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Diacylglycerol causes Ca release from the platelet dense tubular system: comparisons with Ca release caused by inositol 1,4,5-triphosphate[1]

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Platelet activation is often associated with an increase in the cytosolic free Ca concentration that is due in part to Ca release from the dense tubular system. The present studies examine whether the diacylglycerol formed by phosphoinositide hydrolysis during platelet activation contributes to this process. The effect of diacylglycerol on the dense tubular system was tested using platelets that were permeabilized with saponin and then allowed to accumulate ^{45}Ca. A synthetic diacylglycerol, 1-oleoyl-2-acetoyl glycerol (OAG), released up to 70% of the ionophore A23187-releasable ^{45}Ca, a fraction identical to that discharged by inositol 1,4,5-triphosphate (IP_3) under the same conditions. ^{45}Ca release was half-maximal at 40 μM OAG and 1 μM IP_3. The response to OAG was not inhibited by aspirin and could not be reproduced by the addition of a phorbol ester, which suggests that it involves neither arachidonic acid metabolism nor protein kinase C activation. The time course of OAG-induced ^{45}Ca release, which was slower than IP_3-induced ^{45}Ca release, corresponded to the time course of conversion of the OAG to 1-oleoyl-2-acetoyl phosphatidic acid (OAG-PA). When either OAG-PA or lysophosphatidic acid was added to the saponin-treated platelets, the extent of ^{45}Ca release was similar to that observed with OAG, but both the OAG-PA and the lysophosphatidic acid were 5 to 10 times more potent than OAG on a molar basis. These data suggest: (1) that the Ca release caused by diacylglycerol is actually due to formation of phosphatidic acid and/or lysophosphatidic acid, (2) that these molecules are not acting as simple Ca ionophores and (3) that diacylglycerol metabolites may augment the changes in Ca homeostasis caused by IP_3 during platelet activation.

One of the earliest responses in platelets to agonists such as thrombin is the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to form inositol 1,4,5-triphosphate[2] (IP_3) and diacylglycerol [1-4], a process that appears to involve a guanine nucleotide binding protein or G protein [5,6]. Recent studies have shown that both of the products of phosphoinositide hydrolysis serve as secondary messengers during platelet activation. IP_3 causes Ca release from the platelet dense tubular system, contributing thereby to the increase in the cytosolic free Ca concentration that accompanies platelet activation [7-9]. Diacylglycerol causes granule secretion and the exposure of fibrinogen receptors on the platelet surface, events that appears to be mediated entirely or in part by protein kinase C [10-12]. It also appears that there may be some degree of cross-over between the events intiated by IP_3 and diacylglycerol. For example,

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[2]OAG,1-oleoyl-2-acetoyl glycerol; OAG-PA, 1-oleoyl-2-acetoyl phosphatidic acid; IP_3, inositol 1,4,5-triphosphate; TPA, 12-O-tetradecanoyl phorbol-13-acetate

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the Ca released into the cytosol by IP_3 promotes secretion [7,13]. In addition, Ware and co-workers [14] have recently demonstrated that the membrane-permeable diacylglycerols, such as 1-oleoyl-2-acetoylglycerol (OAG), can cause an increase in the cytosolic free Ca concentration in platelets. This increase, which was detected using platelets loaded with aequorin, did not appear to be dependent upon platelet aggregation, secretion or arachidonic acid metabolism and was only partially inhibited by removing extracellular Ca with EGTA. This suggests that OAG, like IP_3, may be able to cause Ca release from the platelet dense tubular system. Similar results were obtained when the aequorin-loaded platelets were incubated with 12-O-tetradecanoyl phorbol-13-acetate (TPA), another activator of protein kinase C, suggesting that activation of this enzyme may be required for OAG-induced Ca release. In contrast to the results obtained by Ware, however, Rink and co-workers [15] had previously reported that TPA and OAG have no effect on the cytosolic free Ca concentration in platelets loaded with another fluorescent Ca probe, quin2, a distinction that may be related to the higher Ca buffering capacity of quin2 compared to aequorin or to differences in the distribution of aequorin and quin2 within the cell [14].

The purpose of the present studies was to address the issue of whether diacylglycerol contributes to intracellular Ca mobilization in platelets using an alternative approach to directly detect Ca release from the dense tubular system. Previous studies have shown that low concentrations of saponin will permeabilize the platelet plasma membrane without disturbing the function or integrity of the dense tubular system [7,13]. When the saponin-treated platelets are incubated with ^{45}Ca and ATP, tracer accumulates in the dense tubular system, but not the dense granules. Under these conditions, the addition of exogenous IP_3 rapidly discharges 70-80% of the sequestered ^{45}Ca with a K_m of approximately 1 μM [7]. Qualitatively similar results are obtained when endogenous phosphoinositide hydrolysis and IP_3 formation are stimulated in the permeabilized platelets by the addition of thrombin or nonhydrolyzable GTP analogs such as Gpp(NH)p [6]. In the present studies, we have used saponin-permeabilized platelets (1) to examine whether diacylglycerol and TPA can independently cause Ca release from the dense tubular system, (2) to determine whether diacylglycerol or its metabolites can enhance the ability of IP_3 to cause Ca release and (3) to examine the mechanism by which such Ca release might occur.

Experimental Procedures

Platelet preparation: Human platelets were resuspended in buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO4, and 20 mM HEPES, pH 7.4 at a final concentration of 1.4×10^9/ml [7]. In the studies of secretion, the platelets were loaded with [^{14}C]serotonin (New England Nuclear) by addition of the labeled compound to platelet-rich plasma [7]. When noted, cyclooxygenase was inhibited by incubating the platelet-rich plasma with 1 mM aspirin for 30 min or by adding 20 μM indomethacin to the washed platelet suspension. Successful inhibition of prostaglandin synthesis was confirmed by measuring arachidonic acid-induced platelet aggregation [7].

Studies of Ca release from saponin-treated platelets: IP_3-induced ^{45}Ca release from the platelet dense tubular system was measured as previously described [7]. Briefly, the washed platelet suspension was diluted to one fourth of the original concentration using buffer containing 160 mM KCl, 5.3 mM $MgCl_2$, 3.3 mM ATP and 13.3 mM HEPES, pH 7.1. EGTA and $CaCl_2$ were added to give a final free Ca concentration of 0.1 μM [16]. Afterwards, saponin (final concentration 15 μg/ml) and ^{45}Ca (10-20 μCi/ml) were added. After a 20 min loading period at 37°, the platelets were again diluted 4-fold with buffer containing 120 mM KCl, 4 mM $MgCl_2$, 20mM HEPES, pH 7.1 and the agonist to be studied. The amount of tracer remaining in the cells 4 min after addition of the agonist was measured in aliquots of platelet suspension which were diluted in wash buffer (130 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES, 5 mM EGTA, pH 7.1) and filtered through 0.45 μ HAWP filters (Millipore Corp.). ^{45}Ca retained by the filters was measured in a scintillation counter. The fraction of ^{45}Ca released was determined by comparing ^{45}Ca loss in the presence of an agonist with the amount of ^{45}Ca released under the same conditions by the Ca ionophore, A23187. This latter value was typically 85% of the total.

Serotonin release from saponin-treated platelets: [^{14}C]Serotonin-loaded platelets were diluted to $3.5x10^8$/ml using buffer containing 160 mM KCl, 5.3 mM $MgCl_2$, 3.3 mM ATP, 20 μM EGTA and 13.3 mM HEPES, pH 7.1 and preincubated with saponin (13-15 μg/ml) for 1 min before the addition of an agonist. The reaction was terminated after 10 min by sedimenting the platelets in an Eppendorf microcentrifuge. Serotonin release was quantitated by measuring ^{14}C in aliquots of the supernate.

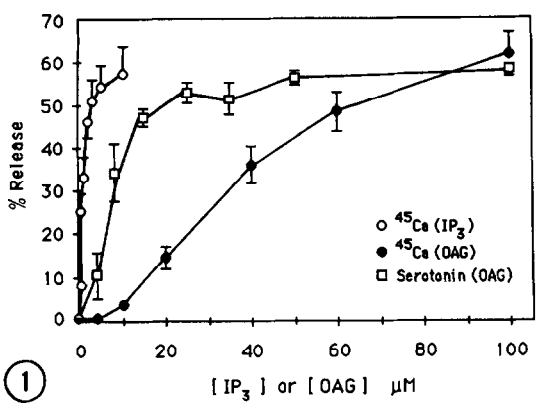
Phosphatidic acid formation: Phosphatidic acid formation from OAG was detected by a modification of a procedure described previously [6]. In brief, washed platelets were pre-incubated for 20 min under the same conditions as in the Ca release assay, except that the ^{45}Ca was omitted from the buffer and the ATP concentration was reduced to 1 mM. Immediately afterwards, 200 μCi of γ-^{32}P-labeled ATP (specific activity 10-40 Ci/mmol, New England Nuclear) was added. Two min later the platelet suspension was diluted 4-fold as in the Ca release assay and OAG (final concentration 100 μM) was added. At various times 1 ml aliquots of the reaction were removed and the lipids extracted with 5 ml of chloroform:methanol:concentrated HCl (100:100:0.6). Thirty min later, the phases were split by the addition of 1.5 ml of 5 mM EGTA in 1 N HCl. The bottom layer was collected, dried under nitrogen, resuspended in a small volume of chloroform:methanol (2:1) and spotted onto an activated silica gel 60 plate (E. Merck) for thin layer chromatography. ^{32}P-labeled phosphatidic acid was isolated using a double one-dimensional system as described by Kennerly, et al. [17]. A sample of ^{32}P-labeled 1-oleoyl-2-acetoyl phosphatidic acid (OAG-PA) prepared by Dr. Donald Kennerly (University of Texas Health Science Center, Dallas, TX) was used as a reference standard. The amount of radioactivity in the phosphatidic acid fraction was quantitated by liquid scintillation counting.

Other materials. IP_3, OAG, PGI_2, cAMP, dibutyryl cAMP, and A23187 were obtained from Sigma Chemical Co., St. Louis, MO. The OAG was stored in hexane. Immediately prior to use it was evaporated under nitrogen and reconstituted in dimethyl formamide at a final concentration of 25 mM. This stock was then diluted to 1 mM in water to form a 1 mM working stock. Dimethyl formamide alone at the same final concentration had no effect. OAG-PA was prepared by Avanti Polar Lipids. Lysophosphatidic acid from egg lecithin was obtained from Serdary Research Laboratories and contained a mixture of fatty acids. The lysophosphatidic acid was purified to homogeneity by thin layer chromatography and quantitated by phosphate assay [18] before use.

Results

Ca release in response to diacylglycerol and IP_3. In order to test the ability of OAG to cause intracellular Ca mobilization, washed platelets were permeabilized with saponin and then, in the presence of ATP and Mg, allowed to accumulate ^{45}Ca. Under the conditions used, essentially all of the sequestered ^{45}Ca entered the dense tubular system. Little, if any, entered the platelet storage granules or mitochondria [7]. At the end of the loading period, OAG was added and ^{45}Ca release was measured. As is shown in Figure 1, 60 to 70% of the sequestered ^{45}Ca that could be released by the Ca ionophore A23187 was discharged in response to the OAG. Release was half-maximal at approximately 40 μM OAG. This dose/response curve for OAG is similar to that obtained by Ware [14] for OAG-induced changes in the cytosolic free Ca concentration. In contrast to the results obtained by those investigators, however, phorbol ester at concentrations as high as 200 nM failed to cause Ca release: 0 ± 2%, n = 2. The same or lower concentration of TPA did, however, cause platelet aggregation and completely inhibited thrombin-induced phosphoinositide hydrolysis (not shown) [19].

Figure 1 also shows the dose/response curves for IP_3-induced ^{45}Ca release and OAG-induced ^{14}C-serotonin release obtained under the same conditions as the the OAG-induced ^{45}Ca release. The maximum fraction of the sequestered ^{45}Ca that could be release by IP_3 was similar to that obtained with OAG, which suggests that both molecules discharge Ca from the same pool. However, on a molar basis, IP_3 was approximately 40 times more potent than OAG since half-maximal ^{45}Ca release required only 1 μM IP_3 as compared to 40 μM OAG. Previous studies from this laboratory have shown that the dose/response curve for IP_3-induced serotonin secretion is identical to that for IP_3-induced ^{45}Ca release [7]. In contrast, OAG-induced serotonin release required 5-6 times less OAG than did ^{45}Ca release (Figure 1). The



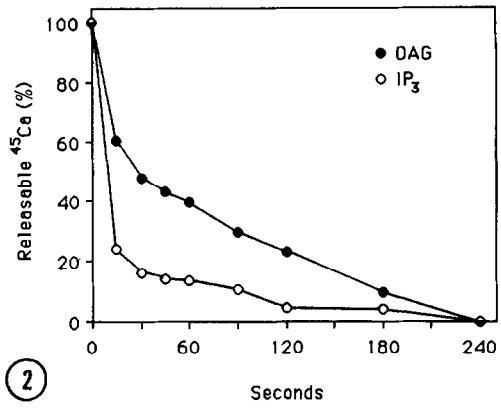


Figure 1. A comparison of OAG-induced Ca release with OAG-induced secretion and IP_3-induced Ca release. For the Ca release studies, saponin-treated platelets were preincubated with ATP and ^{45}Ca to allow tracer uptake into the dense tubular system. ^{45}Ca release was measured as described in the Methods section using various concentrations of OAG (closed circles) and IP_3 (open circles). The results shown are the mean ± sem for 10 studies and are expressed as a fraction of the ^{45}Ca releasable with 10 µM A23187 (approximately 85% of the total). For the serotonin release studies, the platelets were pre-labeled with ^{14}C-serotonin. Serotonin release from the washed, saponin-treated platelets was measured 10 min after the addition of OAG. The results shown are the mean ± sem for 3 studies expressed as a fraction of intracellular ^{14}C-serotonin (open squares).

Figure 2. The time course of OAG and IP_3-induced Ca release. Saponin-treated platelets were pre-labeled with ^{45}Ca as described in Figure 1. At the end of the loading period (time zero), 100 µM OAG (closed circles) or 10 µM IP_3 (open circles) was added. The results shown are the mean of 6 studies expressed in each case as the fraction of the maximum amount of ^{45}Ca releasable by each agonist that was still present at any given time.

differences between OAG and IP_3 also extend to the rate at which ^{45}Ca was released. The studies shown in Figure 2 compare the time course of ^{45}Ca release in response to maximum concentrations IP_3 and OAG. The rate of ^{45}Ca release caused by OAG was relatively rapid, but distinctly slower than the rate of ^{45}Ca release caused by IP_3.

Arachidonic acid metabolites such as thromboxane A_2 (TxA_2) have been shown to cause phosphoinositide hydrolysis and Ca mobilization in both intact platelets and platelets permeabilized with saponin [20-22]. In order to determine whether the ^{45}Ca release observed with OAG involves activation of the arachidonic acid pathway, similar studies were performed in platelets preincubated with either aspirin or indomethacin to inhibit cyclooxygenase. These inhibitors have previously been shown to have no effect on IP_3-induced ^{45}Ca release or on OAG-induced serotonin secretion and fibrinogen binding [7,12,20]. The data in Figure 3A show that OAG-induced ^{45}Ca release was only minimally affected by aspirin and indomethacin. Under the same conditions, arachidonic acid-induced aggregation was inhibited completely (not shown).

OAG-induced ^{45}Ca release was also unaffected by preincubating the the platelets with PGI_2 and dibutyryl cAMP under conditions in which these compounds inhibited secretion in response to OAG (Figure 3B). The mechanism by which OAG causes ^{45}Ca release from the platelet dense tubular system does not, therefore, appear to be sensitive to changes in platelet cAMP levels.

Phosphatidic acid and lysophosphatidic acid. Platelets contain diacylglycerol kinase and phospholipase A_2 which can convert diacylglycerol to phosphatidic acid and lysophosphatidic acid, respectively [23]. Since both of these molecules have been shown to cause platelet aggregation and Ca release from platelet

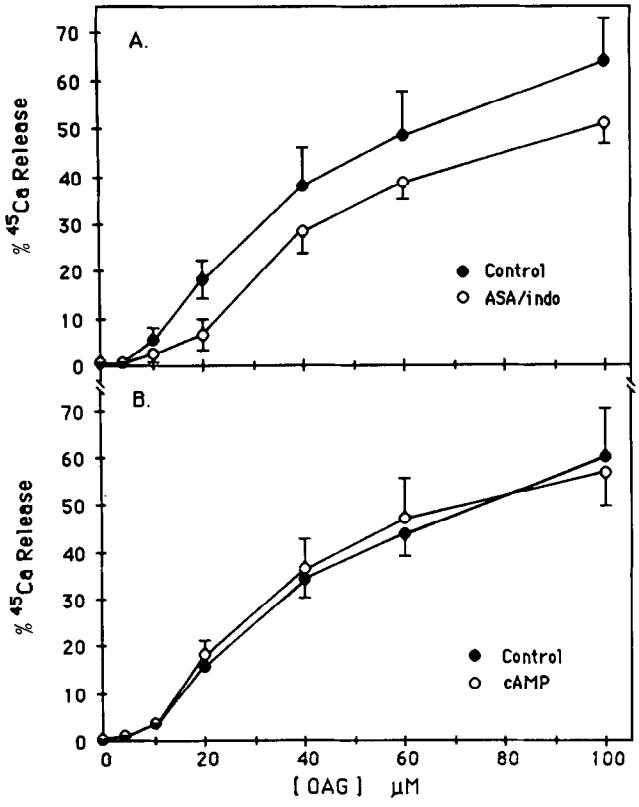


Figure 3. The effect of cyclooxygenase inhibitors and cAMP on OAG-induced Ca release and secretion. In (A) is shown the dose/response curves for OAG-induced ^{45}Ca release from platelets preincubated with (open circles) or without (closed circles) 1 mM aspirin or 20 µM indomethacin. The results shown are the mean ± sem of 5 studies. In (B) is shown the dose/response curve for OAG-induced ^{45}Ca release from control platelets (closed circles) and from platelets in which the cAMP concentration was increased by incubation with 1 µM PGI_2 and 1 mM dibutyryl cAMP for 5 min before adding the saponin (open circles). The results shown are the mean ± sem of 3 studies.

membrane vesicles [24-26], we (1) examined whether 1-oleoyl-2-acetoyl phosphatidic acid (OAG-PA) was formed in the saponin-permeablized platelets and (2) compared the ability of OAG, OAG-PA and lysophosphatidic acid to cause ^{45}Ca release from saponin-treated platelets. The data in Figure 4 document

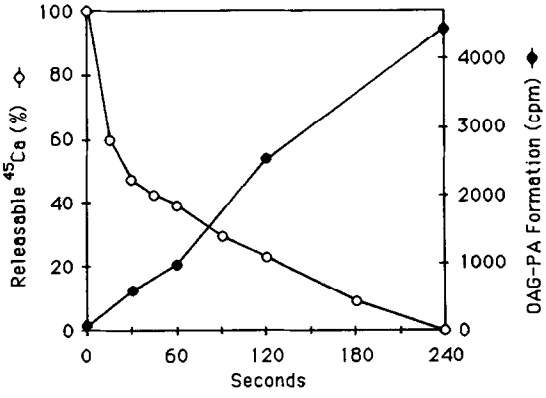


Figure 4. A comparison of the time course of OAG-PA formation and OAG-induced Ca release. [^{32}P]-OAG-PA formation (closed circles) was detected by thin layer chromatography in platelets incubated with OAG, saponin and [^{32}P]-ATP. The time course of OAG-induced ^{45}Ca release (open circles) was measured as described in Figure 2. The results shown are the mean of 2 studies.

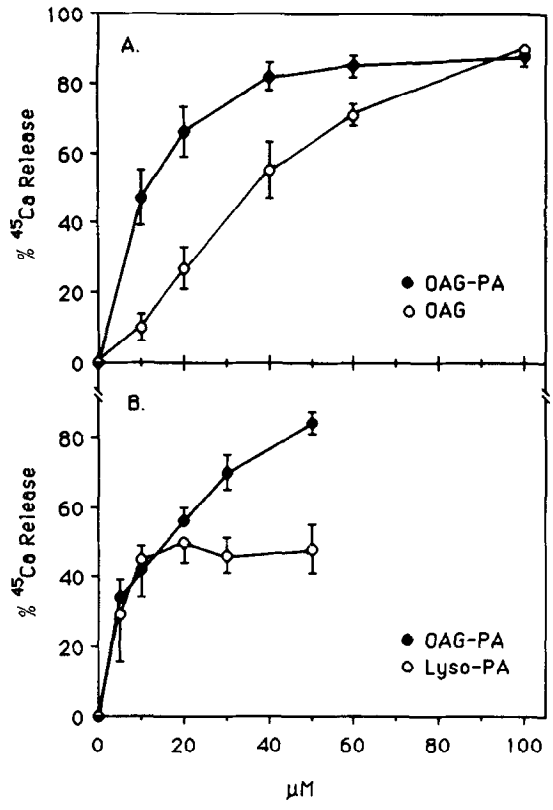


Figure 5. Ca release by OAG, OAG-PA. (A) The dose/response curves for ^{45}Ca release from saponin-treated platelets stimulated by OAG (open circles) or OAG-PA (closed circles) were determined as described in Figure 1. The results shown are the mean ± sem of 3 studies. (B) Dose/response curves for ^{45}Ca release from saponin-treated platelets stimulated by OAG-PA (closed circles) or lysophosphatidic acid (open circles). The results shown are the mean ± sem of 3 studies.

the formation of [^{32}P]-OAG-PA in permeabilized platelets incubated with OAG and ATP-γ-^{32}P. The [^{32}P]-OAG-PA was detected by thin layer chromatography as a distinct band with an R_f between phosphatidic acid and lysophosphatidic acid. The R_f of this band was identical to that of a reference sample of known [^{32}P]-OAG-PA. The time course of [^{32}P]-OAG-PA formation was similar to that for OAG-induced ^{45}Ca release. Since this suggests that OAG-PA formation occurs sufficiently early to participate in the ^{45}Ca release observed in response to OAG, we compared the dose/response curves for ^{45}Ca release in response to OAG and exogenous OAG-PA (Figure 5A). The maximum extent of ^{45}Ca release was the same for both species. However, half-maximal ^{45}Ca release required approximately 4 times less OAG-PA than OAG (10 μM vs. 40 μM). Because the *sn*-2 position of OAG-PA is occupied by the two carbon acetyl group, OAG-PA closely resembles 1-oleoyl-lysophosphatidic acid. In the ^{45}Ca assay, lysophosphatidic acid gave similar results to those obtained with OAG-PA except that the maximum extent of ^{45}Ca release was not as great (Figure 5B).

Finally, we examined whether the presence of a submaximal concentration of phosphatidic acid might affect the ability of IP_3 to cause ^{45}Ca release. In these studies ^{45}Ca release in response to IP_3 was measured in the presence of OAG-PA. In the absence of OAG-PA, IP_3 released 85% of the sequestered, ionophore-releasable ^{45}Ca with a K_m of approximately 1 μM. In the absence of IP_3, 5 μM OAG-PA released approximately 50% of the sequestered ^{45}Ca. This value increased steadily with the addition of low

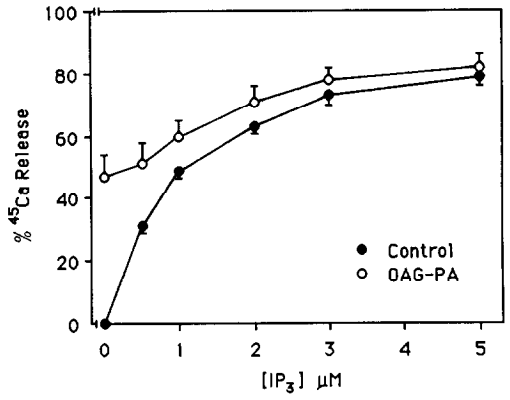


Figure 6. The effect of lysophosphatidic acid on IP_3-induced Ca release. IP_3-induced ^{45}Ca release was measured as described in Figure 1 in the absence (closed circles) or presence (open circles) of 5 μM lysophosphatidic acid. The results shown are the mean ± sem of 3 studies.

concentrations of IP_3. However, the OAG-PA appeared to cause neither an increase in the maximum extent of ^{45}Ca release nor a shift in the K_m of the IP_3 response to lower IP_3 concentrations (Figure 6).

Discussion

Quiescent platelets maintain a low cytosolic free Ca concentration and a steep plasma membrane Ca gradient in part by sequestering Ca within the dense tubular system, a structure derived from megakaryocyte endoplasmic reticulum. With many agonists, platelet activation is associated with the release of a substantial fraction of the sequestered Ca into the cytosol. It has recently been established that in platelets, as in other tissues, IP_3 produced by phosphoinositide hydrolysis plays a key role in this process [7-9]. The purpose of the present studies was to determine whether, as is suggested by studies with aequorin-loaded platelets [14], the diacylglycerol formed by phosphoinositide hydrolysis also contributes to intracellular Ca mobilization. In order to allow impermeant molecules access to the cytosol and the dense tubular system, the studies were performed with platelets selectively permeabilized with saponin. The results show that the synthetic diacylglycerol analog, OAG, is able to release Ca from the dense tubular system. OAG was used as the model for diacylglycerol because of its greater solubility in water than, for example, the naturally-occurring 1-stearoyl-2-arachidonyl-glycerol. The latter would be expected to form lipid vesicles larger than the holes created by saponin in the platelet plasma membrane. The ^{45}Ca release caused by OAG was half-maximal at 40 μM OAG, which compares favorably with the peak concentration of 50-60 μM diacylglycerol estimated from studies with thrombin-stimulated platelets reported by Rittenhouse [1]. At its maximum, OAG released approximately 70% of the ionophore-releasable ^{45}Ca from the saponin-treated platelets, a value similar to that observed with IP_3 under the same conditions.

Thus far, these results are consistent with those obtained with aequorin-loaded platelets. In contrast to those results, however, the ability of OAG to cause Ca release was not duplicated by the phorbol ester TPA even at concentrations which the TPA had other demonstrable effects on platelets. Therefore, it appears unlikely that the effects of OAG that we observed are mediated by protein kinase C. It also appears unlikely that these effects involve the arachidonic acid pathway since preincubating the platelets with aspirin or indomethacin to inhibit cyclooxygenase had only a small effect on ^{45}Ca release. What, then, is the mechanism by which OAG causes ^{45}Ca release? Several pieces of evidence suggest that this effect of OAG

is a consequence of its conversion into OAG-PA , a molecule which is structurally similar to lysophosphatidic acid. First, the time course of OAG-induced ^{45}Ca release was consistent with the time course of OAG-PA formation. Second, on a molar basis, OAG-PA and lysophosphatidic acid were 5 to 10 times more potent than OAG as stimulii for ^{45}Ca release. Third, the maximum extent of ^{45}Ca release in response to OAG-PA was similar to OAG, suggesting that a common pool of Ca had been discharged. The observed ability of OAG-PA and lysophosphatidic acid to cause ^{45}Ca release did not appear to be related to the capacity of either molecule to act as a Ca ionophore since in both cases the maximum extent of ^{45}Ca release was less than that obtained with the Ca ionophore A23187 (see also ref. [26]).

In some respects the ability of the diacylglycerol metabolites to cause Ca release compared favorably with the ability of IP_3 to release Ca from the permeabilized platelets. As was already noted, the maximum extent of ^{45}Ca release caused by OAG, OAG-PA and IP_3 were similar. Although the concentration of OAG-PA required to cause half-maximal ^{45}Ca release (5 μM) was 5 times higher than the concentration of IP_3 required for half-maximal release (1 μM), this difference is partially ameliorated by the fact that more diacylglycerol is formed during platelet activation than IP_3 [1,27]. Bishop and Bell [28] have recently argued that a large fraction of the diacylglycerol produced during platelet activation is metabolized to phosphatidic acid. Thus, even though the time course of ^{45}Ca release in response to OAG was slower than the time course of response to IP_3, these observations suggest that metabolites of diacylglycerol such as phosphatidic acid and lysophosphatidic acid may participate in agonist-induced changes of Ca homeostasis, either by amplifying the effects of submaximal concentrations of IP_3 or by producing a sustained release of Ca after IP_3 is transformed to inactive metabolites.

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