Calcium modulates the generation of inositol 1,3,4-trisphosphate in human platelets by the activation of inositol 1,4,5-trisphosphate 3-kinase

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We observed that more total inositol trisphosphate (InsP_3) was formed when human platelets were stimulated with agonists (15-hydroxy-9,11- α -prosta-5,13-dienoic acid or thrombin) in the presence of extracellular Ca^{2+} than in its absence. Analysis of the InsP_3 by h.p.l.c. indicated that the increased InsP_3 formed in the presence of extracellular Ca^{2+} was primarily the 1,3,4-trisphosphate [$\text{Ins}(1,3,4)\text{P}_3$]. In addition, more inositol 1,3,4,5-tetrakisphosphate (InsP_4) was formed in the presence of extracellular Ca^{2+} . Experiments conducted with electrically permeabilized platelets demonstrated that conversion of [^3H] $\text{Ins}(1,4,5)\text{P}_3$ to [^3H] InsP_4 in platelets was Ca^{2+} -dependent, with half-maximal conversion observed at approx. $2.5\ \mu\text{M}\text{-Ca}^{2+}$. By contrast, dephosphorylation of [^3H] InsP_4 to [^3H] $\text{Ins}(1,3,4)\text{P}_3$ was not activated by Ca^{2+} . A partially purified preparation of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase from human platelets was found to be insensitive to Ca^{2+} , but addition of calmodulin restored Ca^{2+} -sensitivity to the kinase, increasing its activity about 5-fold. These results show that in human platelets the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ is regulated by Ca^{2+} -calmodulin, and suggest that the metabolites of $\text{Ins}(1,4,5)\text{P}_3$ may also have important second-messenger functions in platelets, and are consistent with the hypothesis that the activation of phospholipase C is not dependent on extracellular Ca^{2+} .

INTRODUCTION

Agonist-dependent stimulation of a wide variety of cells results in mobilization of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). Michell (1975) and Michell *et al.* (1981) proposed that increased metabolism of phosphoinositides might be responsible for this increase in intracellular Ca^{2+} . In the last few years, the evidence for this hypothesis has been strengthened by the observation that $\text{Ins}(1,4,5)\text{P}_3$, the product of phosphodiesteritic cleavage of $\text{PtdIns}(4,5)\text{P}_2$, can mobilize Ca^{2+} from intracellular reservoirs (Streb *et al.*, 1983). Berridge (1984) has proposed that stimulation of certain receptors leads to activation of a specific phospholipase C that preferentially cleaves $\text{PtdIns}(4,5)\text{P}_2$, releasing both diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$. These metabolites lead to the activation of protein kinase C and the release of stored Ca^{2+} , respectively.

The metabolism of $\text{Ins}(1,4,5)\text{P}_3$ was at first thought to involve only dephosphorylation to inositol via $\text{Ins}(1,4)\text{P}_2$ (Downes *et al.*, 1982). However, subsequent studies have shown that in many cells, in addition to the dephosphorylation pathway, $\text{Ins}(1,4,5)\text{P}_3$ metabolism involves phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ to InsP_4 , followed by dephosphorylation to $\text{Ins}(1,3,4)\text{P}_3$ (Irvine *et al.*, 1984, 1985; Burgess *et al.*, 1985). We have shown that blood platelets metabolize $\text{Ins}(1,4,5)\text{P}_3$ via the latter pathway and that $\text{Ins}(1,3,4)\text{P}_3$ is the major form of InsP_3 found in human platelets (Daniel *et al.* 1987a). Since this pathway consumes ATP, one can suggest that the metabolites of this pathway may be important to

platelet function and might be second messengers with functions complementary to that of $\text{Ins}(1,4,5)\text{P}_3$. In fact, Irvine & Moor (1986) have shown that InsP_4 may regulate Ca^{2+} entry into sea-urchin eggs.

The present study was undertaken when we observed that both the total InsP_3 formed and the intracellular Ca^{2+} concentration in stimulated platelets was greater and was maintained for longer periods when external Ca^{2+} was present. We show here that the apparent Ca^{2+} -sensitivity of total InsP_3 formation is primarily accounted for by Ca^{2+} -sensitive $\text{Ins}(1,4,5)\text{P}_3$ metabolism. Investigations using permeabilized platelets demonstrated that conversion of $\text{Ins}(1,4,5)\text{P}_3$ into $\text{Ins}(1,3,4,5)\text{P}_4$ was dependent on Ca^{2+} , whereas dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ was not. The $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity present in lysed cells was Ca^{2+} -dependent and was not enhanced by addition of calmodulin. However, after chromatography on DEAE-cellulose, the kinase was Ca^{2+} -insensitive. Addition of calmodulin activated the purified kinase and restored Ca^{2+} -sensitivity. These data are consistent with a regulation of inositol polyphosphate metabolism through a Ca^{2+} -calmodulin-dependent activation of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase by influx of external Ca^{2+} .

MATERIALS AND METHODS

Materials

InsP_3 , PP_1 , 2,3-diphosphoglycerate, 2,3-diphosphoglycerate phosphatase (rabbit muscle) and inorganic

Abbreviations used: $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; InsP_3 , inositol trisphosphate (isomeric form unspecified); $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; InsP_4 , inositol 1,3,4,5-tetrakisphosphate; 9,11- α -PGH₂, 15-hydroxy-9,11- α -prosta-5,13-dienoic acid; fMet-Leu-Phe, *N*-formylmethionyl-leucylphenylalanine.

pyrophosphatase (baker's yeast) were obtained from Sigma Chemical Co. Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 were obtained from Calbiochem. [3H]Ins(1,4,5) P_3 and [3H]Ins(1,3,4,5) P_4 were obtained from Amersham, and [^{32}P]P $_i$ was obtained from ICN. Fura-2 and its acetomethoxy ester were obtained from Molecular Probes. Bovine thrombin was from Armour Pharmaceutical. 9,11-Azo-PGH $_2$ is a synthetic analogue of PGH $_2$ (Corey *et al.*, 1975), and was given by Dr. Donald Harris of the Squibb Institute for Medical Research. Prepacked 25 cm Partisil 10 SAX columns were obtained from Whatman. Water and methanol were h.p.l.c. grade.

Preparation of platelets

Human blood was taken by venipuncture from informed healthy volunteers into acid/citrate/dextrose (Aster & Jandl, 1964). Platelet-rich plasma obtained by centrifugation at 180 *g* for 15 min at ambient temperature was again centrifuged (800 *g*, 15 min, ambient temperature). The platelet pellet was resuspended in 0.2 vol. of autologous platelet-poor plasma and incubated with [^{32}P]P $_i$ (0.25 mCi/ml) for 1 h at 37 °C. In some experiments fura-2 acetomethoxy ester (5 μ M) was added after 30 min and the incubation continued for another 30 min. The platelets were separated from unincorporated [^{32}P]P $_i$ by centrifugation at 800 *g* for 15 min, and resuspended in a Ca $^{2+}$ -free Tyrode's buffer containing 0.2% albumin, 10 mM-glucose and 10 mM-Hepes (pH 7.3). The cell suspension was adjusted to a final concentration of 2.5×10^8 cells/ml.

Measurement of intracellular free Ca $^{2+}$ with fura-2

Fura-2 fluorescence was monitored continuously by using settings of 340 nm (excitation) and 510 nm (emission). Fura-2 fluorescence signals were calibrated by the method of Pollack *et al.* (1986). F_{max} was determined by lysing the cells with 50 μ M-digitonin in the presence of 1 mM-CaCl $_2$; F_{min} was determined by addition of 10 mM-EGTA and 20 mM-Tris base. Correction was made for the presence of external dye.

Preparation of electrically permeabilized platelets

Platelet-rich plasma was centrifuged (800 *g*, 15 min, ambient temperature), and the cells were resuspended in a buffer containing 140 mM-NaCl, 14 mM-MgSO $_4$, 5 mM-glucose, 1 mM-EGTA, 0.37% bovine serum albumin, 25 mM-Pipes, 5 mM-Hepes and 28.5 mM-NaHCO $_3$ (pH 7.2). This cell suspension was subjected to ten electrical discharges of a 4 μ F capacitor at a field strength of about 20000 V/cm in a home-made apparatus similar to that described by Knight (1981). The cells were separated from the suspension buffer by centrifugation (800 *g*, 20 min, 4 °C) and resuspended in a buffer containing 140 mM-potassium glutamate, 17 mM-MgCl $_2$, 10 mM-EDTA and 10 mM-Hepes (pH 7.2). Centrifugation was found to be preferable to gel-filtration (Haslam & Davidson, 1984). The latter technique resulted in aggregation of the platelets on the gel-filtration column. Platelets permeabilized and isolated in this manner were found to behave identically with platelets isolated by gel-filtration (Daniel *et al.*, 1987b).

Preparation of partially purified Ins(1,4,5) P_3 3-kinase

Human blood (500 ml) was collected into 0.1 vol. of 3.8% (w/v) sodium citrate. Platelet-rich plasma was prepared by centrifugation at 150 *g* for 15 min. The

platelet-rich plasma was cooled on ice and 5 mM-EDTA (final concn; pH 7.4) was added. The platelet suspension was centrifuged at 900 *g* for 30 min at 4 °C, and the pellet was washed twice with a buffer containing 134 mM-NaCl, 2 mM-EDTA, 10 mM-glucose and 20 mM-Hepes (pH 7.4). The platelet pellet was resuspended in 20 ml of a buffer containing 5 mM-MgCl $_2$, 4 mM-EDTA, 10 μ M-leupeptin, 100 μ M-phenylmethanesulphonyl fluoride, 100 μ M-benzamidine, 2 mM-dithiothreitol and 25 mM-Hepes (pH 8.0). The cells were lysed by three cycles of freezing and thawing, and the unbroken cells and particulate material were removed by centrifugation at 100000 *g* for 30 min at 4 °C.

The supernatant was applied to a DEAE-cellulose column (Whatman DE-52; 5 ml bed vol., flow rate 1 ml/min) equilibrated with 5 mM-MgCl $_2$ /4 mM-EDTA/10 μ M-leupeptin/2 mM-dithiothreitol/10 mM-Tris/HCl (pH 8.0). The column was washed with the equilibration buffer until protein was no longer detected as monitored by the A_{280} . The KCl concentration of the elution buffer was increased in 0.05 M increments to a final concentration of 0.5 M. Two 2.5 ml fractions were collected at each concentration of KCl.

Ins(1,4,5) P_3 3-kinase activity was assayed by measurement of Ins P_4 by h.p.l.c. The assay mixture (100 μ l) contained 17 mM-MgCl $_2$, 5 mM-ATP, 10 mM-EDTA, 5 mM-2,3-diphosphoglycerate, 5 mM-PP $_i$, 4 mM-CaCl $_2$, 50 mM-potassium glutamate (pH 8.0) and 25 μ l of each fraction. The samples were preincubated at 37 °C, and the reaction was initiated by addition of 20 μ M-Ins(1,4,5) P_3 containing 20 nCi of [3H]Ins(1,4,5) P_3 . After 10–30 min, the reaction was terminated by addition of an equal volume of cold 1.2 M-HClO $_4$. The amount of Ins P_4 formed was determined by an h.p.l.c. procedure similar to that described below, except that a 20 min gradient was used. All fractions were assayed in the presence and absence of 5 μ g of calmodulin.

Assay of the Ca $^{2+}$ -dependence of Ins(1,4,5) P_3 3-kinase

The assay system was the same as that used during kinase purification, except that different concentrations of CaCl $_2$ (100 μ M–4 mM) were added to each sample. The amount of free Ca $^{2+}$ present at each concentration of total CaCl $_2$ was estimated by measuring the relative fluorescence of fura-2 free acid that had been added to the buffer. This method gave only approximate values for free Ca $^{2+}$ concentrations above 5 μ M; however, Ins(1,4,5) P_3 3-kinase was maximally activated in this range.

Measurement of Ins P_3 by electrophoresis

Neutralized platelet extracts were treated with 2,3-diphosphoglycerate phosphatase to remove 2,3-diphosphoglycerate (Dangelmaier *et al.*, 1986). Samples were analysed by chromatography on Dowex 1 (formate form), followed by electrophoresis on paper in 0.06 M-sodium oxalate buffer (pH 1.5) at 3000 V for 30 min. The radioactive band which co-migrated with an Ins P_3 standard was cut out and counted for radioactivity by liquid-scintillation spectrometry.

Analysis of inositol polyphosphates by h.p.l.c.

The method for analysis of inositol polyphosphates is as previously described (Daniel *et al.*, 1987a). This method depends on the fact that the γ -phosphate group of ATP is in metabolic equilibrium with the monoester

phosphates of PtdIns(4,5)P₂, which was originally demonstrated by ourselves (Dangelmaier *et al.*, 1986) and confirmed by others (Verhoeven *et al.*, 1987). Briefly, samples containing 0.02 μ Ci of [³H]Ins(1,4,5)P₃ were neutralized to pH 7.5–8.0 by addition of 2 M-K₂CO₃ and treated at 37 °C for 15 min with 50 μ l of inorganic pyrophosphatase/ml (50 units/ml) in 5 mM-MgCl₂ and 1.0 M-Tris/HCl (pH 8.0). After addition of 0.1 vol. of 6.6 M-HClO₄, the supernatant was adjusted to pH 6–7 with 2 M-K₂CO₃ and treated with 200 mg of charcoal (Darco, type G-60) to remove nucleotides (Meek, 1986). Samples were left for 5 min on ice and the charcoal was removed by centrifugation. The clear supernatant was diluted 5-fold with water and passed over a NH₂-bonded-phase column (type LC-NH₂, from Supelco, Bellefonte, PA, U.S.A.). After washing with 5 ml of water, the column was eluted with 1.0 ml of 1.5 M-NH₃ and the eluate freeze-dried in a Savant Speed-Vac Concentrator to a volume of 500 μ l.

Inositol phosphates were separated and analysed on a Waters h.p.l.c. M-6000A apparatus with a Whatman Partisil 10 SAX separating column, which was eluted with a 60 min linear gradient (1.5 ml/min) formed from water as initial buffer, and 1.5 M-ammonium formate, adjusted to pH 3.7 with H₃PO₄, as final buffer. Fractions were collected and analysed for both ³H and ³²P in a Beckman LS 1800 liquid-scintillation counter.

RESULTS

It has been shown by Rink and co-workers (Rink *et al.*, 1982; Hallam & Rink, 1985) that agonist-induced increases in intracellular Ca²⁺ in human platelets are augmented when extracellular Ca²⁺ is added. We determined whether the agonist-induced formation of InsP₃ also was altered in the presence and absence of extracellular Ca²⁺. The amount of [³²P]InsP₃ formed when platelets were stimulated with 1 μ M-9,11-azo-PGH₂ in buffer containing either 1 mM-CaCl₂ or 1 mM-EGTA is shown in Fig. 1(a). The initial rate of formation of InsP₃ was not significantly different in the two circumstances. However, in the presence of extracellular Ca²⁺ more [³²P]InsP₃ was formed, and its amount was maintained above the basal value for a longer period of time. The concentration response curve (Fig. 1b) shows that extracellular Ca²⁺ only caused a significant enhancement of InsP₃ formation at higher concentrations of 9,11-azo-PGH₂ (i.e. 100 nM and above).

In these initial experiments we measured total InsP₃. However, it subsequently became apparent that total InsP₃ represented the sum of the two isomers, Ins(1,4,5)P₃ and Ins(1,3,4)P₃. To address this issue, experiments similar to those of Fig. 1(a) were repeated and the amounts of ³²P-labelled Ins(1,4,5)P₃ and Ins(1,3,4)P₃ were determined with methodology recently developed in our laboratory (Daniel *et al.*, 1987a). Fig. 2 shows the effect of the presence or absence of external Ca²⁺ on the 9,11-azo-PGH₂-induced formation of both Ins(1,3,4)P₃ and InsP₄, as well as on the cytosolic free Ca²⁺ concentration as monitored by fura-2 fluorescence. This experiment demonstrates that external Ca²⁺ has an effect on both the maximum concentration of cytoplasmic Ca²⁺ attained and the length of time for which cytoplasmic Ca²⁺ is increased above the basal concentration. The concentrations of Ins(1,3,4)P₃ parallel cytoplasmic Ca²⁺, suggesting that the formation of Ins(1,3,4)P₃ is

Fig. 1. Effect of external Ca²⁺ on 9,11-azo-PGH₂-induced formation of InsP₃.

Samples of washed ³²P-labelled human platelets were treated at 37 °C with 9,11-azo-PGH₂ in the presence of either 1 mM added CaCl₂ (□) or 1 mM-EGTA (■) (these agents were added 1 min before addition of agonist) and the amount of InsP₃ was determined by paper electrophoresis. The results have been calculated as fold elevation above basal and are shown as means $\pm$ S.D. for three experiments. (a) Time course of InsP₃ formation induced by 100 nM-9,11-azo-PGH₂. (b) Concentration-dependence of InsP₃ formation measured at 30 s after 9,11-azo-PGH₂ addition.

altered by the cytosolic free Ca²⁺ concentration. More InsP₄ also was formed in the presence of external Ca²⁺, but the effect was not as dramatic as that on Ins(1,3,4)P₃ formation. The time course of formation of Ins(1,4,5)P₃ was not changed by addition of external Ca²⁺ (results not shown). These data are representative of three experiments that all gave similar results. In order to show that the effect of external Ca²⁺ was a general response of platelets to agonist-dependent receptor activation, fura-2-loaded platelets were stimulated with thrombin in the presence and absence of extracellular Ca²⁺ (Fig. 3). In three separate experiments both the intracellular Ca²⁺ concentration and the metabolism of Ins(1,3,4)P₃ and InsP₄ were enhanced by the presence of extracellular Ca²⁺.

The concentration of Ins(1,3,4)P₃ at any given time is determined by the rate of its formation from InsP₄ and

Fig. 2. Effect of external Ca^{2+} on 9,11-azo- PGH_2 -induced formation of $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4

Samples of washed ^{32}P -labelled human platelets labelled with fura-2 were treated at 37°C with 100 nM-9,11-azo- PGH_2 . (a) The amounts of $\text{Ins}(1,3,4)\text{P}_3$ ($\square$, $\blacksquare$) and InsP_4 ($\diamond$, $\blacklozenge$) were determined by h.p.l.c. in the presence of either 1 mM added CaCl_2 ($\square$, $\diamond$) or 1 mM-EGTA ($\blacksquare$, $\blacklozenge$) at the indicated times. (b) Record of the fura-2 fluorescence for the 120 s samples of panel (a): upper trace for CaCl_2 , lower trace for EGTA. Abbreviation: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concn.

Fig. 3. Effect of external Ca^{2+} on thrombin-induced formation of $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4

For details, see Fig. 2 legend, except that ^{32}P -labelled platelets were treated with 1 unit of thrombin/ml for the indicated times, and that $\triangle$, $\blacktriangle$ replace $\diamond$, $\blacklozenge$.

the rate of its degradation. Since the concentration of InsP_4 also appeared to be dependent on the cytoplasmic Ca^{2+} concentration, it was possible that the rate of either formation or degradation of InsP_4 determined the amount of $\text{Ins}(1,3,4)\text{P}_3$ present in the platelet. In order to determine whether formation of InsP_4 was Ca^{2+} -dependent, $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was added to electrically permeabilized platelets and formation of $[\text{H}]\text{InsP}_4$ was determined as a function of the free Ca^{2+} concentration. If the phosphatase inhibitors 2,3-diphosphoglycerate and PP_i were omitted, no $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was detected, presumably owing to its rapid degradation to InsP_2 . However, in the presence of phosphatase inhibitors, the formation of InsP_4 was strongly dependent on Ca^{2+} , with half-maximal InsP_4 formed at $2.5 \mu\text{M}\text{-Ca}^{2+}$ (Fig. 4). In order to determine whether InsP_4 phosphatase was also Ca^{2+} -dependent, $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was added to electrically permeabilized platelets and its conversion into $\text{Ins}(1,3,4)\text{P}_3$ was measured. In four separate experiments,

the rate of this reaction was not enhanced over the same range of Ca^{2+} concentrations as used in Fig. 4.

During the course of this work, it became apparent that $\text{Ins}(1,4,5)\text{P}_3$ kinase from other cell types was regulated by Ca^{2+} through a calmodulin-dependent mechanism. In order to confirm that the platelet kinase was Ca^{2+} -regulated and to demonstrate that calmodulin was required for its Ca^{2+} -dependent activation, the kinase was isolated from human platelets. Attempts to use outdated platelet concentrates indicated that the enzyme was labile, and only fresh human blood could be used. Consequently, the amount of material available for study was limited. The kinase was partially purified by chromatography on DEAE-cellulose. When column fractions were assayed in the presence of calmodulin, two main peaks of activity were detected. The material present in the first peak, which was eluted at 0.2–0.25 M-KCl, had over 5-fold greater $\text{Ins}(1,4,5)\text{P}_3$ kinase activity in the presence of calmodulin than in its absence. The

Fig. 4. Ca²⁺-dependence of conversion of [³H]Ins(1,4,5)P₃ into [³H]InsP₄ in electrically permeabilized platelets

Ins(1,4,5)P₃ (20 μM) and [³H]Ins(1,4,5)P₃ (20 nCi) were added to electrically permeabilized platelets containing 10 mM-2,3-diphosphoglycerate and 10 mM-PP_i. After incubation for 15 min at 37 °C, the reaction was terminated with an equal volume of 1.2 M-HClO₄, neutralized, and the amount of [³H]InsP₄ formed was determined by h.p.l.c. with a 20 min gradient (see the Materials and methods section). The results represent means ± S.D. for six determinations.

Fig. 5. Effect of calmodulin on the Ca²⁺-dependence of platelet Ins(1,4,5)P₃ 3-kinase

The Ins(1,4,5)P₃ 3-kinase partially purified on DEAE-cellulose was assayed in the absence (■) and presence (□) of calmodulin (50 μg/ml).

material of the second peak, which was eluted at 0.3–0.35 M-KCl, exhibited approximately the same amount of Ins(1,4,5)P₃ kinase in the presence or absence of calmodulin. The latter peak may represent kinase that has not been separated from calmodulin. The Ca²⁺-dependence of the partially purified kinase is shown in Fig. 5. In the absence of calmodulin, no Ca²⁺-dependent activation of Ins(1,4,5)P₃ kinase activity was apparent. In the presence of calmodulin, the Ca²⁺-dependence of

the kinase was restored, with half-maximal activity occurring at a free Ca²⁺ concentration of 1.3 μM, in reasonably good agreement with the data from Fig. 4.

DISCUSSION

Human platelets, in response to a wide variety of stimuli, rapidly convert PtdIns(4,5)P₂ into diacylglycerol and InsP₃ (Watson *et al.*, 1984, 1985; Rittenhouse & Sasson, 1985; Daniel *et al.*, 1986). The InsP₃ formed represents the sum of two isomers, Ins(1,4,5)P₃ and Ins(1,3,4)P₃ (Daniel *et al.*, 1987a). In addition, InsP₄ is found in thrombin-stimulated platelets indicating that the metabolism of Ins(1,4,5)P₃ is similar to that found in other cells (Daniel *et al.*, 1987a). Thus, in addition to being degraded by phosphatase to Ins(1,4)P₂, Ins(1,4,5)P₃ is phosphorylated by a kinase to InsP₄, followed by cleavage of the 5-phosphate to give Ins(1,3,4)P₃ (Batty *et al.*, 1985; Irvine *et al.*, 1986). At present the role of InsP₄ and Ins(1,3,4)P₃ in platelet function is not known.

Our initial experiments (Fig. 1) indicated that the formation of total InsP₃ was dependent on the external Ca²⁺ concentration. Previous studies from the laboratory of Rink (Rink *et al.*, 1982; Hallam & Rink, 1985) demonstrated that the agonist-dependent increase in cytosolic Ca²⁺ was greater in the presence of 1 mM extracellular CaCl₂ than when 1 mM extracellular EGTA was added. These data and our own results at first suggested that formation of Ins(1,4,5)P₃ might result, at least in part, from a change in intracellular Ca²⁺, in contradiction of current dogma (Berridge & Irvine, 1984). However, with application of improved methodology, we have shown that the isomer of InsP₃ whose formation is Ca²⁺-sensitive is not Ins(1,4,5)P₃, the direct product of phospholipase C-dependent cleavage of PtdIns(4,5)P₂, but rather Ins(1,3,4)P₃. This result complements the study of Lew *et al.* (1986), which showed that fMet-Leu-Phe-stimulated HL-60 cells produced the same amount of Ins(1,4,5)P₃, but less Ins(1,3,4)P₃, if the intracellular Ca²⁺ stores of the cells had been previously depleted. In addition, we have shown that more InsP₄ is formed in agonist-stimulated platelets when 1 mM extracellular Ca²⁺ is present. Our results support their conclusion that, whereas agonist-induced Ins(1,4,5)P₃ formation has a minimal Ca²⁺ requirement, Ins(1,3,4)P₃ formation is not directly dependent on receptor activation, but is dependent on the cytosolic Ca²⁺ concentration.

Rossier *et al.* (1986, 1987), using permeabilized preparations of both adrenal glomerulosa cells and rat aortic smooth-muscle cells, and Biden & Wollheim (1986), using broken preparations of insulin-secreting RINm5F cells, have shown that the conversion of Ins(1,4,5)P₃ into Ins(1,3,4)P₃ is augmented by increasing Ca²⁺ over the physiological range. In addition, Biden & Wollheim (1986) measured an EC₅₀ (concn. giving 50% of maximum effect) of the Ins(1,4,5)P₃ 3-kinase activity for Ca²⁺ of 0.8 μM, which is in reasonable agreement with the values obtained here. Using electrically permeabilized platelets, we have confirmed and extended these observations to show that the conversion of InsP₄ into Ins(1,3,4)P₃ is not enhanced with an increase in Ca²⁺ from 100 nM to 10 μM. Thus the Ca²⁺-dependent phosphorylation of Ins(1,4,5)P₃ by Ins(1,4,5)P₃ 3-kinase is responsible for the Ca²⁺-dependent increase in both InsP₄

and $\text{Ins}(1,3,4)\text{P}_3$. The kinase that catalyses this phosphorylation was first partially isolated from rat brain (Irvine *et al.*, 1986) and found to be independent of Ca^{2+} . Later, partially purified kinase preparations were obtained from a malignant human T-cell line (Imboden & Pattison, 1987) and pig aortic smooth muscle. Both enzymes were found to be regulated by Ca^{2+} . The smooth-muscle enzyme was similar to the platelet kinase which we have isolated, in that the purest preparation of the kinase was no longer Ca^{2+} -dependent and that Ca^{2+} -sensitivity was restored by the addition of calmodulin. In a recent abstract (Johanson *et al.*, 1987), a 4000-fold purification of rat brain $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase was reported. This enzyme was initially activated by Ca^{2+} , but lost Ca^{2+} -sensitivity during purification. Calmodulin restored Ca^{2+} -sensitivity to the kinase. The reason for the discrepancy with the previous study of Irvine *et al.* (1986) is not known, but perhaps calmodulin was lost from this preparation.

The exact role of the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase-dependent pathway for inositol phosphate metabolism in platelets and most other cells is not known. Both the fact that cellular energy, in the form of ATP, is used and the fact that the pathway is regulated by Ca^{2+} -calmodulin suggest that the metabolites formed in the pathway have some importance for the function of the cell. It is tempting to extend to other cells the studies in sea-urchin eggs that indicate that InsP_4 opens Ca^{2+} gates in the plasma membrane (Irvine & Moor, 1986). However, direct evidence for this hypothesis is lacking. It is noteworthy that in our studies the formation of InsP_3 was only dependent on external Ca^{2+} at higher concentrations of 9,11-azo-PGH₂ (Fig. 1b). In addition, half-maximal activity of the kinase required relatively high Ca^{2+} concentrations, of over 1 μM . One can suggest that, in human platelets, this high Ca^{2+} requirement of the kinase may distinguish between strong and weak platelet agonists. High concentrations of strong agonists would be expected to produce more $\text{Ins}(1,4,5)\text{P}_3$ and thus reach the threshold concentration of cytosolic Ca^{2+} that would activate the $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase to the extent required to build significant concentrations of InsP_4 . If InsP_4 does open Ca^{2+} gates at the plasma membrane, more Ca^{2+} would enter the cell and responses would be both increased and prolonged (see, e.g., Figs. 2b and 3b). Weak agonists would not cause a sufficient rise of cytosolic Ca^{2+} to activate $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase significantly, and thus would not produce a prolonged activation of the platelet.

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