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THE EFFECTS OF LYSOPHOSPHATIDYLCHOLINE AND RELATED AMPHIPHILES ON PLATELET CYTOSKELETAL ASSEMBLY

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The effects of lysoPC, four other amphiphiles containing a linear 16 carbon alkane tail and chlorpromazine on platelet cytoskeletal assembly were compared. LysoPC and nonmetabolized amphiphiles all caused time-dependent inhibition followed by potentiation of thrombin-induced aggregation, serotonin secretion and cytoskeletal assembly in gel-filtered platelets, a result which ruled out hydrolysis of the amphiphiles as the mechanism of the time dependence. Hexadecanesulfonate was superior as a potentiator and cetyltrimethyl ammonium bromide (CTAB) was a better inhibitor. On the contrary, inhibition of platelet activation by arachidonate was not effected in a time-dependent manner and the actin-crosslinking proteins, actin-binding protein and myosin, were selectively prevented from incorporation into cytoskeletal cores, although protein phosphorylation and actin polymerization still occurred. Chlorpromazine also showed this selective inhibition of cytoskeletal assembly. LysoPC at concentrations which have been reported to cause development of filopodia did increase slightly the amount of actin present in Triton X-100-insoluble cores but not protein phosphorylation or incorporation of actin-crosslinking proteins. The effective concentrations of lysoPC and chlorpromazine can be predicted from the Meyer-Overton-Mullins rule of anesthesia which indicates their general effectiveness, but their specific effects only partially overlap.

Introduction

During platelet activation, phospholipid metabolism is markedly enhanced. Investigations on this metabolism have centered on the liberation of arachidonic acid (reviewed in Ref. 1) and the importance of the products of arachidonic acid oxidation to platelet activation is well-established. The arachidonic acid consumed in this metabolism is released from several phospholipid pools by a complex set of reactions which has complicated the assessment of the relative contributions of each pool [2,3]. Two intermediates of the phosphatidylinositol cycle serve as sources of arachidonic acid. Diacylglycerol is hydrolyzed by a lipase [4] and phosphatidic acid is cleaved by specific phospholipase A_2 [5]. Two other phospholipase A_2 enzymes liberate arachidonic acid from phosphatidylinositol [6] and phosphatidylcholine [7], respectively. However, products of phospholipid hydrolysis other than arachidonic acid have active roles in platelet activation. For example, diacylglycerol has been has been implicated in activation of protein kinase C [8]. In fact, the major, measurable byproduct of this arachidonate liberation is lysoPC [9] which can alter platelet

Abbreviations: CTAB, cetyltrimethyl ammonium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; lysoPC, lysophosphatidylcholine.

response, but its function in platelet activation remains unclear. In addition to this endogenously produced lysoPC, Cucuianu [10] has argued that the plasma concentrations of lysoPC are sufficient to alter platelet function.

Joist et al. [11] have extended the studies of the effects of lysoPC on platelet function. They reported the effect was time-dependent, showing both inhibition and potentiation. The later development of the potentiation partly paralleled the hydrolysis of lysoPC and for this reason, they thought the potentiation was produced by the saturated fatty acids derived from the lysoPC. Cucuianu [10], who has continued these investigations, states that plasma lysoPC concentrations range between potentiation and inhibition of platelets with larger deviations in this range in certain disease states. More recently, Kanaho and Fujii [12] have examined the morphological changes induced in platelets by two classes of amphiphiles where the classification was derived from the differences produced in red blood cell morphology. LysoPC and lauric acid transformed normal platelets into spiny discs and spheres with unusually slender filopodia. Neither the time dependency nor the possibility that the morphological changes were produced by the fatty acids from lysoPC catabolism were examined. Chlorpromazine, which represented the second class of amphiphiles, induced swelling and smooth sphere formation but no filopodia.

During normal platelet activation, the development of filopodia depends on the assembly of a cytoskeleton composed primarily of actin filaments associated with actin-binding protein and an alpha-actinin-like protein [13]. In this study, we examined the effects of lysoPC on the assembly of the cytoskeleton before and after activation. To rule out the possibility that results were produced by the saturated fatty acids derived from lysoPC during its metabolism, several closely related, synthetic compounds that would not produce fatty acids were included for comparison.

Methods

The materials used in this study were purchased from the following sources: lysoPC (Avanti Biochemicals); [^{14}C]serotonin (Amersham); [^{32}P]orthophosphate (New England Nuclear); arachidonic acid (NuChek Prep); Hepes, EDTA, EGTA, Tris, Triton X-100, ATP and bovine serum albumin (Sigma); sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide and protein molecular weight standard (Bio-Rad); CTAB and sodium hexadecanesulfonate (Aldrich); and hexadecylsulfobetaine (Calbiochem Behring). CTAB and sodium hexadecanesulfonate were recrystallized from ethanol.

Platelet preparation and labelling with [^{32}P]orthophosphate or [^{14}C]serotonin. Whole blood was freshly drawn from the antecubital vein of volunteers after obtaining informed consent. Platelets were isolated from citrate anticoagulated whole blood as previously described [13]. Platelets suspended at a concentration of $1 \cdot 10^9$/ml in buffer 1 (145 mM sodium chloride/5 mM potassium chloride/0.1 mM magnesium chloride/0.05 mM calcium chloride/5.5 mM glucose/1 mg/ml bovine serum albumin/15 mM Hepes (pH 7.4)) were labeled for 45 min at 37 °C with either 1 mCi/ml [^{32}P]orthophosphate or 0.05 μCi/ml [^{14}C]serotonin. The platelets were then gel filtered at 4 °C on a Sepharose 2B column with bed volume 10-times the applied sample volume. The platelets were collected, counted on a hemocytometer and adjusted to $5 \cdot 10^8$ platelets/ml with buffer 1. After 30 min. at 37 °C, the platelets completely recovered their discoid shape and were utilized in the experiments described in this study. Aggregation and secretion were monitored as detailed previously [13].

Secretion. Secretion of [^{14}C]serotonin from platelets labeled as described above was measured on 0.05 ml time-course aliquots in which secretion was stopped by mixing with 10 vol. 50 mM sodium phosphate (pH 7.4)/0.2% glutaraldehyde. After removing the platelets by centrifugation at 8000 × *g* for 1 min, aliquots of the supernatants were counted for [^{14}C]serotonin. Percent release was calculated by subtracting zero-time values for [^{14}C]serotonin (less than 10% of total values) from the time-course values and dividing these corrected numbers by the total amount in the 0.05 ml platelet aliquot after subtraction of zero-time values.

Phosphorylation. Phosphorylation of platelet actin-binding protein, myosin 20 kDa light chains, and a 40 kDa protein was monitored with time.

Although referred to in this and previous papers as a 40 kDa polypeptide, the actual molecular mass has been estimated at around 47 000 by Lyons and Atherton [14]. This polypeptide migrates anomalously in the electrophoresis system employed in this study and is usually located in the leading or trailing edge of the actin band. Aliquots (0.05 ml) withdrawn during the experimental time course were immediately denatured for 3 min at 100 °C after adding 0.025 ml of a 3-fold concentrated denaturing buffer consisting of 6% SDS/6% 2-mercaptoethanol/30% glycerol/3 mM EDTA/12 mM EGTA/0.03% Bromophenol blue/150 mM Tris-HCl (pH 6.8). These aliquots were stored at −20 °C and reheated for 2 min at 100 °C before electrophoresis. Electrophoresis on 6–17.5% polyacrylamide gradient gels was performed as described by Studier [15]. After soaking the gels for 30 min at 60 °C in 10% trichlororacetic acid, the gels were subsequently fix-stained with Coomassie blue, destained, dried, autoradiographed and the ^{32}P cpm/gel band was quantified as previously described [16].

Cytoskeletal core isolation and quantification. Isolation of platelet cytoskeletal cores by extraction with 1% Triton X-100 with 5 mM EGTA present, was performed according to the procedure of Phillips et al. [17] with slight modifications [16]. Cytoskeletal cores were denatured for electrophoresis with a volume-denaturing buffer equivalent to the original platelet suspension volume and 0.04-ml aliquots were electrophoresed as described above. Supernatant fractions which were diluted 2-fold by the extraction buffer were denatured by the addition of 1/5 vol. 5-fold concentrated denaturing buffer and 0.1-ml aliquots, equivalent to 0.04 ml of the original platelet suspension, were electrophoresed as described above. After fix-staining and destaining, the cytoskeletal core and supernatant lanes were scanned on a LKB UltroScan Laser Densitometer in order to quantify protein distribution between the fractions.

Amphiphile assays. The concentrations of the hexadecanesulfonate in platelets and their supernates were estimated by the partitioning of the anionic detergent-cationic methylene blue salt into chloroform [18]. The counterpart assay of CTAB as cationic CTAB-Bromophenol blue salt was not possible because of the low recoveries of CTAB from the albumin-containing buffer solutions and from the platelet pellets. The assay for hexadecanesulfonate is described briefly as follows. After incubation of platelet suspension with amphiphile, the mixture was centrifugally separated for 5 min at approx. $1000 \times g$. To 1 ml of supernate was added 0.7 ml chloroform, 1 ml of H_2O and 25 µl of a solution of 1 g methylene blue in 1 l 0.4 mM HCl. The platelet pellet was extracted with 0.7 ml chloroform and 0.35 ml methanol before addition of 2 ml H_2O and the methylene blue. The cloudy chloroform layers were recentrifuged for 3 min at $5000 \times g$. Standards in water were similarly extracted and platelet pellets containing no hexadecanesulfonate were also extracted to correct for endogenous negatively charged amphiphiles that would cary dye into the organic phase. The methylene blue transferred to the chloroform was monitored at 655 nm.

Results

Similar to what had been previously reported for lysoPC, hexadecanesulfonate and CTAB displayed a complex pattern of effects on thrombin-induced activation of gel-filtered platelets. The potentiations and inhibitions were complicated

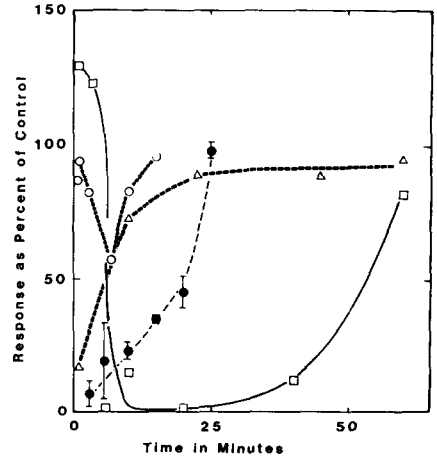


Fig. 1. Comparison of the effect of CTAB and hexadecanesulfonate on thrombin-induced activation of gel-filtered platelets. Gel-filtered platelets were incubated at 37 °C with amphiphile for the designated time period before challenge with 0.2 U/ml thrombin or 0.25 U/ml thrombin with lysoPC. The effects on aggregation are designated thus: 2.5 µM CTAB (○), 5 µM CTAB (△), 30 µM hexadecanesulfonate (□) and 60 µM lysoPC (●).

functions of time (Fig. 1), when these compounds were incubated with platelets for various time periods before challenge with thrombin. Hexadecanesulfonate was superior at the initial potentiation and appeared to require a longer period of incubation before expressing an inhibitory effect even at higher doses. As the concentrations of the amphiphiles were increased, the inhibition was expressed earlier and the adjustment period, during which the platelets partially recovered their activity, was extended. Recovery and potentiation of secretion often paralleled that of aggregation, but in some platelet preparations, secretion remained depressed. In the experiment depicted in Fig. 1, after the 12-min incubation with 2.5 and 5 μM CTAB, platelet secretion had not only recovered but was stimulated to over 100% of the control value. In contrast to these marked changes in the effects of the amphiphiles with time on the thrombin-induced activation, the time dependence of inhibition by CTAB of arachidonate-initiated aggregation was much less apparent (not shown). The relative inhibition by selected amphiphiles were compared with platelets challenged with 10 μM arachidonate after a 1-min incubation with inhibitor. Hexadecylsulfobetaine and CTAB were more effective than chlorpromazine and lysoPC, followed by hexadecanesulfonate. CTAB was about three-times more potent than lysoPC.

Neither CTAB nor hexadecanesulfonate could be hydrolyzed to fatty acids, the products suggested by Joist et al. [11] as the mediators of potentiation. However, the recovery of activity with time might still be metabolic. Both of these detergents are readily assayed in aqueous solutions based on their ability to transfer into an organic phase when paired with an organic dye of opposite charge. Albumin and platelets prevented the assay of CTAB, but hexadecanesulfonate was assayed. Platelet suspensions incubated with 30 μM hexadecanesulfonate for 3 and 45 min at 37°C were centrifuged and the supernates and pellets were assayed separately. The amount of detergent in the supernate, was 90.7 $\pm$ and 86.8 $\pm$ 4.4%, respectively, of the total amount added for the 3 and 45 min samples. The platelet pellets from both samples contained 7.3 + 0.4 and 8.0 + 1.0%, respectively, of the total hexadecanesulfonate after correction for some dye binding by platelets com-

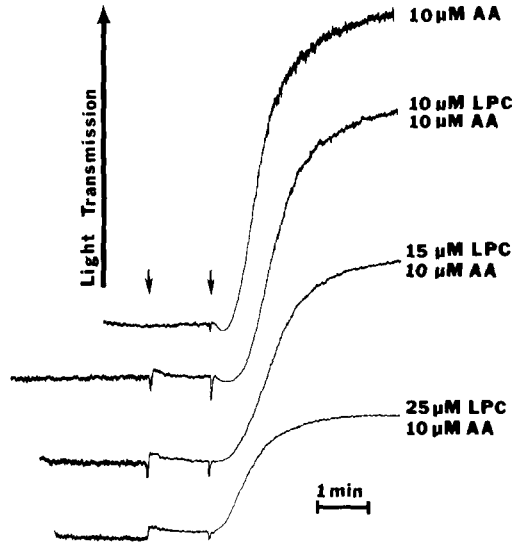


Fig. 2. Aggregometer tracings of gel-filtered platelets. Starting from the top, platelets were activated with 10 μM arachidonate (AA) (added at second arrow) with prior addition of 0, 10, 15, or 25 μM lysoPC (LPC) (added at the first arrow). A 1-min time interval is indicated by the bar.

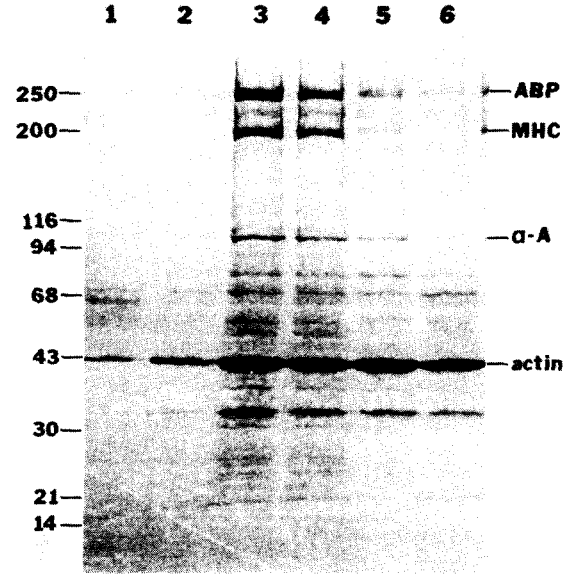


Fig. 3. The effect of increasing concentration of lysoPC on cytoskeletal development. SDS-polyacrylamide gel electrophoresis of cytoskeleta cores isolated from equivalent numbers of platelets after: lane 1, no treatment; lane, 2, 1 min with 25 μM lysoPC; or pretreated 1 min with the indicated concentrations of lysoPC before activation for 1 min with 10 μM arachidonate; lane 3, no lysoPC; lane 4, 10 μM lysoPC; lane 5, 15 μM lysoPC, and lane 6, 25 μM lysoPC. Cytoskeletal cores were isolated and denatured as described in Methods. ABP, actin-binding protein; MHC, myosin heavy chain.

ponents. The similarity in the values for the 3 and 45 min samples suggests that metabolism of the antagonists is not the reason for the time dependency of inhibition and recovery of platelet function.

The effects of CTAB and lysoPC on cytoskeletal assembly were examined next. After activation of untreated platelets, a stable cytoskeletal core was isolatable from Triton X-100-lysed platelets as has been demonstrated before [17]. Increasing dose of lysoPC reduced not only the extent of aggregation (Fig. 2) but the amount of actin-binding protein and myosin heavy chain incorporated into the cytoskeleton (Fig. 3). At concentrations that blocked aggregation almost completely, these proteins were also prevented from being incorporated into the cores. In contrast, actin and to a lesser extent a 34 kDa peptide (possibly platelet tropomyosin) remained in the cores at these same inhibitory concentrations of lysoPC. The incorporation of actin in the core was dependent on platelet activation, since only a small increase in actin in the core was noted after treatment with lysoPC (comparison between control, lane 1, and treated, unactivated sample, lane 2). The effect of the other amphiphiles on the incorporation of the crosslinking proteins (actin-binding protein and myosin heavy chain) into cytoskeletal cores were quantified (Table I). Little cytoskeletal core was detectable in the control platelets or those treated with any of the amphiphiles. After activation, those samples in which aggregation was blocked also showed a near complete inhibition of the increase in actin-binding protein and myosin heavy chain in the core as compared to the activated control. Much more of the actin

TABLE I

EFFECTS OF AMPHIPHILES ON INCORPORATION OF CYTOSKELETAL PROTEINS INTO CORES

The area under the densitometric tracing of the bands for each protein incorporated into the activated control core was arbitrarily assigned as 100%. The areas were linear with a 4-fold reduction in the amount of control sample applied to the gel and a standard deviation of ±7% was obtained for replicate samples. The dashes represent percent areas below an estimated level of significance which was 6, 4 and 10 for actin-binding protein, myosin heavy chain and 34 kDa peptide, respectively. The samples and their respective controls were activated with either 10 μM arachidonate or 0.25 U/ml thrombin. Unless otherwise specified, the amphiphiles were incubated for 2 min with the platelets before sampling or activation.

Sample	Percentage incorporation compared to activated control			
	Actin	Actin-binding protein	Myosin heavy chain	34 kDa peptide
Unactivated control	15	–	–	–
15 μM CTAB	16	–	–	–
+ arachidonate	60	12	8	48
30 μM lysoPC	27	–	–	22
+ arachidonate	48	17	5	42
30 μM hexadecanesulfonate	10	–	–	–
+ arachidonate	104	96	80	102
25 μM chlorpromazine	14	–	–	12
+ arachidonate	67	22	12	52
25 μM CTAB + thrombin	68	54	58	60
25 μM CTAB [a] + thrombin	90	91	101	73

[a] This sample was incubated for 30 min at 37 °C with CTAB before activation. To the sample above, the amphiphile was added only 2 min before challenge, similar to all the other preceding samples.

and 34 kDa peptide were retained in treated cores. Hexadecanesulfonate in this series of experiments showed a slight enhancement of aggregation but little effect on the incorporation of the proteins into the cytoskeletal core.

In thrombin-activated platelets, CTAB did not prevent selectively the incorporation of actin-binding protein and myosin heavy chain into the activated cores (Table I). Furthermore, after the platelets recovered partially from the CTAB treatment with respect to thrombin activation, the full assembly of the cytoskeletal cores was nearly restored. This finding would appear to rule out a direct effect of the amphiphiles on the proteins, since the inhibition persisted with respect to arachidonate activation (not shown). Therefore, amphiphiles appeared to affect an activation process, and protein phosphorylation of these cytoskeletal proteins was considered a prime candidate.

However, protein phosphorylation was almost as intense after arachidonate activation, despite near complete inhibition by lysoPC of aggregation (Fig. 4). The prevention of aggregation alone (even with EGTA) prevents dephosphorylation [16] and is not a specific effect of lysoPC (compare also Fig. 4a and c). The maximum phosphorylation whether or not lysoPC is present indicates that lysoPC is not inhibitory. This lack of effect was most evident with phosphorylation of the 40 kDA protein but more importantly, the same extensive phosphorylation of actin-binding protein and myosin light chain, both normally incorporated into the cytoskeletal cores, was detected (Fig. 5). Nor was the phosphorylation of these proteins initiated by levels of lysoPC that caused shape change. Therefore, the platelets were responding to the agonists but certain processes other than phosphorylation were blocked selectively.

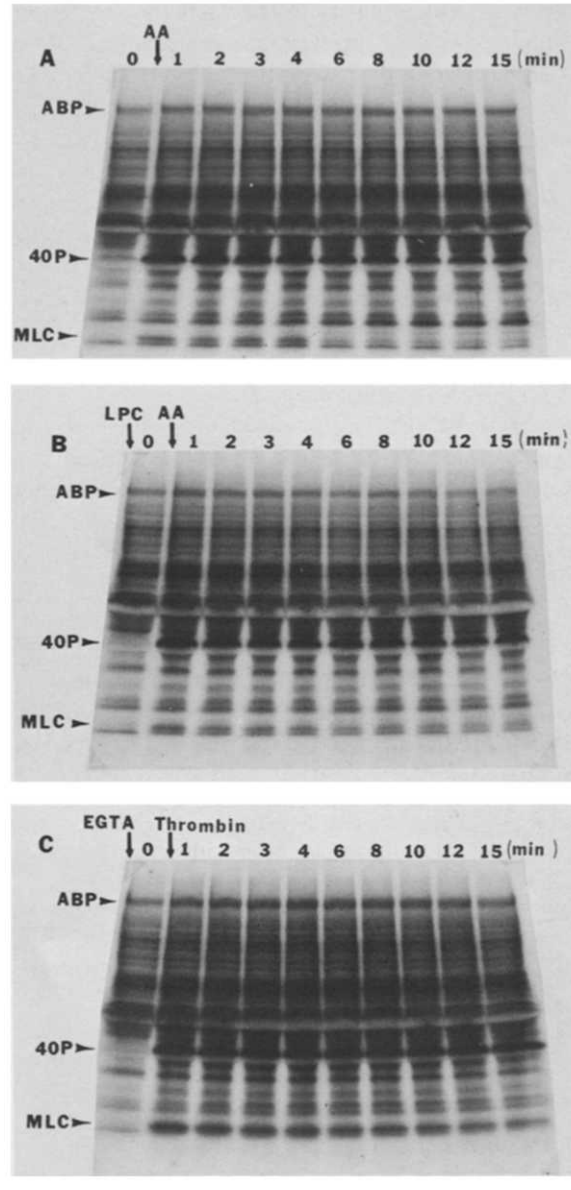
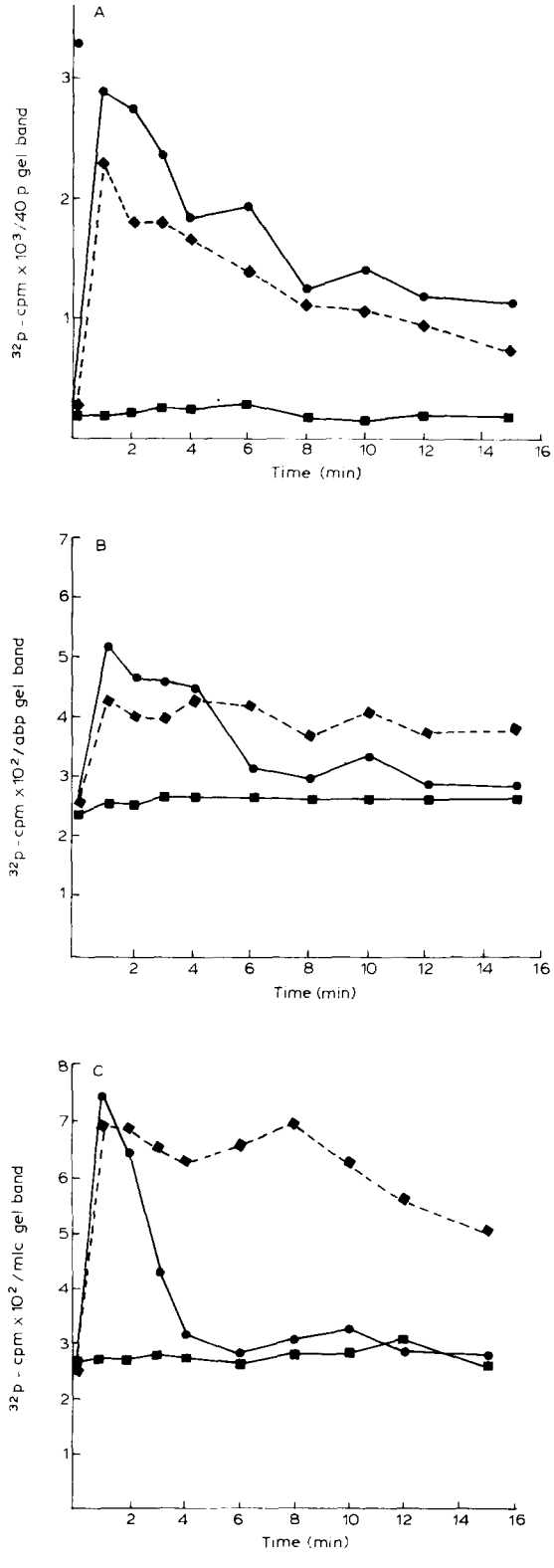


Fig. 4. Autoradiograms of the time-course of protein phosphorylation after platelet activation. The radiolabeling of the platelet nucleotides, the processing of the samples taken at the indicated times after activation, the electrophoresis of the samples in polyacrylamide gradients in the presence of SDS and mercaptoethanol, and the autoradiography of these gels are described in Methods. The time-courses are (A) after activation with 10 μM arachidonate, (B) pretreatment for 2 min with 25 μM lysoPC before activation with 10 μM arachidonate and (C) pretreatment for 2 min with 5 mM EGTA before activation with 1 U/ml thrombin. The positions of actin-binding protein (ABP), the phosphorylated 40 kDA protein (40P) and myosin light chain (MLC) are marked. Other abbreviations are the same as Fig. 2.

Discussion

The time dependence of the effect of lysoPC was previously attributed to the hydrolysis of lysoPC to free fatty acids which in turn produced the potentiation [11]. This seems unlikely, since similar results were obtained with CTAB and hexadecanesulfonate. These compounds could pro-



voke slow processes that produced the time-dependent effects, but diffusion of the amphiphiles into different areas within the cell [19] would be an alternate explanation. Such an explanation could account for the faster action of CTAB whose quaternary amine headgroup would be more soluble than the sulfonate ion in the hydrophobic core of the membrane, thus increasing its transmembrane diffusion rate. A similar explanation has been given for the differences between ionizable and quaternary amine derivatives of local anesthetics, where the ionizable amine is the more rapid diffusable moiety [20]. Furthermore, ethyl lidocaine, a quaternary amine, but not its tertiary amine derivative, lidocaine, produces a time-dependent effect on platelets [21].

As mentioned earlier, lysoPC causes the development of abnormally slender filopodia [12]. These filopodia are not only morphologically distinct from the filopodia normally produced during platelet activation, but they are not associated with actin-binding protein-crosslinked action filaments. In fact, lysoPC prevented this type of cytoskeletal assembly after activation induced by arachidonate. However, lysoPC did cause a small increase in the amount actin incorporated into the core of non-activated platelets (Table I), whereas hexadecanesulfonate and chlorpromazine, at concentrations that caused shape change, did not cause an increase in core actin. Whether this small increase in core actin is related to the slender filopodia was not resolved in this study.

Inhibition by the amphiphiles is unrelated to shape change. This point was first made by Joist et al. [11]. In our experiments with 10 μM

Fig. 5. The Effect of lysoPC on protein phosphorylation induced by arachidonic acid. Phosphorylation time-courses of (A) 40 kDa protein (40P), (B) actin-binding protein (ABP) and (C) myosin light chain (MLC) in platelets treated with 25 μM lysoPC (■), pretreated 2 min with 25 μM lysoPC before activation by 10 μM arachidonic acid (◆) or activated by 10 μM arachidonic acid without lysoPC (●). Incorporation of ^{32}P cmp/gel band indicated on the left axis was determined as described in Methods for aliquots taken at the times indicated on the bottom axis. Duplicate runs displayed less than 10% variation. In five separate experiments the time of the peak in radioactivity after arachidonic acid activation varied less than 2 min. Samples with lysoPC alone remained at basal levels ($\pm$10%) throughout the time-course.

arachidonate, shape change was often associated with lysoPC inhibition. However, inhibition of aggregation did not depend on shape change in the experiments of lysoPC inhibition of activation at lower doses of arachidonate (not shown) or CTAB blockade of 10 μM arachidonate activation. Furthermore, shape change did not assure inhibition, since 30 μM hexadecanesulfonate induced shape change while producing some potentiation.

The inhibition was markedly selective for the crosslinking of the actin filaments and was unrelated to protein phosphorylation. Myosin incorporation appeared to be most sensitive. However, this selectivity was not readily apparent with thrombin. Therefore, thrombin has a secondary activation route for governing the crosslinking reactions. This secondary process would appear to be the one that is affected in a time-dependent manner, since inhibition of arachidonate activation was nearly time independent.

Most amphiphiles, including detergents, fit the definition of anesthetics and anesthetics are known to affect other tissues beside neural tissues, all at comparable concentrations (reviewed in Ref. 22). The Meyer-Overton-Mullins rule of anesthesia, that basically states the efficacy of an anesthetic depends on the volume occupancy of the amphiphile, indicates the generality of the effect. This volume occupancy, and hence the efficacy of anesthesia, correlates with many changes in membrane properties, including protection against osmotic hemolysis of erythrocytes. From this latter property, effective doses for lysoPC and chlorpromazine are predicted as 25 and 10 μM, respectively [22]. The similarity of these predicted values and the effective concentrations determined here conform with the generality of amphiphilic inhibition of platelet activation that has been reported previously [18]. Kanaho et al. [24] have reached a similar conclusion about the amphiphilic inhibition of platelets.

Underlying the generality of the amphiphilic response is a selectivity of effects on the morphology directly as well as cellular responses. Thus, the reported difference in the morphological effect of the amphiphiles, lysoPC producing filopodia while chlorpromazine merely rounds platelets [12], fall into the selective effects, whereas during inhibition of arachidonate activation by an amphiphile, the disruption of the cytoskeletal assembly appeared to be a general effect, since chlorpromazine showed the same pattern of cytoskeletal disruption as lysoPC.

The complexity of the effects of lysoPC on platelet function including time-dependent potentiation and inhibition of activation, prevents any prediction on the role of endogenously produced lysoPC. Furthermore, the general effectiveness of amphiphiles in modifying platelet responses suggests that other phospholipid metabolic intermediates of platelet activation may also have important modifying functions in the different platelet processes. These modifying functions can be selective not only for the process affected but for type of agonist, as indicated by the selective disruption by amphiphiles of the cytoskeletal assembly induced by arachidonate but not by thrombin.

Acknowledgements

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References

1 Carroll, R.C. and Cox, A.C. (1983) Surv. Synth. Path. Res. 2, 21–33
2 Hofmann, S.L. and Majerus, P.W. (1982) J. Biol. Chem. 257, 14359–14364
3 Lapetina, E. (1982) Trends Pharmacol. Sci. 3, 115–118
4 Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Proc. Natl. Acad. Sci. USA 76, 3238–3241
5 Billah, M.M., Lapetina, E.G. and Cautrecasas, P. (1981) J. Biol. Chem. 256, 5399–5403
6 Lapetina, E.G., Billah, M.M. and Cautrecasas, P. (1981) J. Biol. Chem. 256, 11984–11987
7 Kannagi, R., Koigumi, K. and Masuda, T. (1981) J. Biol. Chem. 256, 1177–1184
8 Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 91, 1218–1224
9 McKean, M.L., Smith, J.B. and Silver, M.I. (1981) J. Biol. Chem. 256, 1522–1524
10 Cucuianu, M.P. (1983) Ann. N.Y. Acad. Sci. 414, 57–65
11 Joist, H.H., Dolezel, G., Cucuianu, M.P., Nishizawa, E.E. and Mustard, J.F. (1977) Blood 49, 101–112
12 Kanaho, Y. and Fujii, T. (1982) Biochem. Biophys. Res. Commun. 106, 513–519

13 Carroll, R.C., Butler, R.G., Morris, P.A. and Gerrard, J.M. (1982) Cell 30, 385–393
14 Lyons, R.M.N. and Atherton, R.M. (1979) Biochemistry 18, 544–552
15 Studier, J.W. (1973) J. Mol. Biol. 79, 237–248
16 Carroll, R.C. and Gerrard, J.M. (1982) Blood 59, 466–471
17 Phillips, D.R., Jennings, L.K. and Edwards, H.H. (1980) J. Cell Biol. 86, 77–86
18 Murkejee, P. (1956) Anal. Chem. 28, 870–873
19 Pagano, R.E., Longmuir, K.J., Martin, O.C. and Struck, D.K. (1981) J. Cell Biol. 91, 872–877
20 Ritchie, J.M., Ritchie, B. and Greengard, P. (1965) J. Pharmacol. Exp. Ther. 150, 152–159
21 Anderson, E.R., Foulks, J.G. and Godin, D.V. (1981) Thrombos. Haemostas. 45, 18–23
22 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
23 Jain, M.K., Eskow, D., Kuchibhotla, J. and Colman, R.W. (1978) Thrombos. Res. 13, 1067–1075
24 Kanaho, Y., Kometani, M., Sato, T. and Fujii, T. (1983) Thrombos. Res. 31, 817–831