How does fluoroaluminate activate human platelets?

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Platelet activation induced by NaF or fluoroaluminate (AlF_4^-) was studied. The latter has been described to substitute for the γ -phosphate group of the GTP molecule. With 10 mM-NaF, a concentration unable to induce any measurable Ca^{2+} mobilization (as measured with Indo 1), addition of AlCl_3 potentiated platelet aggregation, thromboxane synthesis, diacylglycerol formation and p43 phosphorylation, without any increase in intracellular Ca^{2+} . Neither phosphoinositide hydrolysis nor phosphatidic acid formation could be detected. AlF_4^- induced the release through a granule centralization within a microtubule bundle, although no myosin light-chain phosphorylation could be detected. Addition of flurbiprofen (10 μM) resulted in only partial inhibition of diacylglycerol formation, with no effect on the release reaction or on p43 phosphorylation. The present results suggest that AlF_4^- does not stimulate a G-protein governing the phosphoinositide-specific phospholipase C. The AlF_4^- -induced diacylglycerol formation is discussed. Moreover, these results bring evidence that there is no correlation between granule centralization and myosin light-chain phosphorylation.

INTRODUCTION

Human platelet responses to a number of physiological agonists occur through activation of the polyphosphoinositide specific phospholipase C. This generates the two messenger molecules, namely diacylglycerol (DAG), the protein kinase C activator, and InsP_3 , the intracellular Ca^{2+} -mobilizing agent (for review see Rendu *et al.*, 1987b). More recently, it has been proposed, although not yet demonstrated, that the effect of an agonist on the platelet surface could first involve a guanine-nucleotide-binding protein (G-protein) regulating the PtdInsP_2 -specific phospholipase C (Crouch & Lapetina, 1988; Brass *et al.*, 1988).

Until now the implication of G-proteins in human blood platelets was studied by using either permeabilized platelets (Haslam & Davidson, 1984; Shattil & Brass, 1987; Brass *et al.*, 1988) or isolated platelet membranes (O'Rourke *et al.*, 1987) and/or under stimulation with pertussis toxin, which ADP-ribosylates the G-protein. However, these approaches are limited for the understanding of physiological events required for platelet function. More precisely, the prominent role of α -granule adhesive proteins, which become expressed on the surface during the secretory process in intact platelets (Leung, 1984), can no longer be taken into account in permeabilized platelets. Pertussis toxin has itself recently been shown to activate platelets, and one has to be cautious in its use (Banga *et al.*, 1987).

Since the discovery that fluoride, in combination with AlCl_3 , activates adenylate cyclase and cyclic GMP phosphodiesterase, many reports have demonstrated that it can modulate G-proteins (Sternweis & Gilman, 1982; Bigay *et al.*, 1985; Kanaho *et al.*, 1985). Addition of AlCl_3 , at concentrations comparable with those present in the blood circulation (Sternweis & Gilman, 1982), to fluoride allows the formation of fluoroaluminate (AlF_4^-).

AlF_4^- substitutes for the γ -phosphate of GTP, and as a consequence activates G-proteins (Bigay *et al.*, 1985). Thus AlF_4^- represents a useful tool with which to study the involvement of G-proteins in the activation of intact platelets.

For this purpose, using AlF_4^- as the inducer, we studied thoroughly the transducing system of activation in intact human platelets, in parallel with the physiological responses. The present paper describes this stimulation and suggests another pathway than phosphoinositide metabolism.

MATERIALS AND METHODS

Materials

5-Hydroxy[^{14}C]tryptamine (CFA 170; 2 GBq/mmol) and $\text{H}_3^{32}\text{PO}_4$ (P32-S1; 0.37 GBq/ml) were from ORIS (CEA, Saclay, France). [U- ^{14}C]Arachidonic acid (NEC 756; 14.4 GBq/mmol) was from NEN (Boston, MA, U.S.A.). Metrizamide was from Nyegaard (Oslo, Norway). All other chemicals were of the purest grade available and were obtained commercially.

Preparation of platelets

Blood was anticoagulated with ACD-C (13 mM-citric acid, 12 mM-citrate, 11 mM-dextrose) and obtained from human volunteers who had not ingested any drug. Platelet-rich plasma (PRP) was obtained by centrifugation for 10 min at 100 g. PRP was incubated for 90 min with 5-hydroxy[^{14}C]tryptamine (0.6 μM) in order to follow the release, or with [^{32}P]P_i (37 MBq/10 ml of PRP) to measure phosphorylations, or with 0.2 μM -[^{14}C]arachidonic acid to measure DAG formation. At the end of incubation, platelets were isolated on metrizamide gradients as previously described (Rendu *et al.*, 1987a). The so-called 'metrizamide-gradient

Abbreviations used: AlF_4^- , fluoroaluminate; TX, thromboxane; DAG, diacylglycerol; PA, phosphatidic acid; PRP, platelet-rich plasma.

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platelets' obtained were resuspended in 140 mM-NaCl/3 mM-KCl/0.5 mM-MgCl₂/5 mM-NaHCO₃/10 mM-glucose/10 mM-Hepes, pH 7.35. Platelet suspensions were prewarmed for 5 min at 37 °C in the presence of saline (0.9 % NaCl) or NaF without stirring, and transferred to the cuvette of an aggregometer where saline or AlCl₃ (10 µM) was added. Aggregation was recorded, and at the desired time samples were transferred to ice-cold 0.1 M-EDTA for release analysis, or to mixtures of methanol/chloroform for phosphorylations or DAG measurements as described below.

Electron microscopy

Samples were transferred to the fixative (1.25 % glutaraldehyde in White's saline) and post-fixed in 1 % OsO₄. After fixation and washing, the samples were dehydrated and embedded in Epon 812 in accordance with standard methods. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL 1200 EX electron microscope.

Ca²⁺ mobilization

Cytoplasmic free Ca²⁺ was determined after addition of NaF or AlF₄⁻ to unstirred samples of washed platelet suspension. Platelets in PRP were loaded with 15 µM Indo-1 acetoxyethyl ester for 15 min at 37 °C, centrifuged at 1100 g for 10 min and resuspended in the same buffer as above. Metrizamide was omitted because of its interference with the fluorescence measurements. Fluorescence was measured in a Jobin-Yvon JY3D (Longjumeau, France) spectrofluorimeter (excitation at 331 nm, emission at 410 nm).

Release of granule constituents

Supernatants from 5-hydroxyl[¹⁴C]tryptamine-labelled samples were analysed for 5-hydroxytryptamine content by counting radioactivity of incorporated 5-hydroxyl[¹⁴C]tryptamine for β-thromboglobulin by using a radioimmunoassay kit (IM 88; Amersham International, Amersham, Bucks., U.K.), and for β-N-acetylglucosaminidase with p-nitrophenol as substrate (Dangelmaier & Holmsen, 1980). Thromboxane B₂ (TXB₂) was determined on a sample of ³²P-labelled platelets in the organic mixture before the phase partition, by enzyme immunoassay with acetylcholinesterase-TXB₂ as label (Pradelles *et al.*, 1985).

Phosphorylations

Protein and lipid phosphorylations were measured on samples as previously described (Rendu *et al.*, 1987a). Briefly, activation was stopped by the addition of 3.75 vol. of methanol/chloroform/12 M-HCl/0.1 M-EDTA (20:40:1:2, by vol.) to each ³²P-labelled sample. Chloroform and water (1.25 vol. of each) were added, followed by vigorous vortex-mixing.

The aqueous phase was discarded, proteins concentrated at the interphase were solubilized with 2 % SDS in 0.625 M-Tris/20 % (v/v) glycerol/0.01 % Bromophenol Blue and analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, with a 13 %-acrylamide resolving gel and 5 % stacking gel. After staining with Coomassie Blue, dried gels were exposed to Kodak XAR films and autoradiographs scanned with a LKB Ultrascan.

The lower chloroform phase was evaporated, washed as described by Jolles *et al.* (1981) and resuspended

in chloroform/methanol/water (75:25:2, by vol.). Phosphoinositides and phosphatidic acid (PA) were separated by t.l.c. on silica plates with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, by vol.) as the migration solvent. Individual [³²P]phospholipids were detected by autoradiography, recovered by scraping the identified spots and counted for radioactivity.

DAG measurements

Activation of [¹⁴C]arachidonic acid-labelled platelets was stopped by addition of 3.75 vol. of chloroform/methanol (1:2, v/v), and they were transferred in 1.37 vol. of chloroform/methanol (5:6, v/v). Extraction was performed in chloroform/0.2 % formic acid (1.25 vol. of each), three times. The organic phase was evaporated to dryness under N₂, then resuspended in chloroform with addition of diolein. DAG was separated by one-dimensional t.l.c. on silica plates with hexane/diethyl ether/formic acid (40:10:1, by vol.) as the migration solvent. After detection by iodine vapour, [¹⁴C]DAG was scraped off and analysed by scintillation counting.

Statistical analysis

The paired Student's *t* test was used to test the significance of the differences between the responses induced by AlF₄⁻ or by NaF.

RESULTS

Aggregation and release

At low concentrations (5–10 mM), NaF preincubated for 5 min with platelets without stirring induced only slight morphological changes (Fig. 1B) as compared with control platelets (Fig. 1A). Under stirring in the cuvette of the aggregometer, no aggregation was measured with 5 mM-NaF. Aggregation required at least 10 mM-NaF to occur, but with no additional shape change. Addition of 10 µM-AlCl₃, conditions in which the entity AlF₄⁻ is formed, resulted in either aggregation with a non-aggregating NaF concentration or increased aggregation. At 1 min after addition of AlCl₃, most of the granules were concentrated in the centre of the cell, but some were extruding their contents by direct exocytosis (Fig. 1C). Long pseudopods were emitted, and small aggregates consisting of two to three platelets were observed. Full aggregation was reached between 2 and 5 min (Fig. 1D), and the intensity of aggregation greatly differed from one donor to the other.

All platelet responses were measured in the same conditions, i.e. 5 min of preincubation of platelets with NaF before addition of saline or 10 µM-AlCl₃ in the cuvette of the aggregometer. No significant differences in the release of different granule constituents or in the TXB₂ synthesis were observed whether NaF was preincubated for 5 min or not. However, the potentiation by AlCl₃ was more evident when NaF had been preincubated for 5 min with platelets. This agrees with an early report on NaF-induced aggregation and release, which were shown to occur after a lag of at least 6 min (Mürer, 1968), the time required for NaF to penetrate the cell (Mürer *et al.*, 1981).

The release of the different granule constituents is shown in Table 1. No lysis of the platelets occurred, as monitored by the measurement of the cytosolic lactate

Fig. 1. Aggregation curves and electron micrographs of human platelets in presence of NaF or AlF_4^-

Curve 1, 5 mM-NaF; curve 2, 5 mM-NaF + 10 μM - AlCl_3 ; curve 3, 10 mM-NaF + 10 μM - AlCl_3 . A: Control 'metrizamide-gradient' platelets. B: Platelets fixed after 5 min of incubation at 37 °C without stirring in presence of 10 mM-NaF. C: Platelets preincubated with 5 mM-NaF and stirred for 1 min in the cuvette of the aggregometer in presence of 10 μM - AlCl_3 . Granules are either centralized within the microtubule bundle ($\downarrow$), or in contact with the plasma membrane ($\nearrow$) or with the open canalicular system ($\nwarrow$). Long pseudopods are emitted. Great vacuoles can be seen, some of which contain a granule (∇). D: Platelet aggregate obtained 5 min after addition of AlCl_3 . Bars = 1 μM .

Table 1. Platelet release reaction measured in presence of NaF or AlF_4^-

Platelet lysis (cytosolic lactate dehydrogenase: LDH) and release from dense bodies (5-hydroxy[^{14}C]tryptamine: 5-HT), α -granules (β -thromboglobulin: βTG), and lysosomes (β -*N*-acetylglucosaminidase: NAG) were measured 2 min after addition of $10\ \mu\text{M}$ - AlCl_3 or saline. Results are expressed as a percentage of total platelet content, and are the means of 7–20 experiments. ^a $P < 0.01$ (very significant), ^b $P < 0.015$ (significant), ^c $P < 0.03$ (significant). For other results, differences were marginally significant, with $0.03 < P < 0.08$.

[NaF] (mm)	$10\ \mu\text{M}$ - AlCl_3	5-HT		βTG		NAG		LDH	
		–	+	–	+	–	+	–	+
0		5	6	7	10	2	6	7	7
5		7	15 ^b	26	24	4	6	8	10
10		11	20 ^a	20	23 ^c	6	13 ^c	7	8
20		17	28 ^a	32	31	10	12	7	12
40		19	39 ^a	45	41	12	14	7	10

Table 2. Platelet thromboxane synthesis measured in presence of NaF or AlF_4^-

Platelets were prewarmed with NaF for 5 min without stirring before addition of saline or $10\ \mu\text{M}$ - AlCl_3 . After stirring for 2 min in the cuvette of the aggregometer platelets were transferred in ice-cold 0.1 M-EDTA, centrifuged, and the supernatant was analysed for TXB_2 . Results are expressed as ng of TXB_2 formed by 10^9 platelets. Results are means $\pm$ S.E.M. of 3–5 experiments ($*P = 0.0096$).

[NaF] (mm)	TXB_2 (ng/ 10^9 platelets)	
	–	+ $10\ \mu\text{M}$ - AlCl_3
0	3 $\pm$ 1	7 $\pm$ 1
5	13 $\pm$ 1	37 $\pm$ 1*
10	16 $\pm$ 2	77 $\pm$ 3*

dehydrogenase activity, which remained at the basal level with NaF concentrations lower than 20 mM. Release of 5-hydroxytryptamine from dense bodies increased with NaF concentration and was significantly larger in the presence of AlCl_3 . This potentiation by AlCl_3 was less evident on the maximum release of α -granules and lysosomes, as shown by the release of β -thromboglobulin and β -*N*-acetylglucosaminidase respectively. The potentiating effect of AlCl_3 on other granule release was observed only on the kinetics at shorter times after AlCl_3 addition. At 30 s after AlCl_3 addition, the amounts of β -thromboglobulin and β -*N*-acetylglucosaminidase released were 26 ± 2 and $3.7 \pm 0.6\%$ of total respectively (means $\pm$ S.E.M.) compared with 18 ± 3 ($P = 0.013$) and $4.5 \pm 0.8\%$ ($P = 0.05$) with NaF alone (10 mM).

TXB_2 formation induced by NaF or AlF_4^- is shown in Table 2. NaF alone (5 mM) or in the presence of AlCl_3 induced TXB_2 synthesis of 13 ± 1 or 37 ± 1 ng/ 10^9 with platelets respectively. This potentiation was more evident with 10 mM-NaF, since NaF or AlF_4^- respectively induced the synthesis of 16 ± 2 and 77 ± 3 ng/ 10^9 platelets.

Ca²⁺ mobilization

We performed three experiments on platelets from three different donors. Under our experimental

Fig. 2. Variations of ^{32}P associated with phosphoinositides (PPI) and PA during NaF- or AlF_4^- -induced platelet aggregation

Concentrations used: 10 mM-NaF, $10\ \mu\text{M}$ - AlCl_3 . Results are expressed as the ratio of the radioactivity in a given phospholipid to that in PA at zero time and are means of five experiments.

conditions, i.e. Ca^{2+} omitted from the final suspending medium, preincubation of platelets with less than 10 mM-NaF, followed by addition of AlCl_3 , resulted in no increase in cytoplasmic free Ca^{2+} above the basal level.

Phosphoinositide metabolism

NaF (5 and 10 mM) alone preincubated for 5 min with platelets resulted in a modification of the distribution of ^{32}P associated with the polyphosphoinositides: it decreased phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) by $12 \pm 1\%$, increased phosphatidylinositol 4-phosphate (PtdIns 4P) by $10 \pm 3\%$, and decreased phosphatidylinositol (PtdIns) by $15 \pm 5\%$. Thus after preincubation with NaF, ^{32}P -PtdIns(4,5)P₂-PtdIns4P and -PtdIns represented 22, 32 and 19% of total ^{32}P -labelled phospholipids. No further variations were observed with stirring for 2 min in the aggregometer. No ^{32}P PA formation was observed in either case with NaF concentration lower than 20 mM, and during this time.

After addition of AlCl_3 (Fig. 2), a 30% decrease in ^{32}P associated with PtdIns(4,5)P₂ was observed in the first 15 s. After this time it was maintained at that level without any resynthesis, as observed with physiological agonists such as thrombin and collagen. During the same time period, ^{32}P associated with both PtdIns4P and PtdIns

Fig. 3. DAG formation during NaF- or AlF_4^- -induced platelet aggregation

Saline or $10 \mu\text{M}$ - AlCl_3 was added to 5 mM- or 10 mM-NaF. Results are expressed as percentages of the DAG value measured in control platelets and are means of three experiments (bars represent S.E.M.): $P < 0.005$.

Fig. 4. Autoradiograms of phosphorylated proteins from platelets aggregated with 5 mM-NaF alone or in presence of $10 \mu\text{M}$ - AlCl_3

increased by 15% in the first 25 s, whereupon it reached a plateau. No $[^{32}\text{P}]\text{PA}$ formation was observed during the first 90 s after addition of AlCl_3 . Significant $[^{32}\text{P}]\text{PA}$ formation ($P = 0.0005$) was only measured with NaF concentrations over 20 mM or at longer times of incubation with AlCl_3 ($P = 0.03$).

DAG formation

This was investigated with both inducers, NaF and AlF_4^- (Fig. 3). NaF preincubated for 5 min with platelets did not induce any formation of DAG. After 2 min of stirring in the aggregometer, 5 mM-NaF had no effect and 10 mM-NaF induced a DAG formation of twice the control value. Addition of $10 \mu\text{M}$ - AlCl_3 to 5 mM-NaF induced a DAG synthesis of 2.5 times the control value. Similarly, addition of $10 \mu\text{M}$ - AlCl_3 to 10 mM-NaF accelerated and increased the synthesis up to 3.7 times the control value in the first 1 min of aggregation, where DAG was at its maximum value.

Fig. 5. Phosphorylation of p43 induced by NaF in presence of saline or $10 \mu\text{M}$ - AlCl_3

Results are given as the ratio of radioactivity measured under stimulation to that of unstimulated platelets, and are means of 3–5 experiments (bars represent S.E.M.): $*P = 0.02$.

Table 3. Effect of flurbiprofen on NaF- or AlF_4^- -induced DAG formation

Flurbiprofen ($10 \mu\text{M}$) was added to platelets 10 min before incubation with 10 mM-NaF, and DAG was measured after 2 min in the aggregometer. Results are expressed as in Fig. 3, and are given as means $\pm$ S.E.M. ($*P < 0.01$).

	DAG (% of control)	
	–	$10 \mu\text{M}$ -Flurbiprofen
NaF	190 ± 10	$130 \pm 30^*$
AlF_4^-	360 ± 10	$275 \pm 15^*$

Protein phosphorylation

Under physiological activation, two proteins appear to be phosphorylated, namely the myosin light chain, p20, and the main substrate for protein kinase C, p43. During NaF- or AlF_4^- -induced activation, no myosin light-chain phosphorylation was ever detected (Fig. 4). With 5 mM-NaF, p43 phosphorylation was 2.2 times that of the resting state, and 3.2 times in the presence of AlCl_3 . The same potentiation was observed with 10 mM-NaF, since p43 phosphorylation induced by either NaF or AlF_4^- was respectively 4 and 6.6 times the control value (Fig. 5). Moreover, this phosphorylation was extremely rapid, being detected before the onset of release in the first seconds after addition of AlF_4^- (Fig. 4).

Effect of flurbiprofen

Addition of $10 \mu\text{M}$ -flurbiprofen, a cyclo-oxygenase inhibitor, to platelets 10 min before addition of NaF completely prevented TXB_2 synthesis. In these conditions no change in the release reaction in response to either NaF or AlF_4^- was observed. The diacylglycerol formation was decreased by 30% as shown in Table 3. However, the p43 phosphorylation occurred normally in the presence of flurbiprofen.

DISCUSSION

The present report describes the biochemical and physiological platelet responses to AlF_4^- , an activator of G-protein. All platelet responses were measured on the same sample. The NaF concentrations that we used (lower than 10 mM) induced no detectable intracellular Ca^{2+} mobilization, in agreement with previous results (Poll *et al.*, 1986; Kienast *et al.*, 1987). The addition of AlCl_3 to these low NaF concentrations resulted in no detectable increase in intracellular Ca^{2+} . Thus the platelet activation here described occurred in the absence of any detectable increase in basal Ca^{2+} .

AlF_4^- induced aggregation, with typical morphological changes, the cells becoming spherical and emitting pseudopods, with granule centralization. As shown in the original reports (Mürer 1968; Mürer *et al.*, 1981; Mürer & Daniel, 1983), the process was slower than with physiological inducers (Rendu *et al.*, 1987b; Legrand *et al.*, 1986). Cells were slightly swollen, and considerable vacuolization was observed, as in the presence of 4β -phorbol 12 β -myristate 13 α -acetate (Ishihara *et al.*, 1985).

Al^{3+} ions potentiated the platelet responses induced by NaF, suggesting that the active species was AlF_4^- . This potentiating effect was particularly evident on TX synthesis, DAG formation and p43 phosphorylation. However, no significant PA synthesis was observed with either NaF or AlF_4^- . This was not the result of a putative inhibitory effect of NaF on the DAG kinase, since PA could be measured with higher NaF concentrations from 20 mM and over, or for times of stimulation longer than 2 min (results not shown). Thus, under the conditions described in this report, the DAG kinase seems to be delayed or to require a higher Ca^{2+} concentration than the basal level. Some decrease in PtdInsP_2 was measured, but was not followed by a resynthesis, as usually observed during phosphoinositide turnover induced by physiological agonists. This decrease in PtdIns(4,5)P_2 could thus correspond to a perturbation of the 'futile cycle' [i.e. the equilibrium between PtdIns , PtdIns4P and PtdIns(4,5)P_2]. This is already suggested by the perturbation of the labelling of the phosphoinositides, and could be a consequence of an NaF effect on glycolysis (Mürer *et al.*, 1981), since a diversion of ATP has been shown to decrease the amount of PtdIns(4,5)P_2 (Vickers *et al.*, 1982). Neither NaF or AlF_4^- induced any inositol phosphate formation (results not shown). This agrees with a previous report showing that low NaF concentration did not induce any inositol phosphate production (Kienast *et al.*, 1987). Altogether, these results suggest that AlF_4^- did not activate the phospholipase C hydrolysing the polyphosphoinositides.

In the absence of phosphoinositide hydrolysis, the Ca^{2+} mobilizing molecule Ins(1,4,5)P_3 should not be formed and Ca^{2+} should not be liberated from the dense tubular system. As a consequence, no phosphorylation of the myosin light chain could be detected. A direct relationship between myosin light-chain phosphorylation and granule centralization thus remains questionable.

NaF induced DAG synthesis and phosphorylation of the main substrate for protein kinase C, p43, both stimulated by Al^{3+} ions. Thus AlF_4^- induced DAG formation in the absence of detectable Ca^{2+} mobilization. The origin of DAG cannot be related to the PtdIns(4,5)P_2 breakdown. The AlF_4^- -induced DAG formation was not transient, as it is usually shown under thrombin stimu-

lation, where it is phosphorylated to the corresponding PA or metabolized by DAG lipase. In the present study the DAG measurement may have been performed at too short times of stimulation to observe the further decrease. This is supported by the fact that PA could be detected only with longer times of stimulation. Preincubation of platelets with flurbiprofen, a cyclo-oxygenase inhibitor, at a concentration which completely blocked TX synthesis, resulted in only 30% inhibition of DAG formation. This result suggests that the TX formed, shown to activate phospholipase C (Siess *et al.*, 1985), co-operatively promotes the DAG formation by only 30%. The DAG formed in the presence of flurbiprofen thus accounts for the main bulk, and originates from another source of phospholipids than phosphoinositides. DAG could originate from phosphatidylcholine (Bocckino *et al.*, 1985; Besterman *et al.*, 1986; Rosoff *et al.*, 1988), as demonstrated in response to phorbol ester (Daniel *et al.*, 1986; Liscovitch *et al.*, 1987), and/or as a consequence of a phospholipase D activity (Bocckino *et al.*, 1987a,b), demonstrated in platelets (Rubin, 1988). In such a situation the PA formed is non-radioactive and would have been undetected with the ^{32}P -labelling that we used.

A puzzling result is the TX formation induced by NaF potentiated by Al^{3+} ions, and in the absence of detectable Ca^{2+} mobilization. Platelet TXs are oxygenation products of arachidonic acid liberated mainly from membrane phospholipids by a Ca^{2+} -dependent phospholipase A_2 (Purdon *et al.*, 1987) or from DAG after activation of the DAG lipase (Irvine, 1982). This raises the possibility that AlF_4^- could have an effect at the level of these enzymes which does not require higher Ca^{2+} concentrations than basal.

In conclusion, the present results from studies using AlF_4^- suggest a new pathway for activating platelets directly through DAG formation and protein kinase C activation. At present, no clear-cut conclusions about phosphoinositide involvement in fluoride-induced platelet activation can be drawn. AlF_4^- could act on a G-protein modulating the hydrolysis of phosphatidylcholine (Exton, 1988), giving rise to DAG formation, through a phospholipase, either C or D. However, if AlF_4^- substitutes for the γ -phosphate of GTP, the GDP-AlF_4^- could mimic GTP and have a direct effect. Indeed in a wide range of secretory cell types, GTP has been proposed to be fusogenic, by direct activation of the exocytotic mechanism (Barrowman *et al.*, 1986), in a way similar to poly(ethylene glycol) (Dawson *et al.*, 1987), with no involvement of a G-protein (Chueh & Gill, 1986). Thus one needs to be more cautious in using AlF_4^- as a tool for studies dealing with platelet G-proteins regulating phosphoinositides.

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