Biochem. J. (1989) 257, 715–721 (Printed in Great Britain)

Release of Ca^{2+} by inositol 1,4,5-trisphosphate in platelet membrane vesicles is not dependent on cyclic AMP-dependent protein kinase

Flavia O'ROURKE, George B. ZAVOICO* and Maurice B. FEINSTEIN
Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032, U.S.A.

In contrast with previous reports, it was found that membrane-protein phosphorylation by the catalytic subunit (CS) of cyclic AMP-dependent protein kinase had no effect on Ca^{2+} uptake into platelet membrane vesicles or on subsequent Ca^{2+} release by inositol 1,4,5-trisphosphate (IP_3). Furthermore, IP-20, a highly potent synthetic peptide inhibitor of CS, which totally abolished membrane protein phosphorylation by endogenous or exogenous CS, also had no effect on either Ca^{2+} uptake or release by IP_3. Commercial preparations of protein kinase inhibitor protein (PKI) usually had no effect, but one preparation partially inhibited Ca^{2+} uptake, which is attributable to the gross impurity of the commercial PKI preparation. IP_3-induced release of Ca^{2+} was also unaffected by the absence of ATP from the medium, supporting the conclusion that Ca^{2+} release by IP_3 does not require the phosphorylation of membrane protein.

INTRODUCTION

In platelets IP_3 formed in response to certain stimuli is believed to be a second messenger that can release Ca^{2+} from intracellular membrane stores (O'Rourke *et al.*, 1985; Adunyah & Dean, 1985; Authi & Crawford, 1985). The catalytic subunit (CS) of cyclic AMP-dependent protein kinase was reported to be facilitatory, or even necessary, for Ca^{2+} release by IP_3 from isolated platelet membrane vesicles (Enouf *et al.*, 1987). This conclusion was based on two observations; first, IP_3 released more Ca^{2+} when vesicles were exposed to CS during Ca^{2+} uptake, and secondly, a protein inhibitor of cyclic AMP-dependent protein kinase, originally described by Ashby & Walsh (1972), counteracted these effects of exogenous CS. When tested in the absence of exogenous CS the protein kinase inhibitor (PKI) completely abolished the release of Ca^{2+} by IP_3 (Enouf *et al.*, 1987). From the latter finding it could be concluded that endogenous cyclic AMP-dependent protein kinase was necessary for the Ca^{2+}-releasing action of IP_3. PKI has also been reported to suppress Ca^{2+} uptake strongly (Hettasch & LeBreton, 1987; Adunyah & Dean, 1987), and in one case actually to cause substantial Ca^{2+} loss from vesicles (Hettasch & LeBreton, 1987). The latter observation conflicts with the inhibition of Ca^{2+} release by PKI described by Enouf *et al.* (1987).

Other results all implicate cyclic AMP-dependent kinase in the regulation of Ca^{2+} uptake also. Several laboratories have observed that CS stimulated Ca^{2+} uptake by the platelet-membrane vesicles that are believed to be derived from the dense tubular system of platelets (Kaser-Glanzmann *et al.*, 1977, 1979; Adunyah & Dean, 1987; Hettasch & LeBreton, 1987; Enouf *et al.*, 1987). The effects of CS on Ca^{2+} uptake and release were correlated with the phosphorylation of a 23 kDa polypeptide. It was suggested that this polypeptide may be phospholamban (Kaser-Glanzmann *et al.*, 1979; Adunyah & Dean, 1987), a known promoter of Ca^{2+} transport in cardiac-muscle membranes, but that identity was disputed (LePeuch *et al.*, 1983).

The hypothesis that CS promotes Ca^{2+} release by IP_3 runs counter to the effects of cyclic AMP on free intracellular Ca^{2+} concentrations in intact platelets. Dibutyryl cyclic AMP, and various prostaglandins and forskolin, which stimulate adenylate cyclase, strongly antagonize and reverse the Ca^{2+} mobilization produced by platelet agonists (Rink & Smith, 1983; Yamanishi *et al.*, 1983; Feinstein *et al.*, 1983, 1985). Furthermore, Ca^{2+} release by IP_3 was reported not to require ATP (Adunyah & Dean, 1986*b*; Meyer *et al.*, 1988), and did not display the temperature-sensitivity expected of an enzymic process (Adunyah & Dean, 1986*b*). Because of these conflicting findings, we have investigated the effects of CS on the Ca^{2+} uptake and release processes. In addition to PKI, we also employed a highly purified peptide inhibitor of CS. The synthetic peptide termed IP-20 (see the Materials and methods section for its amino acid sequence) represents the active site of the skeletal-muscle protein inhibitor of CS. It is a 20-amino-acid peptide originally produced by digestion of purified skeletal muscle PKI with *Staphylococcus aureus* V8 protease (Cheng *et al.*, 1986). IP-20 binds to the catalytic subunit of the protein kinase, but not the holoenzyme, and inhibits CS competitively, with a K_i of 2.3 nM (Cheng *et al.*, 1986). Its high potency and purity ($>97\%$) make synthetic IP-20 clearly preferable to the impure commercial PKI preparations as an antagonist of CS.

MATERIALS AND METHODS

Isolation of platelet membrane vesicles responsive to IP_3

Platelet membranes were prepared by differential centrifugation of sonicated platelets on a KCl/Percoll

Abbreviations used: CS, catalytic subunit of cyclic AMP-dependent protein kinase; PKI, protein kinase inhibitor of CS; IP-20, polypeptide inhibitor of CS derived from the active site of PKI; IP_3, inositol 1,4,5-trisphosphate.

* Present address: Department of Hematology, Brigham and Women's Hospital, Boston, MA 02115, U.S.A.

gradient as described by O'Rourke *et al.* (1985). The vesicles were resuspended at 0.4 mg of protein/ml in medium containing 100 mM-KCl, 20 mM-Hepes, 5 mM-$MgCl_2$ and 0–20 mM-phosphate (as described in the text), pH 7.1.

Ca^{2+} uptake by membrane vesicles

Vesicles were incubated in the medium described above plus 0.200 mM-$CaCl_2$, 1 μCi of $^{45}CaCl_2$/ml, 0.568 mM-EGTA, 1.9 mM-disodium ATP and 0–20 mM-potassium phosphate, pH 7.1, at 23 °C (total volume 1.1 ml). The calculated free [Ca^{2+}] was 1.1 μM (O'Rourke *et al.*, 1985). Samples (100 μl) of the suspension were taken at specified times, $^{45}Ca^{2+}$ uptake was 'quenched' with formaldehyde/EDTA solution, and vesicle $^{45}Ca^{2+}$ was measured by liquid-scintillation counting (see below).

Assay for release of Ca^{2+} by IP_3

After preloading of vesicles with $^{45}Ca^{2+}$ for 60 min, an additional 0.4 mM-K–EGTA was added to stop further Ca^{2+} uptake, followed by addition of IP_3. Samples of membrane suspension (100 μl) were removed at specified times and added to polycarbonate tubes (Beckman 7 mm × 20 mm) containing 25 μl of ice-cold quench medium, consisting of 0.633 M-formalin and 50 mM-K–EDTA, pH 7.1. Control experiments showed that the quench medium immediately stops Ca^{2+} uptake, prevents Ca^{2+} efflux and does not cause Ca^{2+} efflux. The membranes were centrifuged at 430000 ***g*** for 2 min at 4 °C in a Beckman TL-100 ultracentrifuge. Portions (10 μl) of supernatant were transferred to a scintillation vial, and the remainder of the supernatant was vacuum-aspirated by using a fine-bore glass pipette. The pellets were extracted with 50 μl of 25% trichloroacetic acid and transferred to scintillation vials for assay of radioactivity.

Phosphorylation of platelet vesicle proteins by CS

The membrane vesicles were incubated with CS in essentially the same medium as was used for Ca^{2+} uptake and IP_3-induced Ca^{2+} release. Phosphorylation was initiated by addition of 50 μM-ATP containing 2.5 μCi of [^{32}P]ATP per 100 μl of membrane suspension. Additions of IP_3 or CS were made as indicated in the legends to the Figures. When used, kinase inhibitors were added for 15 min before CS. The reactions were stopped after 10 min with 20 μl of 20% (v/v) SDS/25 mM-dithiothreitol and heated in a boiling-water bath. The proteins were electrophoresed on 5–15%-polyacrylamide gradient slab gels containing 0.1% SDS, and phosphoproteins were identified by radioautography.

Materials

[γ-^{32}P]ATP was obtained from New England Nuclear. The catalytic subunit (CS) of cyclic AMP dependent protein kinase from bovine heart was purchased from Sigma (P2645): lots 36F-9525, 63F-3931 and 106F-9660 with activities of 34, 26 and 36 units/μg of protein respectively, and containing 0.16–0.2% protein; one phosphorylating unit transfers 1 pmol of [γ-^{32}P]ATP to hydrolysed and partially dephosphorylated casein per min at pH 6.5 at 30 °C. CS was reconstituted in deionized water containing 50 mg of dithiothreitol/ml to a concentration of 0.5 mg of protein/ml. The protein kinase inhibitors (PKI) used were type II from bovine heart (Sigma, P-8140) and type III from pig heart (Sigma, P-0393). The last two preparations inhibited 0.9 and 0.5 phosphorylating unit of kinase per μg of protein respectively. They contained 40% and 70% protein respectively, with the balance consisting of glycerophosphate and EDTA salts. The peptide inhibitor (IP-20) of cyclic AMP-dependent protein kinase was obtained from Bachem, Torrance, CA, U.S.A. (PPHO70 lot no. 781C); 1 μg inhibits approx. 5000 phosphorylating units of cyclic AMP-dependent kinase. This peptide has the amino acid sequence Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp (Cheng *et al.*, 1986).

RESULTS

Previous studies of Ca^{2+} transport in platelet membranes have mostly used a standard membrane filtration technique to measure uptake of $^{45}Ca^{2+}$. In our hands this method proved to be unacceptably variable and unreliable, largely owing to significant non-specific binding of $^{45}Ca^{2+}$ to filter membranes, especially as the phosphate concentration in the medium was increased. We therefore developed an alternative procedure that permitted accurate measurement of Ca^{2+} uptake and release by IP_3. This method employs formaldehyde as a reaction 'quenching' agent combined with EDTA to remove surface-bound Ca^{2+}, followed by rapid high-speed centrifugation (Beckman TL-100 ultracentrifuge). This quenching reagent immediately stopped $^{45}Ca^{2+}$ influx, and did not permit any efflux or response to IP3. The $^{45}Ca^{2+}$ content of vesicles remains stable for at least 1 h in the formaldehyde/EDTA solution. In contrast, vesicles quenched in ice-cold Ca^{2+}-free medium initially have a significantly larger amount of surface-associated $^{45}Ca^{2+}$, which dissociates from the membranes over a 30 min period (Fig. 1*a*). The formalin/EDTA solution immediately removes this surface-bound Ca^{2+}. The membrane pellets obtained by centrifugation at 450000 ***g*** for 2 min contain no significant extravesicular trapped Ca^{2+}, since there was no further decrease in vesicle $^{45}Ca^{2+}$ after two washes of the pellets in Ca^{2+}-free medium. The time course of Ca^{2+} efflux from platelet vesicles in response to IP_3, measured by this method, is shown in Fig. 1(*b*). The presence of EGTA in the medium along with IP_3 prevented re-uptake of released Ca^{2+} and allowed measurement of the maximal extent of Ca^{2+} release by IP_3.

Effects of CS on Ca^{2+} uptake by platelet vesicles

Commercial preparations of CS, widely used in this and most previous studies, have a very high content of phosphate (9.84–19.9% of freeze-dried powder), and the uptake of Ca^{2+} is markedly stimulated by phosphate. In the absence of phosphate, Ca^{2+} uptake by platelet vesicles was never more than 15 nmol/mg of protein, comparable with values obtained by Hack *et al.* (1986) and Adunyah & Dean (1986*a*) under similar conditions. Ca^{2+} accumulation increased to 120–150 nmol/mg of protein when the medium contained 20 mM-phosphate and free Ca^{2+} was buffered with EGTA to 1 μM, a concentration attained intracellularly in stimulated platelets (in contrast, earlier studies on platelet vesicles have mostly used unbuffered higher Ca^{2+} concentrations in the range 5–60 μM). CS concentrations in the range of 100–200 units/ml can contribute several millimolar phosphate to the medium. This has a demonstrable stimulatory

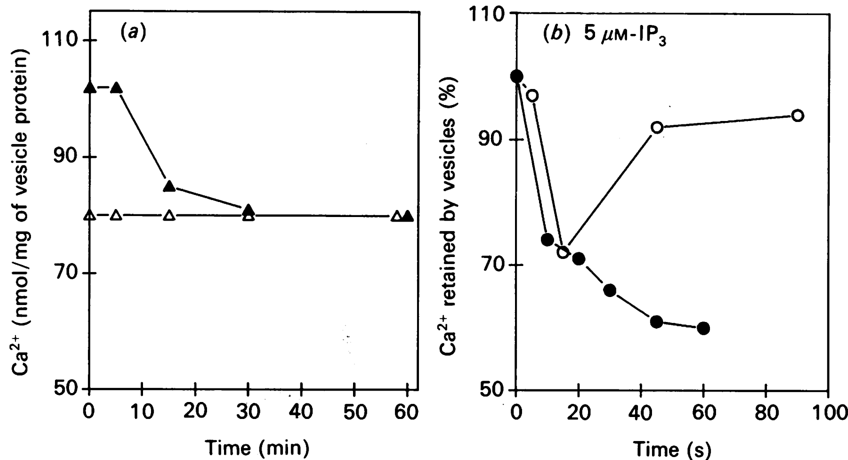

Fig. 1. Stability of intravesicular $^{45}Ca^{2+}$ in formaldehyde/EGTA-treated vesicles

(*a*) Vesicles were preloaded with $^{45}Ca^{2+}$ for 60 min and then added to Ca^{2+}-free buffer (▲) or to formaldehyde/EGTA solution (△). Samples were taken at indicated times for measurement of vesicle $^{45}Ca^{2+}$. (*b*) Time course of release of $^{45}Ca^{2+}$ from platelet vesicles. Vesicles preloaded with $^{45}Ca^{2+}$ were stimulated with 5 μM-IP_3 in the absence (○) or the presence (●) of 4 mM-EGTA. Samples of the suspension were taken at times indicated, quenched with formaldehyde/EGTA, and analysed for $^{45}Ca^{2+}$.

effect on Ca^{2+} uptake if the medium contains little or no phosphate.

To study effects of CS at low phosphate concentration in the medium, we desalted CS by several cycles of centrifugation in Centricon-10 (Amicon) concentrators with phosphate-free Ca^{2+}-transport buffer containing 50 mM-dithiothreitol. Desalted enzyme completely retained its activity, including the ability to phosphorylate the platelet membrane 23 kDa polypeptide. The desalted CS was without influence on Ca^{2+} uptake by membrane vesicles, irrespective of the phosphate content of the medium (Fig. 2).

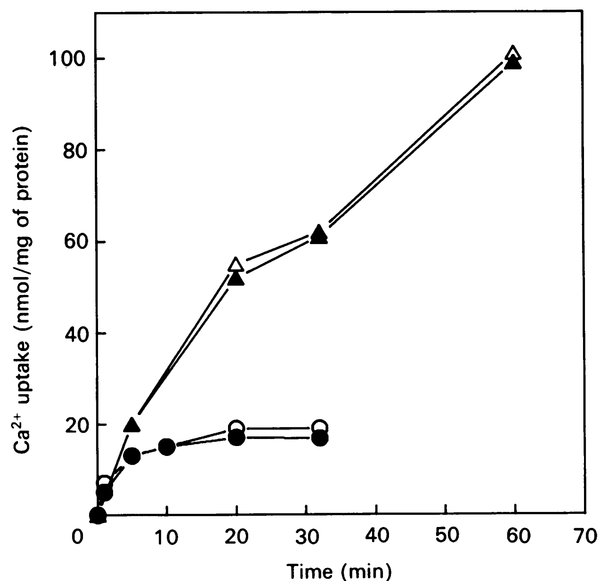

Fig. 2. Effect of CS on Ca^{2+} uptake at 0–20 mM-phosphate in the medium

Ca^{2+} uptake was measured as described in the Materials and methods section in the absence (○, △) or the presence (●, ▲) of 200 units of desalted CS/ml. The phosphate content of the medium was 2 mM (○, ●) or 20 mM (△, ▲). For clarity, experiments at zero phosphate are not shown; Ca^{2+} uptake reached a peak at 3 nmol/mg of protein, and was unaffected by CS.

Ca^{2+} uptake in the presence of PKI and the peptide inhibitor IP-20

Commercial preparations of PKI have been reported to inhibit Ca^{2+} uptake (Hettasch & LeBreton, 1987; Adunyah & Dean, 1987), suggesting that cyclic AMP-dependent protein kinase promotes Ca^{2+} uptake. However, conclusions drawn from these experiments are questionable, because the commercial PKI preparations are grossly impure, exhibiting many polypeptides on SDS/polyacrylamide gels (Fig. 3*a*). In our experiments inhibition of Ca^{2+} uptake by PKI was highly variable, although inhibition of protein phosphorylation by CS was clearly demonstrable (results not shown). One pig heart PKI preparation slightly inhibited Ca^{2+} uptake (Table 1), whereas others from pig or bovine heart of equal potency against CS had virtually no effect on Ca^{2+} uptake (e.g. Fig. 4), suggesting that kinase inhibition was not responsible for the effect on Ca^{2+} uptake.

IP-20, the peptide inhibitor of CS, was without effect on Ca^{2+} uptake in either the presence or the absence of exogenous CS (Fig. 4). The IP-20 completely blocked endogenous protein phosphorylation and that by 125 units of CS/ml, the latter representing an activity far in excess of the endogenous membrane kinase activity (Fig. 3*b*, tracks A–H).

Ca^{2+} release by IP_3 in the presence of CS, PKI and the peptide inhibitor IP-20

Desalted CS (125 units/ml) produced maximal phosphorylation of a 23 kDa membrane polypeptide within 10 min (Fig. 3*b*), but CS (in the absence or presence of inhibitors) did not affect Ca^{2+} release from platelet vesicles (Fig. 4). In the absence of added CS, Enouf *et al.* (1987) reported that PKI (Sigma, type II P 8140) at 80 μg/ml decreased Ca^{2+} release by 50 %, and at 160 μg/ml completely abolished release of Ca^{2+} by 20 μM-IP_3. This effect of PKI was attributed to inactivation of weak endogenous cyclic AMP-dependent kinase activity. In our experiments, the release of Ca^{2+} by IP_3 in

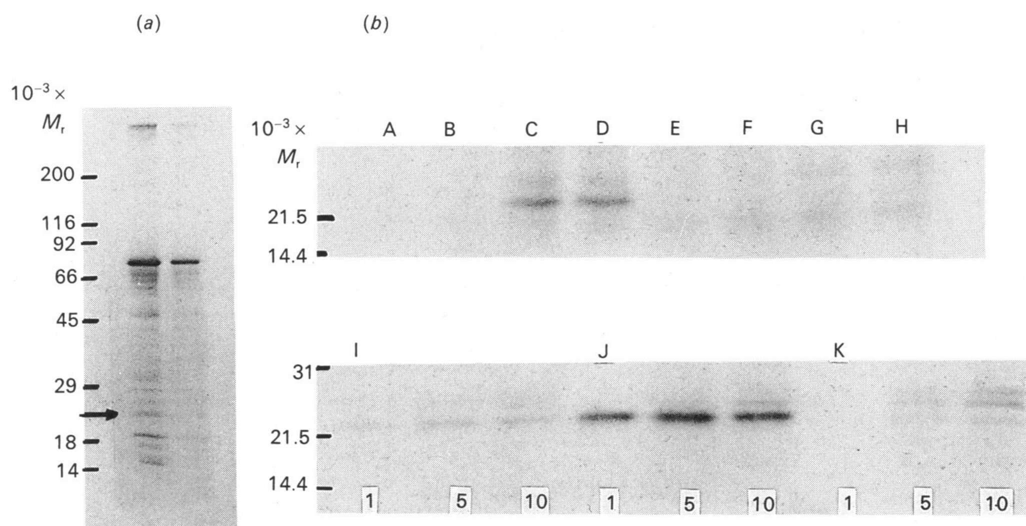

Fig. 3. Polypeptide compostion of type II PKI

(*a*) Type II PKI (20 and 10 μg per lane) was subjected to SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue. At least 20 polypeptide bands are visible; arrow indicates expected position of purified PKI reported by Szmigielski *et al.* (1977). (*b*) Radioautographs of platelet membrane vesicle phosphoproteins. Top panel: membranes were incubated for 10 min with 14 mM-phosphate, 100 μM-$CaCl_2$, 139 μM-EGTA, 100 μM-ATP (20 μCi of [γ-^{32}P]ATP/ml) and 125 units of CS/ml. Duplicate samples were taken for SDS/polyacrylamide-gel electrophoresis and radioautography. CS phosphorylated 23 kDa polypeptide (C, D), which was abolished by 4 μM-IP-20 (A, B). Phosphorylation by endogenous kinase activity (no added CS) (G, H), and inhibition by 4 μM-IP-20 (E, F), are shown. Positions of M_r standards are shown. Bottom panel: time course of membrane phosphorylation in IP_3-treated membranes. Reactions were started by addition of [^{32}P]ATP, and membrane samples were taken at 1, 5 and 10 min intervals as marked and analysed by SDS/polyacrylamide-gel electrophoresis and radioautography: I, phosphorylation without IP_3; J, plus 5 μM-IP_3 added at zero time; K, IP_3 plus 4 mM-EGTA.

the absence of CS was unaffected by 100 μg of type II PKI/ml (Fig. 4). More significantly, a high concentration of IP-20 (4 μM) also had no effect on IP_3-induced Ca^{2+} release (Fig. 4).

ATP and protein phosphorylation are not necessary for IP_3-induced Ca^{2+} release

Responses to IP_3 were measured in the absence of ATP to assess further the possible involvement of protein kinases. In one set of experiments, vesicles were preloaded with $^{45}Ca^{2+}$ plus ATP, and then thoroughly washed to remove the nucleotide. These vesicles released Ca^{2+} normally in response to IP_3. In other experiments, vesicles were passively loaded with $^{45}Ca^{2+}$ without ATP. $^{45}Ca^{2+}$ was added to the medium (devoid of ATP) just before sonication of washed platelets. This permitted $^{45}Ca^{2+}$ to enter the vesicles during the process of membrane disruption and resealing. The vesicles were isolated as usual and washed to remove any extravesicular $^{45}Ca^{2+}$. The vesicles were fully capable of releasing Ca^{2+} in response to IP_3 in the total absence of ATP (Table 2). These experiments show that protein phosphorylation involving an endogenous vesicle kinase is not required for response to IP_3.

In some experiments a polypeptide of approx. 23 kDa was phosphorylated when vesicles were exposed to IP_3 in the presence of [^{32}P]ATP. We do not know if this protein is the same as the substrate for CS. Phosphorylation was totally abolished by EGTA (Fig. 3*b*, tracks I–K), demonstrating that it was dependent on extravesicular Ca^{2+}. Although no phosphorylation was observed in the presence of EGTA, $^{45}Ca^{2+}$ release by IP_3 was actually increased, since EGTA prevents Ca^{2+} re-uptake (e.g. see Fig. 1*b*). These results are in agreement with other findings that ATP is not required for release of Ca^{2+} by IP_3 (Adunyah & Dean, 1986*b*; Meyer *et al.*, 1988).

Table 1. Effects of protein kinase inhibitors on Ca^{2+} uptake and Ca^{2+} release by IP_3

Three batches of platelet vesicles (0.1 mg/ml), (*a*), (*b*) and (*c*), took up $^{45}Ca^{2+}$ for 60 min in the presence of 1.9 mM-ATP and 20 mM-potassium phosphate (free [Ca^{2+}] = 1.1 μM). EGTA (4 mM) was then added, followed by 5 μM-IP_3. The reactions were quenched with formaldehyde (see the Materials and methods section). The concentrations of other ingredients were: CS, 125 units/ml; PKI (type III protein kinase inhibitor, Sigma) 0.33 mg/ml; IP-20 (peptide inhibitor) 4 μM.

	Ca^{2+} uptake (nmol/mg of protein)			IP_3-induced Ca^{2+} release (nmol/mg of protein)		
Batch...	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
Control	112	142	115	33.6	42.6	24.6
+IP-20	119	148	119	35.7	44.4	21.4
+PKI	118	143		21.2	25.7	
+CS	113	148	113	29.4	44.4	20.9
+CS+PKI	122	142		22.0	25.6	
+CS+IP-20		148	120		39.0	24.0

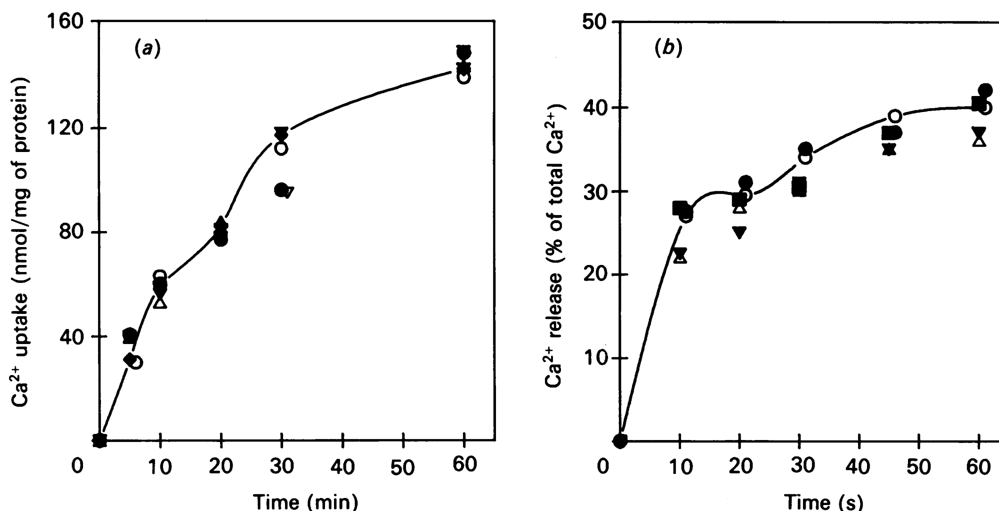

Fig. 4. Effects of IP-20, PKI and CS on time course of $^{45}Ca^{2+}$ uptake and Ca^{2+} release by IP_3

(*a*) Ca^{2+} uptake in the presence of 20 mM-phosphate (◆, continuous line), plus CS (125 units/ml) (○), plus PKI (100 μg/ml) (△), plus 4 μM-IP-20 (●), CS+PKI (▼) and CS+IP-20 (▽). (*b*) Vesicles were preloaded with $^{45}Ca^{2+}$ and then exposed to 5 μM-IP_3. Ca^{2+} release was measured as described in the Materials and methods section. Control (○) release is fitted by the continuous line. Symbols depict Ca^{2+} release in the presence of IP-20 (●), PKI (■), CS (△) and CS+IP-20 (▼). All additions were made 10 min before IP_3.

Table 2. IP_3-induced Ca^{2+} release in the presence or absence of ATP

(*a*) Platelet vesicles minus ATP were sonicated in the presence of $^{45}CaCl_2$ (100 μCi/3 ml of platelet suspension). Vesicle fraction isolated as described by O'Rourke *et al.* (1985) was suspended to 0.5 mg of protein/ml and assayed for IP_3-stimulated Ca^{2+} release at room temperature in the absence of ATP or after addition of 100 μM-ATP. (*b*) Vesicles loaded with $^{45}Ca^{2+}$ were washed at 4 °C three times with ATP-free medium and then tested for Ca^{2+} release by IP_3 (5 μM).

(*a*)	Vesicles	$^{45}Ca^{2+}$ in vesicles		Ca^{2+} released (%)
		Control	$+IP_3$	
	−ATP	4754	3654	24
	+ATP	4912	3920	21

(*b*)	Vesicles	Ca^{2+} in vesicles (nmol)		Ca^{2+} released (nmol)
		Control	$+IP_3$	
	−ATP	80	70.4	9.6
	+ATP	75	65.2	9.8

DISCUSSION

We find no evidence to support the contention that cyclic AMP-dependent protein kinase is either necessary for, or stimulatory to, IP_3-induced Ca^{2+} release from platelet membrane vesicles. This conclusion is based on two observations: (1) desalted catalytic subunit of cyclic AMP-dependent protein kinase did not stimulate Ca^{2+} release by IP_3, and (2) IP-20, the potent polypeptide inhibitor of CS, had no effect on Ca^{2+} release by IP_3 in either the absence or the presence of CS. IP-20 was present at a concentration that completely blocked CS activity far in excess of that present in the membranes. The peptide inhibitor IP-20 represents the active site of the 24 kDa skeletal-muscle protein kinase inhibitor protein (PKI), and is highly specific for the catalytic subunit of the cyclic AMP-dependent protein kinase (Cheng *et al.*, 1986). The failure of the peptide to inhibit Ca^{2+} release by IP_3 makes the involvement of cyclic AMP-dependent protein kinase in this reaction highly dubious.

Enouf *et al.* (1987) reported that a commercial preparation of PKI could abolish release of Ca^{2+} by IP_3. In our hands, one lot of the inhibitor from pig heart (Sigma, type III) only partially decreased both Ca^{2+} uptake and the release of Ca^{2+} by IP_3, but inhibitor from bovine heart (Sigma, type II), the same preparation as used by Enouf *et al.* (1987) (Sigma P 8140), was entirely lacking in either effect. Gel electrophoresis of these PKI preparations reveals them to be highly impure with regard to protein content. They also contain significant amounts of glycerophosphate and EDTA. We cannot account for the lack of effect of type II PKI in our experiments, but it is possible that various lots differ in their content of some inhibitory material. Also, Enouf *et al.* (1987) exposed vesicles to PKI for 80 min, a long time considering that reaction of the kinase inhibitor with CS is virtually immediate [see Table II of Ashby & Walsh (1972)]. Such prolonged interaction might enhance non-specific effects of impurities.

Another very crude commercial preparation of PKI from rabbit muscle (Sigma) was reported to release very rapidly up to 45% of accumulated Ca^{2+} when added to preloaded platelet vesicles (Hettasch & LeBreton, 1987). It is difficult to attribute the rapid release of Ca^{2+} by PKI to inhibition of CS, because it occurred with 55 μM-Ca^{2+} in the medium, a condition in which Ca^{2+} uptake was

completely unaffected by added CS (Hettasch & LeBreton, 1987). Spontaneous release of Ca^{2+} from platelet vesicles does not occur after ATP depletion (Adunyah & Dean, 1986*a*), which suggests that the PKI preparation was able to release Ca^{2+} by a mechanism unrelated to protein phosphorylation. In our experiments 4 μM-IP-20 did not release any Ca^{2+} when added to preloaded membrane vesicles (results not shown). We conclude that inhibition of cyclic AMP-dependent protein kinase is not a mechanism for Ca^{2+} release.

The independence of IP_3-mediated Ca^{2+} release from protein kinase activity is also supported by the lack of requirement for ATP and protein phosphorylation, and is in agreement with other work (Adunyah & Dean, 1986*b*; Meyer *et al.*, 1988). Protein phosphorylation when seen in platelet vesicles exposed to IP_3 was entirely indirect, as it occurred after release of Ca^{2+} from vesicles into the medium and was abolished by EGTA. In contrast, EGTA actually enhanced net release of Ca^{2+} by IP_3, by preventing its re-uptake.

Although the main purpose of our work was to evaluate the role of cyclic AMP-dependent protein kinase in the release of Ca^{2+} by IP_3, the effect of CS on Ca^{2+} uptake was also of interest. The role of protein phosphorylation in Ca^{2+} uptake by platelet vesicles is unclear. Several groups have reported that cyclic AMP-dependent protein kinase stimulates the early phase of Ca^{2+} uptake and the phosphorylation of a 23 kDa polypeptide suggested to resemble phospholamban (Kaser-Glanzmann *et al.*, 1977, 1979; Adunyah & Dean, 1987; Hettasch & LeBreton, 1987). The last two studies used commercial preparations of CS. In contrast, LePeuch *et al.* (1983), using enzyme purified in their laboratory, observed no stimulation of Ca^{2+} uptake by CS, but rather an increase of Ca^{2+} efflux. They also reported that the 23 kDa polypeptide substrate for the enzyme bore no similarity in properties to phospholamban.

We find no evidence of a role for cyclic AMP-dependent protein kinase in Ca^{2+} uptake. When phosphate was low or absent the widely used commercial preparation of CS increased Ca^{2+} uptake, but in our experiments that effect was attributable entirely to the contribution of phosphate anion from the freeze-dried preparations of the enzyme. CS from which phosphate was removed retained its enzymic activity, phosphorylated the 23 kDa polypeptide, but no longer stimulated Ca^{2+} uptake at any concentration of phosphate in the medium.

Adunyah & Dean (1987) found that bovine heart PKI (Sigma, type II) reversed the effect of CS on Ca^{2+} uptake, strengthening the presumed cause-and-effect link between protein phosphorylation and Ca^{2+} uptake. However, close observation of the data (their Fig. 3) reveals that PKI inhibited only the first 25–50 s of $^{45}Ca^{2+}$ uptake. Thereafter, the rate of $^{45}Ca^{2+}$ uptake was the same as in the absence of the inhibitor. This finding suggests that an initial binding reaction, rather than transport, was affected by both the CS and PKI preparations. Our failure to observe such effects may be due to our method of measuring Ca^{2+} uptake, which eliminates surface-bound Ca^{2+}.

Elevation of cyclic AMP concentration represents a powerful mechanism for inhibition of Ca^{2+} mobilization in intact platelets (Rink & Smith, 1983; Feinstein *et al.*,1983; Yamanishi *et al.*, 1983), and is apparently due to interference with the release of internal Ca^{2+} stores and the surface membrane permeability to Ca^{2+}. The inhibition of intracellular Ca^{2+} release can largely be explained by suppression of phosphatidylinositol 4,5-bisphosphate hydrolysis (Imai *et al.*, 1983; Bushfield *et al.*, 1985; Zavoico *et al.*, 1985; Feinstein *et al.*, 1985) and IP_3 production (Watson *et al.*, 1984) by cyclic AMP. In the case of thrombin-induced responses, cyclic AMP may affect G-protein/receptor interactions (Knight & Scrutton, 1984), and there is evidence that it prevents receptor-induced dissociation of G-protein oligomers (Halenda *et al.*, 1986) and/or binding of thrombin to its receptors (Lerea *et al.*, 1987), thereby preventing activation of phospholipase C and subsequent Ca^{2+} mobilization. It is quite possible that cyclic AMP affects free Ca^{2+} concentrations in the cytoplasm by other actions, but these remain unproven. Further experimentation may be profitably directed towards studying the specific role of membrane binding of Ca^{2+} as a mechanism to decrease cytosolic free Ca^{2+}, and the possibility that such a mechanism may be modulated by cyclic AMP.

M.B.F. is supported by N.I.H. grants HL 18937 and HL 37422.

REFERENCES

Adunyah, S. E. & Dean, W. L. (1985) Biochem. Biophys. Res. Commun. **128**, 1274–1280

Adunyah, S. E. & Dean, W. L. (1986*a*) J. Biol. Chem. **261**, 3122–3127

Adunyah, S. E. & Dean, W. L. (1986*b*) J. Biol. Chem. **261**, 13071–13075

Adunyah, S. E. & Dean, W. L. (1987) Biochim. Biophys. Acta **930**, 401–409

Ashby, C. D. & Walsh, D. A. (1972) J. Biol. Chem. **247**, 6637–6642

Authi, K. & Crawford, N. (1985) Biochem. J. **230**, 247–253

Bushfield, M., McNicol, A. & MacIntyre, D. E. (1985) Biochem. J. **232**, 267–271

Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986) J. Biol. Chem. **261**, 989–992

Enouf, J., Giraud, F., Bredoux, R., Bourdeau, N. & Levy-Toledano, S. (1987) Biochim. Biophys. Acta **928**, 76–82

Feinstein, M. B., Egan, J. J., Sha'afi, R. I. & White, J. (1983) Biochem. Biophys. Res. Commun. **113**, 598–604

Feinstein, M. B., Zavoico, G. B. & Halenda, S. P. (1985) in The Platelets: Physiology and Pharmacology (Longenecker, E., ed.), pp. 237–269, Academic Press, New York

Hack, N., Croset, M. & Crawford, N. (1986) Biochem. J. **233**, 661–668

Halenda, S. P., Volpi, M., Zavoico, G. B., Sha'afi, R. I. & Feinstein, M. B. (1986) FEBS Lett. **204**, 341–346

Hettasch, J. M. & LeBreton, G. C. (1987) Biochim. Biophys. Acta **931**, 49–58

Imai, A., Hattori, H., Takahashi, M. & Nozawa, Y. (1983) Biochem. Biophys. Res. Commun. **90**, 92–98

Kaser-Glanzmann, R., Jakabova, M., George, J. N. & Luscher, E. F. (1977) Biochim. Biophys. Acta **466**, 429–440

Kaser-Glanzmann, R., Gerber, E. & Luscher, E. F. (1979) Biochim. Biophys. Acta **558**, 344–347

Knight, D. E. & Scrutton, M. C. (1984) Nature (London) **309**, 66–68

LePeuch, C. J., LePeuch, D. A. M., Katz, S., DeMaille, J. G., Hincke, M. T., Bredoux, R., Enouf, J., Levy-Toledano, S. & Caen, J. (1983) Biochim, Biophys. Acta **731**, 456–464

Lerea, K. M., Glomset, J. A. & Krebs, E. G. (1987) J. Biol. Chem. **262**, 282–288

Meyer, T., Holowka, D. & Stryer, L. (1988) Science **240**, 653–655

O'Rourke, F. O., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) J. Biol. Chem. **260**, 956–962

Rink, T. J. & Smith, S. W. (1983) J. Physiol. (London) **338**, 66*P*–67*P*

Szmigielski, A., Guidotti, A. & Costa, E. (1977) J. Biol. Chem. **252**, 3848–3853

Watson, S. P., McConnell, R. T. & Lapetina, E. G. (1984) J. Biol. Chem. **259**, 13199–13203

Yamanishi, J., Kawahara, Y. & Fukuzaki, H. (1983) Thromb. Res. **32**, 183–188

Zavoico, G. B., Halenda, S., Chester, D. & Feinstein, M. B. (1985) in Prostaglandins, Leukotrienes and Lipoxins (Bailey, J. M., ed.), pp. 345–356, Plenum, New York

Received 6 June 1988; accepted 4 August 1988