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myo-Inositol 1,4,5-trisphosphate stimulates protein phosphorylation in saponin-permeabilized human platelets

(protein kinase C/myosin light chain kinase/1,2-diacylglycerol/calcium mobilization/inositol phospholipids)

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ABSTRACT **In an attempt to establish a system with physiological substrates and phospholipid surfaces to investigate Ca^{2+}- and 1,2-diacylglycerol-dependent protein kinase C activation, saponized platelets were used. Saponin, through interaction with plasma membrane cholesterol, makes cells permeable without major disruption of organelles. Washed platelets, prelabeled with ^{32}P, were treated with 1–50 μg of saponin per ml. Permeabilization was evident at a concentration of 10 μg of saponin per ml, as indicated by the action of extracellular Ca^{2+} on the phosphorylation of the 20,000- and 40,000-Da proteins. These proteins are, respectively, the substrates for myosin light chain kinase and protein kinase C. Activation of these enzymes occurred when the estimated free [Ca^{2+}] was changed from ≈80 nM to 300 nM. The effect of Ca^{2+} on kinase C-induced phosphorylation was potentiated by 1,2-didecanoylglycerol (1 μM). *myo*-Inositol 1,4,5-trisphosphate (5–20 μM) increased phosphorylation of the 20,000- and 40,000-Da proteins. This action was time and concentration dependent. The effect of *myo*-inositol 1,4,5-trisphosphate on the activation of kinase C was additive with 1,2-didecanoylglycerol. The action of *myo*-inositol 1,4,5-trisphosphate could be due to mobilization of Ca^{2+} from platelet organelles and/or to a direct effect on protein kinases.**

myo-Inositol 1,4,5-trisphosphate (IP3) is produced in activated cells, including human platelets, by the phosphodiesteratic cleavage (phospholipase C) of phosphatidylinositol 4,5-bisphosphate (1–6, 27). It has recently been shown that IP3 is able to mobilize Ca^{2+} from the endoplasmic reticulum in pancreatic acinar cells (7), in hepatocytes (8, 9), and in single muscle cells of porcine coronary artery (10). Prior to administration of IP3, these cells were made semipermeable by EDTA or by saponin treatment.

Mobilization of Ca^{2+} can rapidly be detected in stimulated platelets (11). This occurs in parallel with the breakdown of polyphosphoinositides (12–14), appearance of inositol phosphates (1, 4), 1,2-diacylglycerol (15, 16), phosphatidic acid (17, 18), and activation of protein kinase C and myosin light chain kinase, as reflected by the phosphorylation of 40,000- and 20,000-Da proteins, respectively (19–21). Although 1,2-diacylglycerol in synergism with Ca^{2+} is believed to be the endogenous stimulant for protein kinase C (21), no information is available on the possible actions of IP3 on protein phosphorylation. In the present study, we demonstrate that the addition of IP3 to saponin-permeabilized platelets induces phosphorylation of the 20,000- and 40,000-Da proteins.

EXPERIMENTAL PROCEDURES

Most materials were obtained as reported (4, 15, 19, 20). Saponin was from Fisher, forskolin was from Calbiochem–Behring, and inositol 2-monophosphate, phosphatidylinositol, phosphatidylinositol 4-monophosphate, phosphatidylinositol 4,5-bisphosphate, and phospholipase C were from Sigma.

IP3 and inositol 1,4-bisphosphate were prepared essentially as described (22), through strong alkaline hydrolysis of phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 4-monophosphate. For purification, the reactions were neutralized with formic acid and, after dilution, applied to Dowex anion exchange columns (2). Then, 200 mM ammonium formate/100 mM formic acid (16 ml) was applied, followed by H_2O (16 ml); 1 M HCl (8 ml) was then added, and the eluant was diluted at least 1:10 and lyophilized. The identity of the products was checked by cochromatography with ^{32}P standards prepared from erythrocyte ghosts (23) using high-performance liquid chromatography (27) and thin-layer chromatography. Quantitation was by phosphate analysis. Inositol 1-monophosphate was prepared by the action of phospholipase C on phosphatidylinositol and purified as detailed above, except that 60 mM ammonium formate/5 mM sodium tetraborate was substituted for 200 mM ammonium formate/100 mM formic acid.

Human platelets have been isolated from platelet-rich plasma in the presence of prostacyclin and labeled with ^{32}P as has been reported (15, 19). Platelets were finally resuspended in a modified Tyrode Hepes buffer (134 mM NaCl/12 mM $NaHCO_3$/2.9 mM KCl/0.36 mM NaH_2PO_4/1 mM $MgCl_2$/5 mM Hepes/5 mM glucose/1 mM EGTA, pH 7.4) and platelet concentration was adjusted to 7.4×10^8 per ml. Samples (0.5 ml) were placed in aggregometer tubes and preincubated while stirring for 3 min at 37°C. Then, in most experiments, saponin at a final concentration of 10 μg/ml was added for 1 min, followed by addition of specific concentrations of $CaCl_2$, IP3, and 1,2-didecanoylglycerol for another period of 1 min. Measurement of [^{32}P]phosphatidic acid was carried out as described (15, 19). Gels containing 11% sodium dodecyl sulfate/polyacrylamide were used for the separation of proteins (15, 19, 20).

In all experiments with saponin-treated platelets, EGTA was kept constant at 1.0 mM and total Ca^{2+} was varied between 0.1 and 1.1 mM. A concentration of 2 mM Ca^{2+} was used in experiments as detailed in Fig. 1. Free [Ca^{2+}] was calculated as previously reported (24). All experiments are representative of at least five that gave qualitatively similar results. Results are within ±10% of the mean.

RESULTS

Action of Saponin on Human Platelets. Washed human platelets resuspended in Tyrode's solution were treated with 1–50 μg of saponin per ml for 1 or 2 min while stirring in aggregometer tubes. Examination by scanning electron mi-

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Abbreviation: IP3, inositol 1,4,5-trisphosphate.

croscopy (19) of saponin-treated platelets (not shown) revealed the presence of pores on the platelet surface at a concentration of saponin of 20–50 μg/ml. Alteration of the normal platelet appearance was seen at 50 μg of saponin per ml. Pores were not readily evident at lower concentrations of saponin, such as 10 μg/ml, but as demonstrated below these platelets are permeable to Ca^{2+}.

The action of saponin (1–20 μg/ml) in the absence of Ca^{2+} does not induce activation of protein kinases in platelets that have previously been labeled with ^{32}P (Fig. 1). However, treatment of platelets with a concentration of 10 μg of saponin per ml, followed by addition of Ca^{2+} (2 mM), can increase phosphorylation of the 20,000- and 40,000-Da proteins in those platelets; this is accompanied by a slight dephosphorylation of other proteins (Fig. 1). The fact that phosphorylation can still be observed in semipermeabilized platelets under these conditions demonstrates that ATP is at least partially retained in the cells. The explanation for this is not known but may suggest a certain degree of compartmentalization. At 20 μg of saponin per ml, a more marked general dephosphorylation of proteins is seen, due, most probably, to activation of Ca^{2+}-dependent phosphatases and proteases (25) or through a more extensive leakage of ATP.

Exogenous Ca^{2+} (2 mM) does not affect protein phosphorylation in control platelets or in platelets treated with 1 (Fig. 1) or 2–5 μg of saponin per ml (not shown). In platelets that have not been treated with saponin, 1,2-didecanoylglycerol (10 μM) phosphorylates a 40,000-Da protein, and forskolin, an agent that increases cyclic AMP levels, phosphorylates a 50,000-Da protein (Fig. 1). Saponin (1–20 μg/ml) does not induce formation of phosphatidic acid in the absence or presence of 2 mM exogenous Ca^{2+} (Fig. 1), thereby demonstrating that it does not lead to phospholipase C activation.

Effect of Different Concentrations of Ca^{2+} on the Phosphorylation of Proteins in Platelets Permeabilized with Saponin. Platelets prelabeled with ^{32}P and resuspended in a Tyrode Hepes buffer containing a fixed concentration of 1 mM EGTA were treated with 10 μg of saponin per ml for 1 min. Different concentrations of Ca^{2+} were then added for 1 min and protein phosphorylation was determined. It was repeatedly observed that when the Ca/EGTA ratio was changed from 0.5 to 0.8, an activation of protein kinase C and myosin light chain kinase was observed, as reflected by phosphorylation of the 40,000- and 20,000-Da proteins (Fig. 2). Ca^{2+}-induced phosphorylation of other proteins is also observed, especially between the 40,000- and 20,000-Da proteins. The estimated concentrations of free [Ca^{2+}] that correlate with a Ca/EGTA ratio of 0.5 and 0.8 are ≈80 and ≈300 nM, respectively. It is known that the concentration of platelet cytosolic Ca^{2+} is ≈80 nM and a change to ≈500 nM induced by the Ca^{2+} ionophore ionomycin is correlated with the shape change of platelets (11, 26). Fig. 3 shows the dose–response relationships of Ca^{2+}-induced protein phosphorylation. Furthermore, it shows that pretreatment of platelets with forskolin, which increases cyclic AMP levels and phosphorylates a 50,000-Da protein (ref. 21; Fig. 1), does not affect the direct action of Ca^{2+} on the phosphorylation of the 20,000- and 40,000-Da proteins. 1,2-Didecanoylglycerol directly stimulates phosphorylation of the 40,000-Da protein in the absence of free Ca^{2+} but not that of the 20,000-Da protein in both saponin-treated (Fig. 3) and nontreated platelets (Fig. 1). A change of the estimated free [Ca^{2+}] from 80 to 300 nM potentiates the effect of 1,2-didecanoylglycerol on the phosphorylation of the 40,000-Da protein (Fig. 3).

Effect of IP3 on Protein Phosphorylation of Platelets Permeabilized with Saponin. IP3 (10 μM) stimulates phosphorylation of the 20,000- and 40,000-Da proteins, especially at a Ca/EGTA ratio ranging from 0.8 to 1.0 (0.3–7 μM estimated free [Ca^{2+}]), as shown in Fig. 4. A small but reproducible effect of IP3 on 40,000-Da protein phosphorylation was ap-

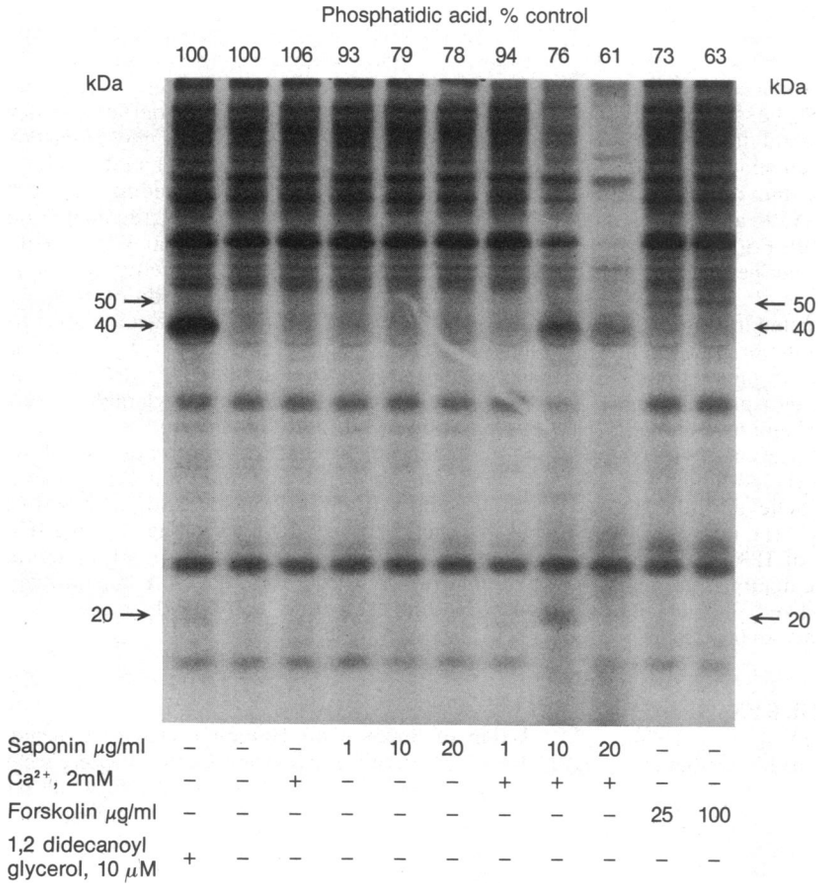

FIG. 1. Effect of Ca^{2+} on protein phosphorylation in platelets made permeable by the action of saponin. Human platelets (0.5 ml, 7.5×10^8/ml) prelabeled with ^{32}P were placed in aggregometer tubes and treated for 1 min without or with 1, 10, or 20 μg of saponin per ml. Subsequently, Ca^{2+} (2 mM) was added for 1 min. Protein phosphorylation and phosphatidic acid were measured. The effects of 1,2-didecanoylglycerol and forskolin were also studied on platelets that were not treated with saponin.

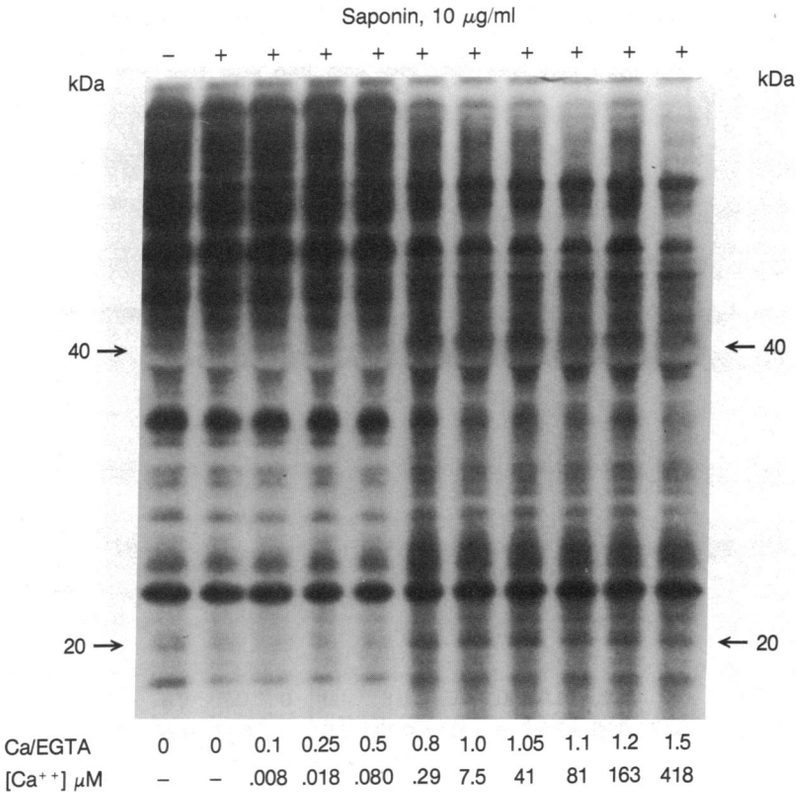

FIG. 2. Effect of different concentrations of Ca^{2+} on protein phosphorylation of saponin-treated platelets. Platelets (as in Fig. 1) resuspended in Tyrode Hepes buffer containing 1 mM EGTA were treated with 10 μg of saponin per ml for 1 min and then with different concentrations of Ca^{2+}, as indicated, for 1 min. The estimated free [Ca^{2+}] was calculated as described (24).

parent in the absence of exogenous Ca^{2+} (Fig. 4). An additive effect of IP3 and 1,2-didecanoylglycerol on phosphorylation of the 40,000-Da protein was also observed (Fig. 4). This potentiation is most apparent at a Ca/EGTA ratio of 0.7 or 0.8. At a ratio of 1.0, protein dephosphorylation prevails (Fig. 4), thereby mimicking the effect of 20 μg of saponin per ml in the presence of 2 mM Ca^{2+} (Fig. 1); this therefore suggests that IP3 may be evoking sufficient Ca^{2+} mobilization under these conditions to activate phosphatases or proteases (25). This action of IP3 on protein phosphorylation is concentration dependent (Fig. 5). Fig. 6 shows a time course of the actions of 5 μM IP3 and 1 μM 1,2-didecanoylglycerol measured at a Ca/EGTA ratio of 1.0, with maximal phosphorylation reached at about 20 sec. Again, it shows the additive actions of IP3 and 1,2-didecanoylglycerol on the kinase C phosphorylation of the 40,000-Da protein.

Inositol 1,4-bisphosphate (20 μM), inositol 1-monophosphate (20 μM), and inositol 2-monophosphate (20 μM) do not significantly affect protein phosphorylation under the assay conditions used in the present study (not shown).

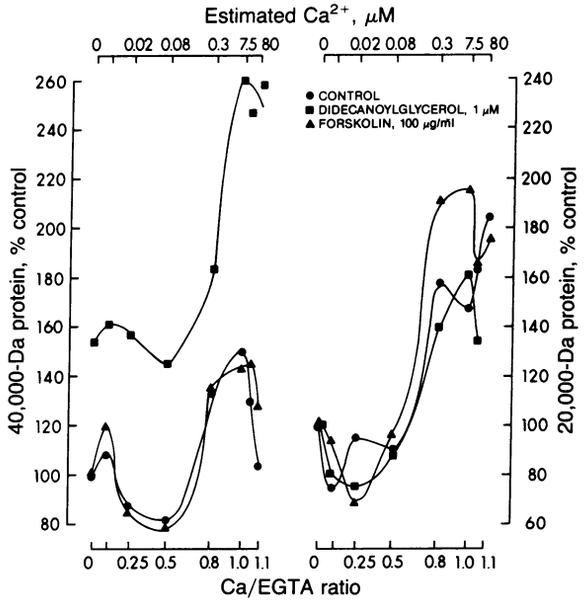

FIG. 3. Effect of Ca^{2+}, 1,2-didecanoylglycerol, and forskolin on protein phosphorylation of saponin-treated platelets. Platelets (as in Fig. 2 or pretreated with 100 μg of forskolin per ml for 2 min) were treated with 10 μg of saponin per ml for 1 min and, subsequently, for 1 min with different concentrations of Ca^{2+} in the absence or presence of 1 μM 1,2-didecanoylglycerol. Other details are as in Figs. 1 and 2.

DISCUSSION

Saponin-treated platelets provide a native environment in which it is possible to study protein phosphorylation with physiological substrates, membrane-bound phospholipids, and a controlled cytosolic Ca^{2+} concentration. In such a system, it is possible to explore the effects on protein phosphorylation of molecules that normally are not permeable. Under those conditions, IP3 provokes increased phosphorylation of the 20,000- and 40,000-Da proteins that are, respectively, the substrates for myosin light chain kinase and protein kinase C. This action of IP3 could be explained on the basis of its known Ca^{2+}-mobilizing properties from the endoplasmic reticulum of liver, pancreas, and smooth muscle (7–10). A similar action of IP3 in platelets might also mobilize Ca^{2+} from the dense tubular system. However, direct evidence that IP3 can mobilize Ca^{2+} in platelets has not yet been provided, and the present observations do not rule out the possibility that IP3 can directly activate protein kinase C, myosin light chain kinase, or an IP3-dependent protein kinase in platelets.

The observation that IP3 possesses biological activity in concentrations that are likely to be reached after receptor activation (7–9) provides support for the hypothesis that it

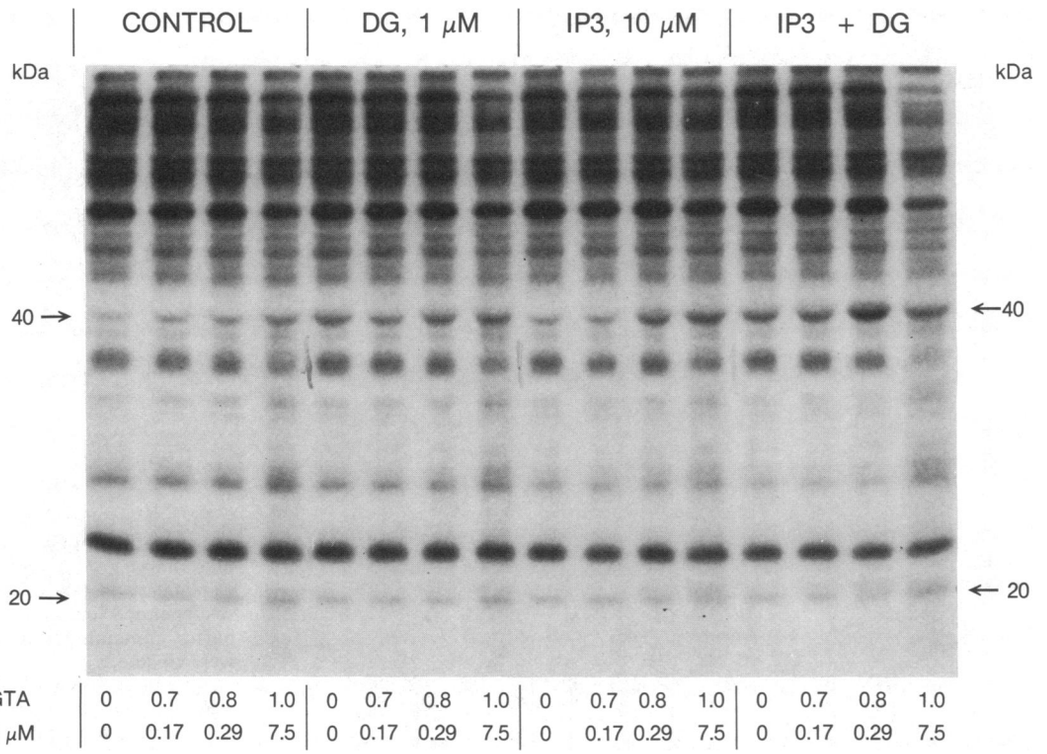

FIG. 4. Effects of IP3 and 1,2-didecanoylglycerol (DG) on protein phosphorylation of saponin-treated platelets. Experimental details are as in Figs. 1–3.

may be a universal second messenger (7–9). In this respect, it is interesting to note that IP3 is additive with 1,2-didecanoylglycerol in eliciting the phosphorylation of the 40,000-Da protein. It would therefore appear that agonists that induce platelet activation through hydrolysis of polyphosphoinositides do so through the additive action of 1,2-diacylglycerol and IP3, the latter possibly by inducing Ca^{2+} mobilization. Others have previously demonstrated that platelet activation can be brought about by the synergistic interaction of Ca^{2+} and 1,2-diacylglycerol (21, 26).

In conclusion, the present study shows that IP3 possesses biological activity in platelets, thereby lending strong support to the notion that IP3 is an important second messenger involved in platelet activation.

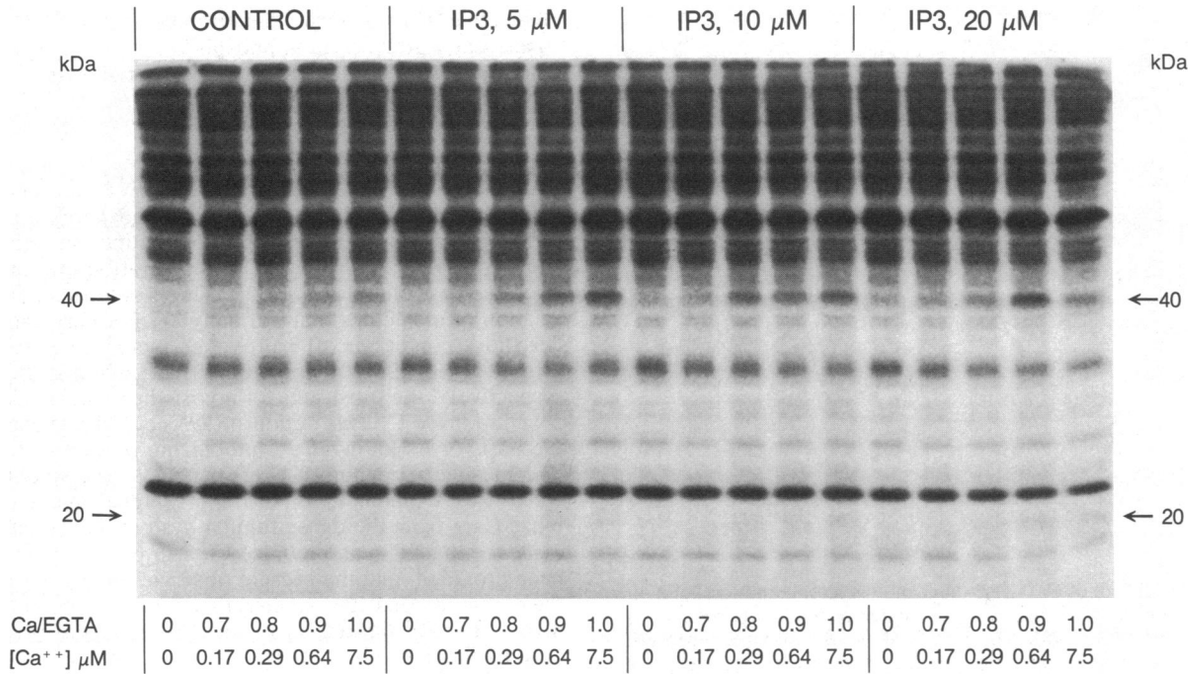

FIG. 5. Effect of different concentrations of IP3 on protein phosphorylation of saponin-treated platelets. Experimental details are as in Figs. 1–3.

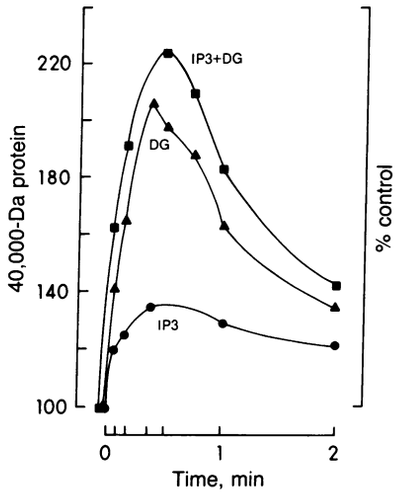

FIG. 6. Time course of effects of IP3 and 1,2-didecanoylglycerol (DG) on phosphorylation of a 40,000-Da protein in saponin-treated platelets. Concentration of IP3 was 5 μM, DG was 1 μM, and Ca/EGTA ratio was 1.0. Experimental details are as in Figs. 1–3.

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