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IONOMYCIN CAN ELEVATE INTRAPLATELET CA^{2+} AND ACTIVATE PHOSPHOLIPASE A WITHOUT ACTIVATING PHOSPHOLIPASE C

S.E. Rittenhouse[1] and W.C. Horne

Hematology Division
Brigham and Women's Hospital/Harvard Medical School
Boston, MA 02115

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SUMMARY: Human platelets exposed to ionomycin, a Ca^{2+} ionophore, exhibit activation of both phospholipases A_2 and C. Such platelets manifest a rise in cytoplasmic Ca^{2+} (monitored by quin 2), a loss in phosphoinositides, formation of lysophosphatidylinositol, thromboxane B_2, phosphatidic acid, and phosphorylated 47 kilodalton protein, and secretion. In the absence of thromboxane formation and secreted ADP, phospholipase C is not activated and the 47 kilodalton protein is not phosphorylated. The elevation in Ca^{2+} is unaffected by inhibition of cyclooxygenase and ADP. Thus, an increase in cytoplasmic Ca^{2+} is not sufficient to stimulate phospholipase C. Further, secretion can occur in the absence of phospholipase C activation and 47 kilodalton protein phosphorylation.

The formation of diacylglycerol (DG)[2] which results from the hydrolysis of phosphoinositides by phospholipase C has been shown to be an early event in human platelet activation (1). DG stimulates protein kinase C, which catalyzes the phosphorylation of a 47kD protein. Such a phosphorylated protein may promote secretion in conjunction with Ca^{2+} (2).

PLC is a Ca^{2+}-dependent enzyme (1,3,4). In our present study with the Ca^{2+} ionophore ionomycin, we have addressed the question of whether PLC is activated by Ca^{2+} in the platelet. We have further determined whether secretion can occur in the absence of 47kD protein phosphorylation.

MATERIALS AND METHODS: Human platelets were obtained freshly from normal human donors and were labeled with (^{32}P)P_i and (^{3}H)arachidonic acid, or (^{14}C)

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[2] Abbreviations used: DG, diacylglycerol; PLC, phospholipase C; kD, kilodaltons; PtdIns, phosphatidylinositol; PtdIns4P,phosphatidylinositol-4phosphate; PtdIns(4,5)P_2, phosphatidylinositol(4,5)bisphosphate; PA, phosphatidic acid; PG, prostaglandin; TXA_2, thromboxane A_2; U46619, (15S)-hydroxy-11α,9α-epoxymethano)prosta-5,13-dienoic acid; CP/CPK, creatine phosphate/creatine phosphokinase; ASA, aspirin; 5HT, 5-hyroxytryptamine (serotonin); AA, arachidonic acid+metabolites.

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serotonin, and washed by Sepharose gel-filtration as described previously (4). Platelets (1-2 x 10^9/ml) were incubated in buffer, pH 7.3 (5) containing 0.25 mM Ca^{2+}, with 0-2 μM ionomycin (E.R. Squibb and Sons) in the presence and absence of aspirin (0.5 mM) and creatine phosphate (5 mM)/creatine phosphokinase (40 U/ml). These inhibitors, respectively, block PG + TXA_2 formation by cyclooxygenase, and remove ADP. Cyclooxygenase metabolites and ADP have both been shown to activate phosphoinositide metabolism in platelets (6,7). In some cases, the PGH_2 analogue U46619 (Upjohn) was added to ionomycin and aspirin-treated platelets. This laboratory has reported recently that U46619 activates PLC in human platelets (5). Platelets were then incubated for various periods at 37°C. Incubations were terminated with (a)$CHCl_3$/MeOH/HCl (20:40:1) and lipids extracted, resolved, and quantitated as described previously (4,5), or (b)EDTA/formaldehyde, and cells were pelleted to allow quantitation of ^{14}C-serotonin released from platelet dense granules (5), or (c) Laemmli reducing buffer and boiling for 4 min (8).

Protein from 5 x 10^7 platelets was resolved by electrophoresis on 7.5% stacking slab gels (8) and stained with Coomassie blue. ^{32}P-labeled proteins were detected by autoradiography on Kodak X-Omat AR film, and ^{32}P-labeled 47kD protein quantitated after digestion with 30% H_2O_2, drying, and scintillation spectrophotometry using Liquiscint fluor (National Diagnostics). TXB_2, the stable metabolite of TXA_2, was assayed by an RIA procedure, as described previously (9). Leakage of lactate dehydrogenase was monitored as an indicator of possible platelet damage in response to ionomycin, and was assayed using a Sigma 226C LDH kit.

Cytoplasmic calcium activity was measured by the fluorimetric method of Rink, et al. (10), using a Perkin-Elmer MPF 44B spectrofluorometer. Concentrated suspensions of platelets, gel-filtered as described above, were incubated for 1 hour at 37°C with 5 μM quin2/acetoxymethyl ester (Lancaster Synthesis, Morecambe, England). The platelets were then gel-filtered to remove unincorporated quin2 ester, and $CaCl_2$ added (final concentration 0.25 mM). These platelets were incubated at room temperature for 30 min, with and without 0.5 mM aspirin. The platelets were diluted to approximately 5x10^7/ml and placed in a siliconized quartz cuvet at 37°. Fluorescence (excitation at 339 nm, emission at 492 nm) was measured before and after the addition of ionomycin. The maximum fluorescence (Fmax) was obtained by lysing the platelets with 0.05% Triton X-100 in the presence of 1 mM $CaCl_2$. The minimum fluorescence (Fmin) was then obtained after adding 3 mM EGTA, 10 mM Tris, pH 8.5. Correction factors for fluorescence of the added reagents and the change in light scattering following lysis of the platelets were determined by measuring the changes in the signal upon the addition of reagents to unlabeled platelets. Cytoplasmic Ca^{2+} concentrations of resting and ionomycin-exposed platelets were calculated using the equation from Tsien, et al. (11), an apparent Kd of 115 nm, and the corrected values for Fmax, Fmin, and the fluorescence of the platelet suspension in the absence and presence of ionomycin.

RESULTS AND DISCUSSION: As illustrated in **Fig.1**, the addition of ionomycin to platelets caused a small transient drop in labeled PtdIns(4,5)P_2, followed by increased labeling of this phospholipid, and increased labeling of PtdIns4P, whether monitored by ^{32}P (shown) or ^{3}H-arachidonic acid (not shown). These responses were blocked by the presence of ASA and CP/CPK. Platelets contain PLC active with respect to PtdIns(4,5)P_2 (4), and it is therefore possible that the decrease in this phosphoinositide is mediated by PLC. No lysoPtdIns(4,5)P_2 was detectable.

In contrast (**Fig.2**), the rise in cytoplasmic Ca^{2+} monitored by quin2 was not significantly affected by the presence of ASA or ASA+CP/CPK. Clearly, the passive flux in Ca^{2+} induced by the ionophore dwarfed any rise in Ca^{2+} attributable to

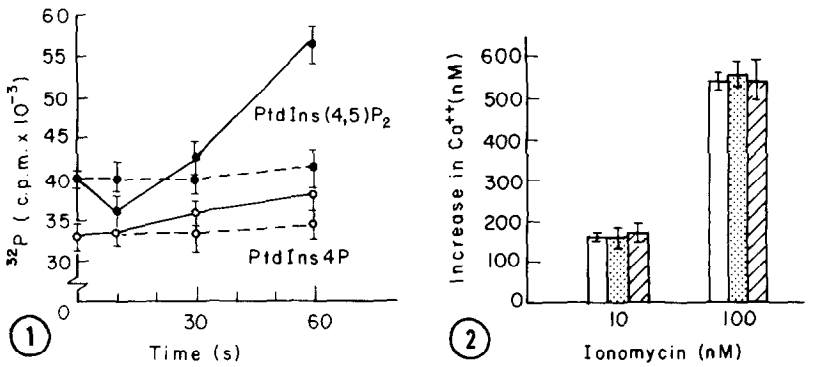

Figure 1 Effect of inhibitors on turnover of phosphoinositides in platelets exposed to ionomycin. Labeled human platelets (10^9/ml) were exposed to buffer or ionomycin (1 μM) for up to 60 s in the presence or absence of ASA+CP/CPK. No significant change was observed in ionomycin-free controls. Results are for a representative experiment of three performed in duplicate.

Figure 2 Effect of inhibitors on ionomycin-induced rise in cytoplasmic Ca^{2+}. Platelets ($5x10^7$/ml) were loaded with quin2 and exposed to ionomycin (open bars), ionomycin+ASA (dotted bars), or ionomycin+ASA+CP/CPK (hatched bars), and the increase in fluorescence was measured vs. ionomycin-free controls. Results are the mean ± S.E.M. of three experiments.

phosphoinositide hydrolysis (12,13). Furthermore, limitation of cytoplasmic Ca^{2+} was not a cause of the inhibition seen in the presence of ASA and CP/CPK.

A platelet protein which migrated near a 45 kD marker protein and corresponded to the 47 kD protein described by other investigators (14,15), was found to be phosphorylated after the addition of 1 uM ionomycin (**Fig. 3**). This phosphorylation was almost completely inhibited by ASA (not shown), and completely blocked by ASA+CP/CPK. Protein kinase C, which phosphorylates the 47 kD protein in platelets (15), has been found to be greatly stimulated by DG, a product of PLC acting on phosphoinositides (1,4). Inhibition by ASA+CP/CPK of the phosphorylation of the 47 kD substrate for this kinase constitutes further evidence in support of the conclusion that PLC is inhibited.

Table 1 summarizes the data illustrating the effect of ASA+CP/CPK on secretion and changes in labeled lipid and protein caused by different concentrations of ionomycin. Release of the cytoplasmic marker lactate dehydrogenase, constituting about 2.2% of the total, did not vary significantly with the different incubation conditions. Thus, the secretion observed could not be attributed to lysis of the platelets. Addition of ionomycin lead to secretion, the accumulation of lysoPtdIns (a marker for PLA_2 activity since no labeled arachidonic acid was found associated

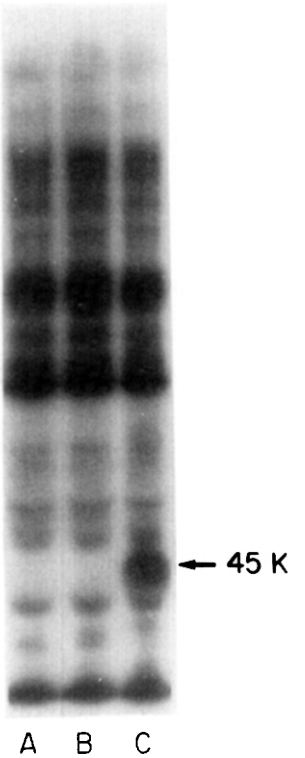

Figure 3 Autoradiographs of ^{32}P-labeled proteins from human platelets. Platelets (10^9/ml) were exposed to buffer (A), ionomycin (1.7 μM)+ASA+CP/CPK (B), or ionomycin alone (C) for 60 s. Proteins from 5x10^7 platelets were resolved by gel electrophoresis.

Table 1. EFFECT OF INHIBITORS ON PHOSPHOLIPASE ACTIVATION, SECRETION, AND PROTEIN PHOSPHORYLATION IN IONOMYCIN-STIMULATED PLATELETS

Ionomycin (μM)	% Change PtdIns	lysoPtdIns	AA	PA	5HT*	47 kD
0.5	-11	150	250	310	28	149
" +ASA+CP/CPK	-7.7	150	260	0	15	0
" " +U46619**	-12	160	260	400	32	210
1.0	-18	260	420	430	37	312
" +ASA+CP/CPK	-13	260	430	0	33	0
2.0	-33	520	1400	690	56	543
" +ASA+CP/CPK	-26	510	1500	0	52	0

* Values for 5HT, a monitor of secretion, refer to % of total 5HT released-control (ionomycin-free) release observed.
**U46619=1 μM.
Labeled platelets (10^9/ml) were incubated with and without ionomycin, in the presence or absence of ASA+CP/CPK at 37°C for 60 s. Lipids were extracted, or secretion determined, or samples were taken for protein resolution (47 kD protein). PA was used as a sensitive means of detecting DG formation (detecting ≤ 1 nmol). TXB_2 generation, in the absence of inhibitors, varied from 0.4 nmol/ml to 3 nmol/ml. Results given are representative of three experiments in duplicate.

with this product), PA (an indirect monitor of PLC), AA, and ^{32}P-labeled 47 kD protein (another indirect monitor of PLC). In one experiment in which DG was measured after a 30 s exposure to 1 μM ionomycin, DG levels (a direct monitor of PLC) increased three-fold, except where ASA+CP/CPK was included in incubations. The levels of PtdIns were decreased, as was transiently the case for PtdIns(4,5)P_2 (**Fig. 1**). At 0.5 μM ionomycin, secretion, protein phosphorylation, and PA formation were inhibited by ASA+CP/CPK. Accumulation of lysoPtdIns and AA were unimpaired and the drop in PtdIns was slightly inhibited. The inhibitions observed could be overcome by the addition of the PG analogue U46619. Higher concentrations of ionomycin did not overcome the inhibition of accumulations of ^{32}P-labeled PA and 47 kD protein, although they did lead to less impaired secretion. At 2 μM ionomycin, ASA partially inhibited PLC-associated responses (not shown), and both ASA and CP/CPK were required to block these responses completely. TXB_2 formation elicited by all concentrations of ionomycin was prevented completely by ASA.

We have thus derived two major conclusions from these studies: first, that although PLC is **dependent** upon Ca^{2+}, it is not **activated** by Ca^{2+}; by contrast, PLA_2 can be so-activated. Second, secretion is not always associated with phosphorylation of the 47 kD protein. Secretion can proceed in the absence of PLC activation and 47 kD phosphorylation, provided that intracellular Ca^{2+} levels are raised sufficiently.

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