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CALMODULIN STIMULATES HUMAN PLATELET PHOSPHOLIPASE A_2

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SUMMARY

Calmodulin is a ubiquitous Ca^{2+}-binding protein, mediating the effect of Ca^{2+} on many enzyme systems and cellular reactions. Phospholipase A_2 (phosphatide-2-acyl-hydrolase, EC 3.1.1.4) which governs the level of arachidonic acid in human platelets, requires Ca^{2+} for maximum activity. Results presented herein suggest that the stimulation of phospholipase A_2 by Ca^{2+} is also mediated through calmodulin. This finding adds to the growing list of enzymes whose activities are regulated by calmodulin.

INTRODUCTION

Phospholipase A_2 (phosphatide-2-acyl-hydrolase, EC 3.1.1.4), which catalyzes the deacylation at the 2 position of phosphoglycerides to produce a free fatty acid and a lysophosphoglyceride, is present in all mammalian tissues (1). The steady state intracellular level of arachidonic acid is generally low (2) and the activity of phospholipase A_2 is believed to be the limiting step in the generation of this fatty acid (3). Arachidonic acid serves as a common precursor of endoperoxides, thromboxanes, and prostaglandins, compounds that figure importantly in platelet aggregation. Using ionophore A23187, Pickett, et al. (4) demonstrated that the mobilization of intracellular Ca^{2+} increased markedly the platelet level of free arachidonic acid, indicating that platelet phospholipase A_2 is stimulated by intracellular Ca^{2+}. Rittenhouse-Simmons, et al. (5, 6) investigated the metal requirements of platelet phospholipase A_2 and showed that Ca^{2+} was required for maximal enzyme activity. The mechanism by which Ca^{2+} stimulates phospholipase A_2 has not been clarified.

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Recent evidence from many laboratories indicates that calmodulin, which was first discovered as an activator of cyclic 3',5'-nucleotide phosphodiesterase (7, 8), is a multifunctional Ca^{2+}-binding protein ubiquitous in eukaryotes. Calmodulin mediates the effects of Ca^{2+} on a variety of enzymes and cellular reactions, including brain adenylate cyclase (9, 10), erythrocyte Ca^{2+}-ATPase (11, 12), myosin light chain kinase (13-16), NAD kinase (17), skeletal muscle phosphorylase kinase (18), Ca^{2+} transport in erythrocytes (19) and sarcoplasmic reticulum (20), phosphorylation of membranes (21, 22), and the disassembly of microtubules (23).

In view of the increasing evidence that calmodulin mediates the effect of Ca^{2+} in many cellular reactions, and the finding that platelet membrane phospholipase A_2 is dependent on Ca^{2+} for maximum activity (5, 6), it was deemed important to examine whether the effect of Ca^{2+} on phospholipase A_2 is mediated by calmodulin. In this report, we present preliminary evidence suggesting that the stimulation of human platelet phospholipase A_2 by Ca^{2+} is indeed mediated through calmodulin.

MATERIALS AND METHODS

Chemicals and Reagents

(1-^{14}C)-2-arachidonyl phosphatidylcholine (55 mCi/mmole) was purchased from Applied Sciences; thin-layer chromatography plates from Brinkman Instruments; (1-^{14}C)-arachidonic acid (56 mCi/mmole) from New England Nuclear; and arachidonic acid from Nuchek Co. Prostaglandins and thromboxane B_2 were gifts from Dr. U. Axen of Upjohn Co.

Preparation of Human Platelet Membranes

Newly expired platelets, collected by centrifugation at 700 x g for 10 min. to remove red blood cells, were sonicated for approximately 20 min. The homogenate was centrifuged at 7,000 x g for 20 min., and the supernatant fluid was centrifuged further at 105,000 x g for 60 min. The sediment was suspended in 50 mM Tris-HCl (pH 8.8) to make a protein concentration about 10 mg/ml. The sample was used fresh or stored at -20°. One unit of human platelets yielded approximately 2 mg of membrane protein.

Assay of Phospholipase A_2 in Platelet Membranes

Each assay mixture (final volume 1 ml) contained 50 muCi of (1-^{14}C)-2-arachidonyl phosphatidylcholine, platelet membranes (approximately 1 mg), 50 mM Tris-HCl (pH 8.8), 0.1 mM Ca^{2+} and 1 uM calmodulin. The substrate was dried under N_2 and sonicated in 20 ul of ethylether to produce an emulsion. The reaction was started by the addition of platelet membranes. After incubation at 37° for 5 min. with constant shaking, the reaction was terminated by the addition of 0.2 ml of ice-cold 0.1 M HCl and 10 ug of arachidonic acid. Carrier arachidonic acid was added to facilitate the isolation of (1-^{14}C)-arachidonic acid. The reaction mixture was extracted 3 times with 2 ml of n-hexane. The organic phase was separated from the aqueous phase by centrifugation at 5,000 x g for 5 min. After phase separation, the tube was

chilled in a methanol-dry ice bath. The hexane phase was removed from the frozen aqueous phase with a Pasteur pipette, and was transferred to a scintillation vial. The extract was evaporated to dryness under a stream of air. The dry lipid residue was dissolved in 5 ml of a Triton X-100, toluene-based scintillation fluid for counting.

Measurement of Thromboxane B_2 Released from Washed Human Platelets

Platelet-rich plasma was passed through a Sepharose-2B column (1.5 x 20 cm) which had been equilibrated with Ca^{2+}-free Tyrode buffer, pH 7.4 (24). The platelets emerged in the void volume of the column, and were well separated from plasma proteins. A fraction of the platelet suspension (0.5 ml) was incubated with (1-^{14}C)-arachidonic acid (2 ug, 0.36 uCi) for 10 min. at 37° with constant stirring in a Payton dual channel aggregometer in the presence or absence of 1 uM calmodulin. After aggregation of the platelets, as monitored by a decrease in light transmission, the reaction was terminated by acidification to pH 3.0 with 0.1 M HCl, and the suspension was extracted 3 times with 2 ml of ethylacetate. The organic phase was pooled and dried under N_2, and the reaction product was separated by thin-layer chromatography, using a solvent system containing ethylacetate:acetic acid (99:1, v/v) (25). The reaction product was identified with an authentic thromboxane B_2 on the same plate (R_f value 0.57). This solvent system clearly separates thromboxane B_2 from PGE_2^1, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$ (25). The radioactive peaks were localized with a Packard 2730 radiochromatogram scanner (Packard Instrument Co.).

Preparation and Assay of Calmodulin

Calmodulin was purified from bovine brain (26, 27). The protein was assayed by its ability to stimulate bovine brain calmodulin-deficient phosphodiesterase (26). The use of bovine brain calmodulin is warranted because it lacks both tissue and species specificity (28).

Protein Determination

Protein was determined according to Lowry (29) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Human platelets contain a considerable amount of calmodulin (30). Four preparations of the platelet membranes which were used as a source of phospholipase A_2 showed a range of 140 to 675 ng of calmodulin per mg of membrane protein. When these platelet membranes were incubated with (1-^{14}C)-2-arachindonyl phosphatidylcholine for 5 min., the reaction mixture generated only one radioactive product which co-migrated with an authentic sample of arachidonic acid on thin-layer chromatography. The release of arachidonic acid in the presence of EGTA reached a plateau level in about 20 min. (Fig. 1). In the presence of Ca^{2+}, the amount of arachidonic acid was increased two-fold, in accord with previous findings that Ca^{2+} stimulates phospholipase A_2 (5, 6). In the presence of exogenous calmodulin, the level of arachidonic acid released was further increased, equivalent to 100-200% above the level obtained in the presence of EGTA. The relatively small increase by exogenous

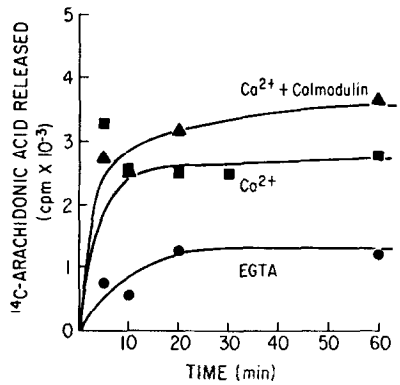

Fig. 1 - Effect of calmodulin and Ca^{2+} on phospholipase A_2 activity in human platelet membranes. Fifty muCi of (1-^{14}C)-2-arachidonyl phosphatidylcholine was dried in a test tube under N_2, and sonicated in 20 ul of ethylether to produce an emulsion. To this tube was added 50 mM Tris buffer (pH 8.8), 1 mg of platelet membranes in a final volume of 1 ml. After incubation at 37° with constant shaking for the times indicated, the amount of arachidonic acid released was determined as described under Materials and Methods. Phospholipase A_2 activity was expressed as arachidonic acid released. EGTA, 1 mM; Ca^{2+}, 0.1 mM; and calmodulin, 1 uM.

calmodulin is presumably due to endogenous calmodulin in the membranes, a phenomenon reminiscent of previous findings on brain adenylate cyclase (9) and erythrocyte plasma membrane Ca^{2+}-ATPase (31). The extent of stimulation of these particulate enzymes by exogenous calmodulin was also small; both preparations retained relatively high levels of endogenous calmodulin. The apparent leveling-off in the release of arachidonic acid after the initial few minutes of incubation probably was due to reacylation. Mammalian tissues contain an active acyltransferase, which catalyzes the reacylation of arachidonic acid to phosphoglycerides. Thus, the level of arachidonic acid in the reaction mixture probably represents an equilibrium between the release from and the reacylation to phosphoglycerides (see Fig. 2).

The activation of phospholipase A_2 by Ca^{2+} and calmodulin was further examined by the use of trifluoperazine, an antidepressive phenothiazine derivative known to block specifically the biological activity of calmodulin (32). In the presence of Ca^{2+}, trifluoperazine is bound to calmodulin with a high affinity, the K_a being 10^7. Calmodulin bound to trifluoperazine is biologically inactive (32). As shown in Table 1, calmodulin itself activated phospholipase A_2; the platelet membranes contained relatively high levels of endogenous Ca^{2+}. Atomic absorption spectrophotometric measurement showed that in 6 different platelet membrane preparations, the Ca^{2+} levels ranged from 0.4 to 0.8 mM. Trifluoperazine and EGTA had no effect on the basal activity of phospholipase A_2; however, they both effectively suppressed the calmodulin-stimulated activity to basal level. Similar observations have been made on adenylate cyclase (33) and cyclic nucleotide phosphodiesterase (32). These

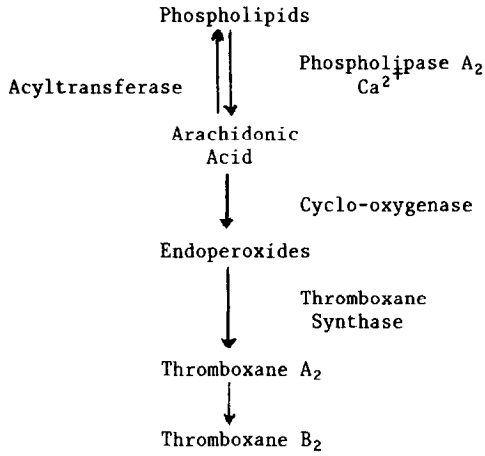

Fig. 2 - A simplified scheme showing the metabolism of arachidonic acid in human platelets. The level of arachidonic acid, which serves as a precursor of endoperoxides and thromboxanes, is governed by its release from and reacylation to phosphoglycerides. Platelet membranes do not further metabolize arachidonic acid. Whole platelets, however, convert arachidonic acid to the endoperoxides and thromboxane A_2. Thromboxane A_2 is unstable, and is readily hydrated nonenzymatically to form the more stable thromboxane B_2.

TABLE 1

EFFECT OF Ca^{2+} AND CALMODULIN ON THE ACTIVITY OF PHOSPHOLIPASE A_2 IN HUMAN PLATELET MEMBRANES

Additions	Activity
	(f mol/mg/5 min.)
None	18.8
EGTA	21.1
Trifluoperazine	18.0
Ca^{2+}	42.3
Calmodulin	38.0
Calmodulin, Ca^{2+}	47.9
Calmodulin, EGTA	18.9
Calmodulin, Ca^{2+}, Trifluoperazine	23.6

Each reaction mixture in a final volume of 1 ml contained 50 muCi of (1-^{14}C)-2-arachidonyl phosphatidylcholine, 50 mM Tris-HCl (pH 8.8), approximately 1 mg of platelet membranes, and where present 0.1 mM Ca^{2+}, 1 uM calmodulin, 1 mM EGTA, or 0.15 mM trifluoperazine. Trifluoperazine was dissolved in 50 mM Tris-HCl (pH 8.8). The reaction mixture was incubated for 5 min. at 37° with constant shaking. Phospholipase A_2 activity is expressed as fmoles of arachidonic acid released per mg protein for 5 min.

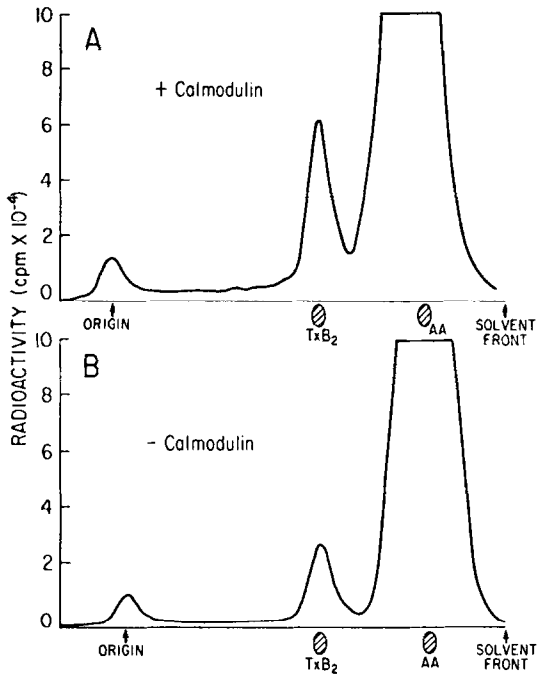

Fig. 3 - Increased formation of thromboxane B_2 by calmodulin in human platelets. Washed platelets (0.5 ml) were incubated with (1-^{14}C)-arachidonic acid (2 ug, 0.36 uCi) at 37°C in the presence (A) or absence (B) of 1 uM calmodulin, with constant stirring (1,200 rpm) in an aggregometer for 10 min. At the end of the incubation, the reaction product was extracted and separated by thin-layer chromatography as described in Materials and Methods. The radioactivity on the thin-layer plates was scanned by a Packard 2730 radiochromatogram scanner. The zone corresponding to TXB_2 was scrapped and extracted with methanol. The extract was dried and the radioactivity was determined in a liquid scintillation counter. Thromboxane B_2 zone in Panel A gave 129,360 dpm, that in Panel B 58,560 dpm.

results support the notion that the stimulation of Ca^{2+} on phospholipase A_2 is mediated through calmodulin.

The stimulation of phospholipase A_2 by calmodulin was corroborated using washed human platelets by measuring the formation of thromboxane B_2 from exogenous arachidonic acid (Fig. 3). After (1-^{14}C)-arachidonic acid was incubated with platelets for 10 min., the reaction was terminated by acidification, and the products were isolated and separated by thin-layer chromatography. A majority of the radioactivity was recovered as unchanged arachidonic acid. One other radioactive spot, identified as thromboxane B_2, was noted. The solvent system used in this experiment clearly separates thromboxane B_2 from PGE_2, $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$, which migrate behind thromboxane B_2 (25). The radioactivity remaining at the origin is presumably phospholipids (34). In the presence of exogenous calmodulin, the amount of thromboxane B_2 formed was increased approximately two-fold. The effect of

exogenous calmodulin on the increased formation of thromboxane B_2 in the intact platelet is of special interest; calmodulin either exerts its effect on the exterior surface of the plasma membrane, or is transported into the platelet and acts on the interior surface. Thrombin, a known physiological agonist of phospholipase A_2, also acts on the intact platelets (35). The arachidonic acid added to the platelets appears to be readily utilized by cyclo-oxygenase and thromboxane synthase (34), neither of them requires Ca^{2+} for activity. Thromboxane synthase catalyzes the synthesis of thromboxane A_2 from the endoperoxides (see Fig. 2). Thromboxane A_2 is short-lived and is rapidly converted non-enzymatically to a more stable product, thromboxane B_2 (36). The increase of thromboxane B_2 from arachidonic acid supports the notion that calmodulin regulates phospholipase A_2 in human platelets.

The effect of Ca^{2+} on phospholipase A_2 has been noted for some time, its mode of action has not been elucidated. Results presented herein suggest that the effect of Ca^{2+}, like the numerous enzymes mentioned under INTRODUCTION, is mediated through calmodulin.

The finding that calmodulin stimulates platelet phospholipase A_2 adds to the growing list of calmodulin-regulated enzymes involved in cellular regulation. The effect and metabolism of cAMP and prostaglandins are interwoven in many instances (37). In controlling the metabolism of cAMP and prostaglandins, calmodulin provides a molecular link between the two classes of cellular regulators, and thus plays a pivotal role in cellular regulation.

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