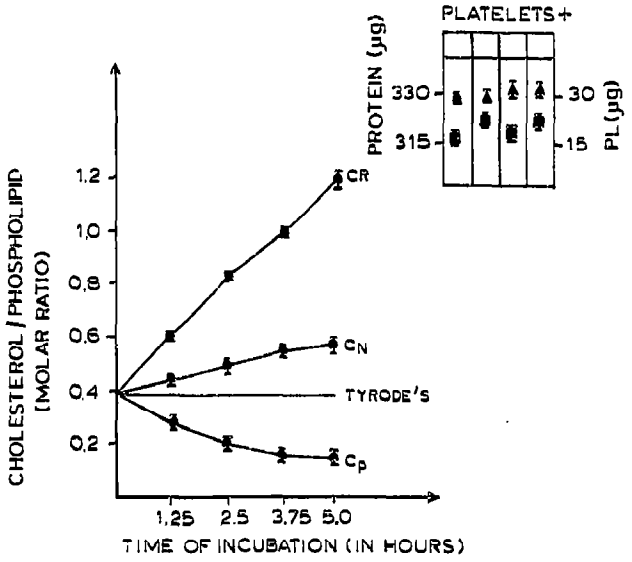

Fig. 1. Effect of incubation of platelets with cholesterol-poor (C_P), cholesterol-normal (C_N) and cholesterol-rich (C_R) liposomes in Tyrode's solution on platelet cholesterol/phospholipid ratio and (inset) phospholipid (▲) and protein (■) content. Values represent mean of 10 subjects ± S.D.

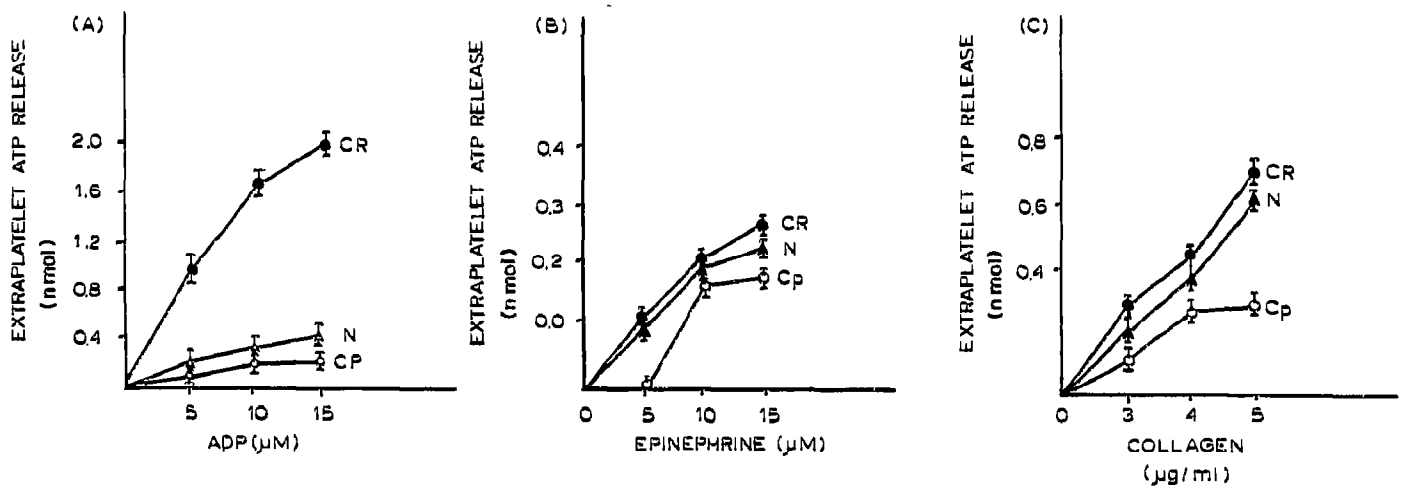

Fig. 2. Effect of different concentrations of agonists on ATP release by cholesterol-enriched platelets having a membrane cholesterol-to-phospholipid ratio of 1.2 (A) ADP. (B) Epinephrine. (C) Collagen. Each value represents mean ± S.D. of 10 subjects. Cr, cholesterol-rich; N, cholesterol-normal; Cp, cholesterol-poor.

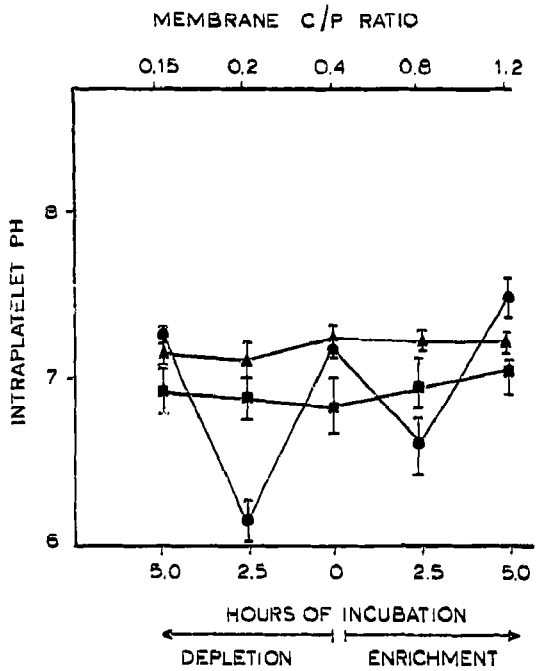

Fig. 3. Plot of intra-platelet pH as a function of platelet membrane cholesterol-to-phospholipid ratio, ranging from 0.15–1.2 (selected from Fig. 1). ● denotes platelets without pretreatment with quinacrine and amiloride; ▲ and ■ denote platelets pretreated with quinacrine and amiloride, respectively. Each point represents mean ± S.D. of experiments done in triplicate.

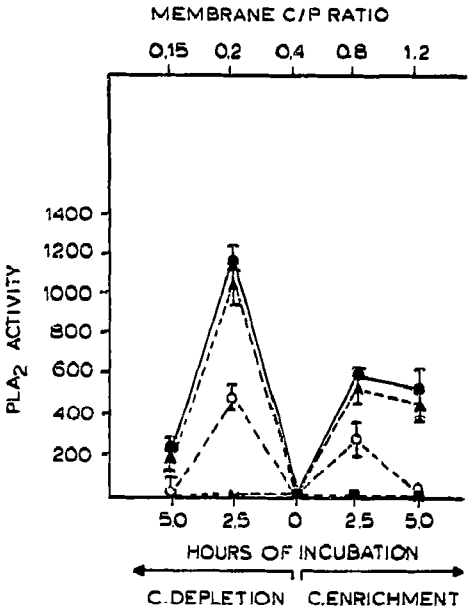

Fig. 4. Plot of phospholipase A_2 (PLA_2) activity as a function of platelet membrane cholesterol-to-phospholipid molar ratio, ranging from 0.15–1.2 (selected from Fig. 1). ● denotes platelets without pretreatment with quinacrine, amiloride and EGTA; ○, ▲ and ■ denote platelets pretreated with quinacrine, amiloride and EGTA, respectively. Each point represents mean ± S.D. of experiments done in triplicate.

to examine the intra-platelet pH, as well as phospholipase A_2 activity, in the presence or absence of quinacrine (a specific inhibitor of phospholipase A_2) or amiloride (a specific inhibitor of Na^+/H^+ exchangers). The results of such a study (Figs. 3 and 4) revealed that, as compared to normal platelets, platelets having; (i) a C/P ratio of 0.2 or 0.8 exhibited a significant increase in phospholipase A_2 activity accompanied by a significant decrease in the intracellular pH; (ii) a C/P ratio of 0.15 showed a slight increase in the phospholipase A_2 activity coupled with nosignificant change in intra-platelet pH; (iii) a C/P ratio of 1.2 showed a significant increase in phospholipase A_2 activity, as well as a slight increase in intra-platelet pH. However, exposure of platelets (pretreated with quinacrine) to cholesterol-poor, cholesterol-normal or cholesterol-rich liposomes for up to 5 h did not result in any significant change in either phospholipase A_2 activity or intra-platelet pH as compared to values found in normal platelets. Further, exposure of platelets (pretreated with amiloride) to cholesterol-poor, cholesterol-normal or cholesterol-rich liposomes for up to 5 h resulted in: (i) no significant change in the intra-platelet pH with respect to values found in normal platelets although the cytoplasmic pH shifted slightly towards the acidic region in all the five types of platelet preparations examined; and (ii) no noticeable effect on the phospholipase A_2 activity stimulated by membrane cholesterol variation. Further, in order to confirm the dose-dependent efficacy of quinacrine on phospholipase A_2 activity, the effect of various concentrations of quinacrine or EGTA upon phospholipase A_2 activity was also studied and the results unambigously depicted that membrane cholesterol-modulated phospholipase A_2 activity was responsible for the observed changes in the cytoplasmic pH (Fig. 5).

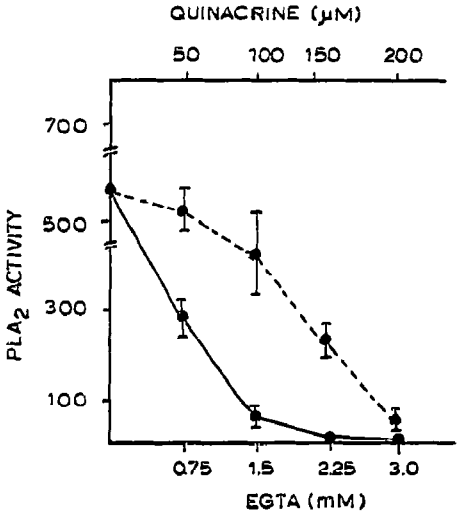

Fig. 5. Effect of different concentrations of quinacrine and EGTA on phospholipase A_2 activity (PLA_2) of cholesterol-enriched platelets having a cholesterol-to-phospholipid ratio of 1.2.●---● and ●—● denote quinacrine and EGTA, respectively. Each value represents mean ± S.D. of experiments done in triplicate.

4. DISCUSSION

Patients with familial hypercholesterolemia have elevated levels of LDL and reduced plasma concentrations of HDL [20,21]. Platelet activity in these patients is elevated and this is evident with regard to platelet adhesion, aggregation, serotonin release and reduced wholeblood bleeding time [20,22,23]. Since platelets are cells without nuclei, the source of their cholesterol must be either the parent megakaryocyte or the plasma, through interaction of lipoproteins that transport cholesterol. It is in this context that the results reported here may be of some importance in understanding the role of membrane cholesterol in platelet activity in general and the hypersensitivity phenomenon in particular. The results reported here clearly indicate that the 'Molecular-switch' through which membrane cholesterol modulates Na^+/H^+ exchange within platelets is phospholipase A_2, and the 'saw-tooth' response curves obtained in the case of Na^+/H^+ exchange, as well as phospholipase A_2 activity as a function of increased or decreased membrane cholesterol content within the platelets, may be arising due to competition between cholesterol and calcium at the calcium-binding site of the phospholipase A_2 molecule. Based upon these results it appears that activation of phospholipase A_2 activity results in accumulation of intra-platelet protons [24], leading to a decreased cytoplasmic pH which may in turn be responsible for the activation of Na^+/H^+ exchangers. These results may also explain why platelets from hypercholesterolemic patients are sensitive to various aggregating agents, because most of these agonists are known to interfere directly or indirectly with Na^+/H^+ exchange. In conclusion we suggest that membrane cholesterol-modulated phospholipase A_2 activity may act as a molecular-switch for the regulation of Na^+/H^+ exchange in cholesterol-enriched platelets. This hypothesis is in agreement with the reported findings that; (i) amiloride-sensitive Na^+ uptake in fibroblasts was found to be stimulated by melittin, which is known to activate phospholipase A_2 [25,26]; and (ii) stimulation of Na^+/H^+ exchange was also reduced by long incubation with dexamethasone, which is known to induce synthesis of lipomodulin, an intrinsic phospholipase inhibitor [25].

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