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STIMULATION OF CALCIUM UPTAKE IN PLATELET MEMBRANE VESICLES BY ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND PROTEIN KINASE

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Summary

The events involved in platelet shape change, aggregation, the release reaction and contraction are thought to be mediated by the availability of Ca^{2+}. Increased cytoplasmic calcium, released from intracellular stores, triggers platelet activity, and increased concentration of adenosine 3',5'-cyclic monophosphate (cyclic AMP) inhibits platelet alterations. We have studied the hypothesis that cyclic AMP may regulate the level of platelet cytoplasmic calcium by stimulating calcium removal by a membrane system. Such a hypothesis would be consistent with the reversibility of most manifestations of platelet activation.

Human platelets were sonicated and unlysed platelets, mitochondria and granules were removed by centrifugation at 19 000 × *g*. Electron microscopy shows that the sediment, after centrifugation of the supernatant at 40 000 × *g* consists to a large extent of membrane vesicles. Such preparations actively concentrate calcium, as measured by the uptake of ^{45}Ca, and also have the maximal calcium-stimulated ATPase activity. Optimal calcium uptake requires ATP and oxalate, and release of calcium from loaded vesicles was stimulated by the calcium ionophore A23187 and inhibited by $LaCl_3$. These data indicate that calcium was being actively concentrated within membrane vesicles.

After washing of such preparations in the absence of ATP, their capacity to take up Ca^{2+} is reduced to an initial value of 2.8 nmol/mg protein per min. In the presence of $2 \cdot 10^{-6}$ M cyclic AMP to which was added a protein kinase preparation from human platelets, up to a 3-fold increase of this rate of uptake was observed.

These results suggest that in platelets, as in muscle, cyclic AMP is a regulatory factor in the control of cytoplasmic calcium. Although the cyclic

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Abbreviation: EGTA, ethyleneglycerol-bis-(β-amino-ethylether)-*N*,*N*'-tetraacetic acid.

nucleotide may have still other functions, it appears likely that the well-known inhibition of many platelet activities by high intracellular cyclic AMP concentrations is directly linked to the stimulation of the removal of Ca^{2+} from the cytoplasm.

Introduction

Blood platelets can be activated by a variety of stimuli to change into a spherical shape with pseudopods, aggregate with other platelets, and release the contents of their storage granules into the surrounding fluid [1]. These events, as well as others involved in platelet activation, initiate the processes of hemostasis and thrombosis and are thought to be mediated by the availability in the cytoplasm of Ca^{2+} [2—5]. An intracellular store of calcium which can be mobilized in the course of platelet activation is suggested by the observations that the platelet release reaction and shape change occur in the absence of extracellular calcium [2—5]. Provided the degree of stimulation does not exceed a critical threshold, shape change and aggregation are reversible and the platelet can resume its disc shape and continue to circulate normally. This is even true for platelets which have undergone the release reaction [6]. The reversibility of these reactions suggests that platelets also have a mechanism for decreasing the concentration of cytoplasmic calcium.

The functional activity of the platelets is closely related to the intracellular cyclic AMP concentration [7]. Increased cyclic AMP levels inhibit platelet activation and agents which activate platelets may decrease cyclic AMP by inducing an inhibition of adenylate cyclase [7,8]. In a variety of cells, cyclic AMP and calcium are interrelated intracellular messengers which mediate cell function [9] and it seemed justified to investigate whether the same is true in platelets. Since platelet membrane fractions can actively concentrate calcium [10,11], we studied the hypothesis that increased cyclic AMP may contribute to the regulation of platelet activity by exerting an effect on intracellular Ca^{2+} levels. In this context it seemed of importance to characterize further the membrane fractions capable of accumulating this cation and to establish beyond doubt that an active pumping mechanism is involved in their activity.

Materials and Methods

Platelet collection and fractionation. Human blood platelets were isolated within 20 h after collection from citrated blood collected for the Central laboratory of the Blood Transfusion Service of the Swiss Red Cross in Berne [12]. The buffy coats were syphoned into a buffered glucose solution and the resulting platelet-rich plasma contained about 20 mM glucose, 12 mM phosphate buffer, pH 6.8, and $3 \cdot 10^9$—$4 \cdot 10^9$ platelets/ml. Platelets were separated from plasma and washed once with 0.15 M NaCl, 0.1% EDTA and then twice with 0.15 M NaCl by centrifugation at room temperature. Washed packed platelets (approx. $5 \cdot 10^{10}$/ml) were resuspended in three volumes of "homogenizing medium" [11] (30 mM KCl, 5 mM $MgCl_2$, 10 mM potassium oxalate and 20 mM Tris · HCl, pH 7.0) to which ATP was added to a final concentration of 2

mM. This suspension was then subjected to maximum intensity sonication (MSE ultrasonic cell disruptor) for 45 s at 4°C.

The disrupted platelets were centrifuged at 19 000 × *g* for 25 min at 4°C. The supernatant fluid was then centrifuged at 40 000 × *g* for 60 min and this sediment was suspended in "homogenizing medium" and re-centrifuged at 12 000 × *g* for 10 min to remove coarse aggregates. The resulting suspension was the standard preparation used in these experiments, which is referred to as the "40 000 × *g* sediment". In some experiments the 40 000 × *g* supernatant fluid was centrifuged at 100 000 × *g* for 60 min. Sediments were resuspended in "homogenizing medium" and the protein concentration was adjusted to 0.4% unless otherwise stated. For the experiments with cyclic AMP, ATP was omitted from the "homogenizing medium" and the 40 000 × *g* sediment was washed once in buffer without ATP in an attempt to diminish endogenous cyclic AMP and protein kinase activity.

Calcium uptake activity. The platelet fraction to be tested (0.4 ml) was added to 3.6 ml of "incubation medium" [11] (100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris · HCl (pH 7.0) and 50 μM $CaCl_2$ containing ^{45}Ca in a final concentration of 0.05 μCi/ml). Since the oxalate concentration in the original suspending "homogenizing medium" was 10 mM, the final oxalate concentration was 1 mM. Samples were stirred continuously at room temperature with a magnetic stirrer and 0.5-ml aliquots were withdrawn at intervals and filtered through membranes of pore size 0.15 μm (type SM 11 308, Sartorius-Membranfilter GmbH, D-3400 Göttingen). ^{45}Ca activity of the filtrate was determined by liquid scintillation counting: 0.1 ml sample plus 11 ml scintillation fluid (1.5 g Permablend III (Packard) + 335 ml xylol + 165 ml Triton X-100; 10 ml of this mixture + 1 ml water) in a Packard Tri-Carb model 2450. The zero time ^{45}Ca activity was determined from 0.1 ml of the incubation mixture without filtration. Control experiments demonstrated that the filter retained no ^{45}Ca from the plain "incubation medium". Calcium uptake was calculated from the decrease of radioactivity in the filtrates and expressed as nmol calcium per mg protein. Protein was estimated by the Biuret method [13].

ATPase assay. Membrane sediments to be assayed for calcium-activated ATPase activity were resuspended in "homogenizing medium" without oxalate and ATP and protein was adjusted to 0.5%.

Then 0.5 mg protein were incubated in a volume of 1 ml containing 93 μmol KCl, 5 μmol $MgCl_2$, 20 μmol Tris · HCl, pH 7.0, 2 μmol ATP and 1 μmol EGTA or varying amounts of $CaCl_2$ for 30 min at 37°C, then the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid and the mixture centrifuged. The released P_i was determined by a slight modification of the method of Rockstein and Herron [14] to make it more sensitive by reduction of the volumes of reagents: 1 ml trichloroacetic acid-supernatant fluid plus 2 ml acid molybdate reagent plus 0.5 ml $FeSO_4$ solution. Absorbance was measured at 720 nm.

Platelet protein kinase. Protein kinase was partially purified from human platelets by the methods of Kaulen and Gross [15] and Booyse et al. [16]. 10 ml of washed packed platelets were resuspended in 30 ml of buffer (20 mM Tris · HCl, 2 mM EDTA, pH 7.4) and sonicated at 4°C for 60 s. This suspension

was centrifuged at 48 000 × *g* for 30 min and the supernatant fluid was adjusted to pH 5.5 with 1% acetic acid and placed in ice for 30 min. This mixture was centrifuged at 48 000 × *g* for 45 min, the supernatant fluid was adjusted to pH 7.0 with 0.1 M Tris, then $(NH_4)_2SO_4$ was added to a final concentration of 32.5% (w/v) and the suspension placed in ice for 30 min. The precipitate was collected by centrifugation at 10 000 × *g* for 30 min and redissolved in 2 ml of 10 mM Tris · HCl, pH 7.4, and dialyzed against this buffer for 36 h at 4° C. Protein kinase activity was determined by phosphorylation of histone by [γ-^{32}P]ATP in the assay procedure of Booyse et al. [16] except that the reaction temperature was 37° C instead of 30° C. The incubation mixture contained 0.15 mg/ml histone, $2 \cdot 10^{-6}$ M cyclic AMP, 0.25 mg/ml protein kinase and 5 μM [γ-^{32}P]ATP (spec. act. 80 Ci/μmol). After the protein was taken through two cycles of precipitation in trichloroacetic acid and redissolving in NaOH, the final NaOH protein solution was neutralized with acetic acid and the radioactivity determined in the scintillation fluid described above. The specific protein kinase activity of this preparation expressed as pmol ^{32}P transferred from [γ-^{32}P]ATP to histone by 1 mg of enzyme protein per min at 37° C was 158 without cyclic AMP and 607 with $2 \cdot 10^{-6}$ M cyclic AMP. This corresponds to a 3.8-fold increase of the phosphate transfer. No phosphorylation of histone occurred with cyclic AMP alone.

Electron microscopy was performed with a Siemens Elmiscope 5300. Fixation and contrasting of preparation was done according to the method described by Baudhuin et al. [17].

Materials. Cyclic AMP was obtained from Fluka AG, Buchs, Switzerland; ^{45}Ca from the Schweizerisches Institut für Reaktorforschung, Würenlingen, Switzerland and [γ-^{32}P]ATP from the Radiochemical Centre, Amersham, U.K. A23187 was a gift from Dr. R.L. Hamill, Eli Lilly, Indianapolis, Ind., U.S.A.

Results

Calcium uptake by platelet fractions

Fig. 1 demonstrates the calcium uptake activity of the whole platelet sonicate and of three fractions separated by centrifugation. The only fraction with an increased specific activity was the 40 000 × *g* sediment. In agreement with the findings of Robblee et al. [11] the calcium uptake was found to be optimal only in the presence of ATP and oxalate. The 40 000 × *g* sediment, used in all subsequent experiments, consisted entirely of membrane vesicles and fragments, with no contamination by mitochondria or granules (Fig. 2). The membrane vesicles of the 100 000 × *g* sediment were smaller and were not active in calcium uptake. The time course of the uptake of calcium was dependent on both calcium and protein concentrations, but with the conditions used in these experiments calcium loading of the vesicles reached a maximum only after 40—60 min. The maximum calcium uptake at 60 min was usually 80—120 nmol/mg protein but in experiments performed over 12 months considerable variations were not unusual. When platelet-rich plasma or washed platelets suspended in buffer (0.12 M NaCl, 0.03 M Tris · HCl, pH 7.4) were allowed to stand overnight at 4° C, there was no change in calcium uptake

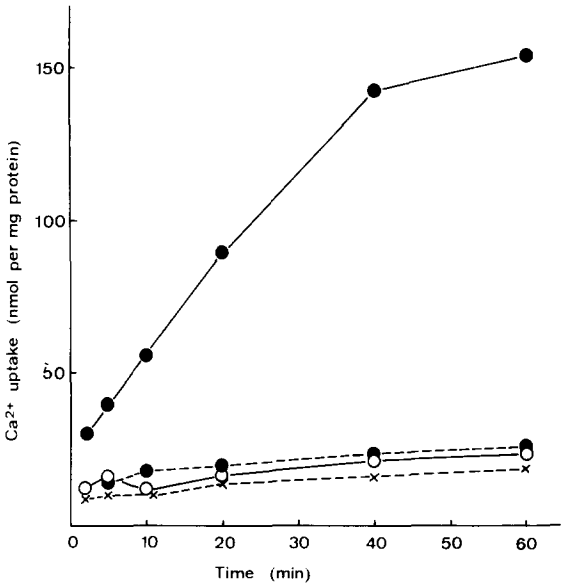

Fig. 1. Calcium uptake by whole platelet sonicate and platelet fractions. Platelets were washed, disrupted by sonication, separated into three sediments by successive centrifugations at 19 000 × *g*, 40 000 × *g* and 100 000 × *g* and then the whole sonicate and sediments were resuspended in "homogenizing medium" to a protein concentration of 1% (except the 40 000 × *g* sediment, which was brought to 0.2%). Calcium uptake was measured as described in Materials and Methods. X- - - - - -X, whole platelet sonicate; ●- - - - -●, 19 000 × *g* sediment; ●———●, 40 000 × *g* sediment; ○———○, 100 000 × *g* sediment.

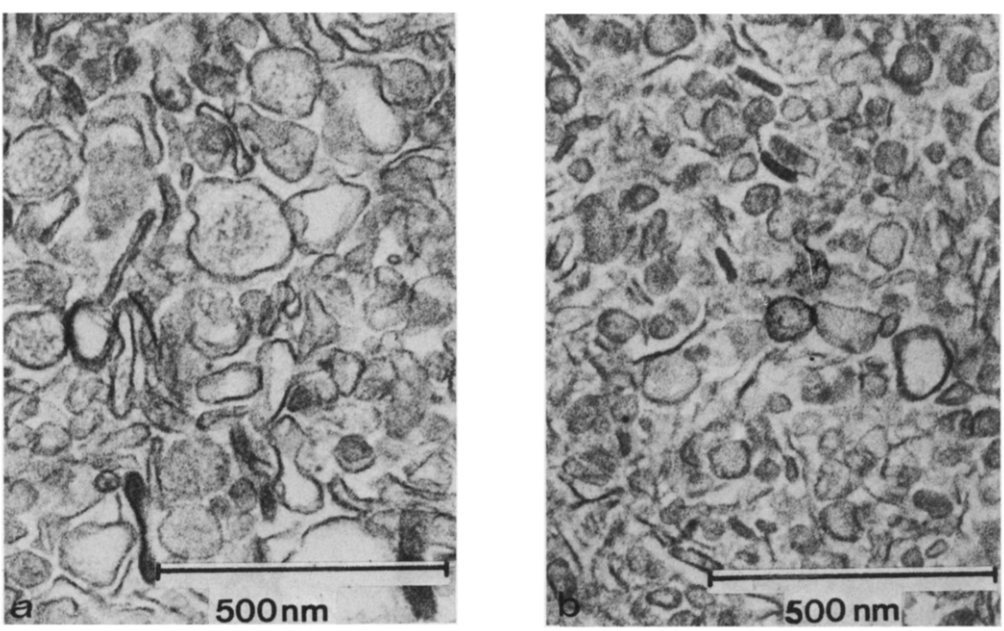

Fig. 2. Electron micrographs of the platelet membrane fractions prepared as described in Materials and Methods. (a) 40 000 × *g* sediment, (b) 100 000 × *g* sediment, × 76 600.

activity. However, if the 40 000 × *g* sediment was kept overnight at 4°C (in "homogenizing medium"), calcium uptake disappeared. Improved stabilization of the calcium uptake activity was achieved by addition of ATP (to a final concentration of 2 mM) to the suspension of washed platelets before sonication and in all subsequent buffers.

Characteristics of calcium uptake by platelet membranes

The calcium-activated ATPase was localized in the same 40 000 × *g* sediment which contained the calcium uptake activity: it is not detectable to any significant extent in the 100 000 × *g* fraction (Fig. 3). A large fraction of calcium taken up by the platelet membrane vesicles was released by the divalent cation ionophore A23187 (Fig. 4). $LaCl_3$ not only decreased calcium uptake by the vesicles (Fig. 5a) but also decreased the release of calcium from the pre-loaded vesicles when they were washed and resuspended in buffer without calcium and ATP (Fig. 5b).

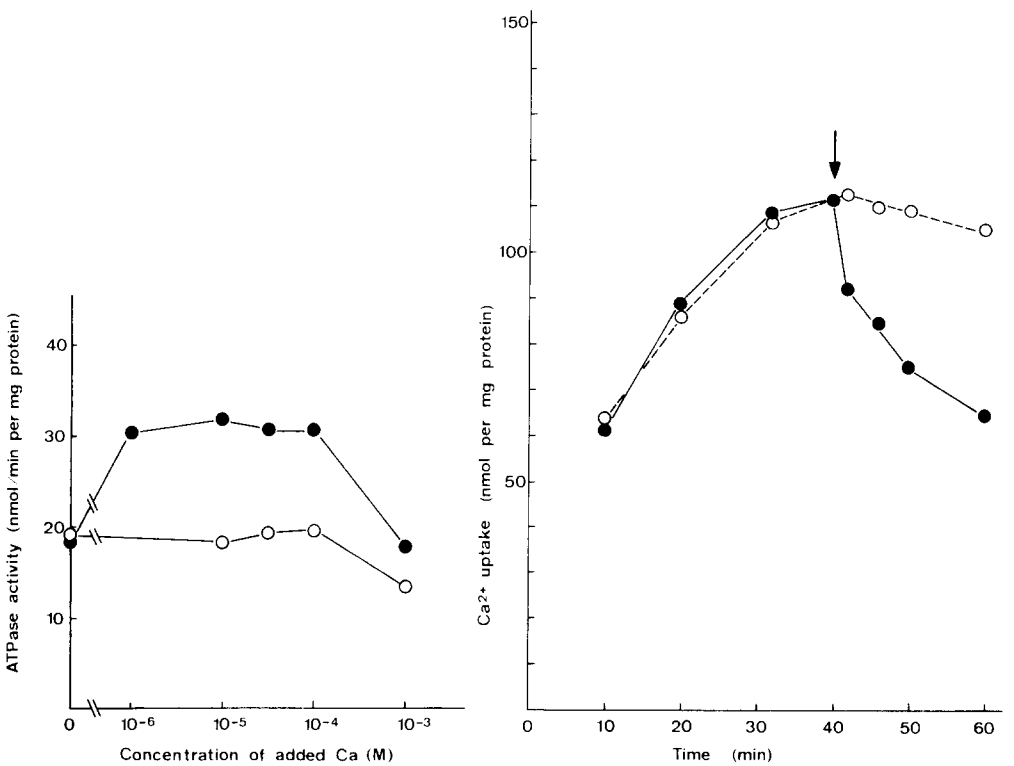

Fig. 3. Activation of Mg^{2+}-ATPase activity in platelet fractions by Ca^{2+}. The 40 000 × *g* and 100 000 × *g* sediments were prepared as described in Materials and Methods. Then 0.5 mg protein of the 40 000 × *g* or the 100 000 × *g* sediment were incubated in a volume of 1 ml containing 93 μmol KCl, 5 μmol $MgCl_2$, 20 μmol Tris · HCl, pH 7.0, 2 μmol ATP and 1 μmol EGTA or varying concentrations of $CaCl_2$ for 30 min at 37°C. The released P_i was determined as described in Materials and Methods. ●——●, 40 000 × *g* sediment; ○——○, 100 000 × *g* sediment.

Fig. 4. Release of calcium from platelet membrane vesicles by ionophore A23187. The 40 000 × *g* sediment was prepared and calcium uptake was measured as described in Materials and Methods. After 40 min incubation (arrow), 4 μl of an ethanol solution of the divalent cation ionophore, A23187, was added to the experimental sample (final A23187 concentration: 3.6 μM) (●——●), and 4 μl of ethanol was added to the control sample (○- - - - -○).

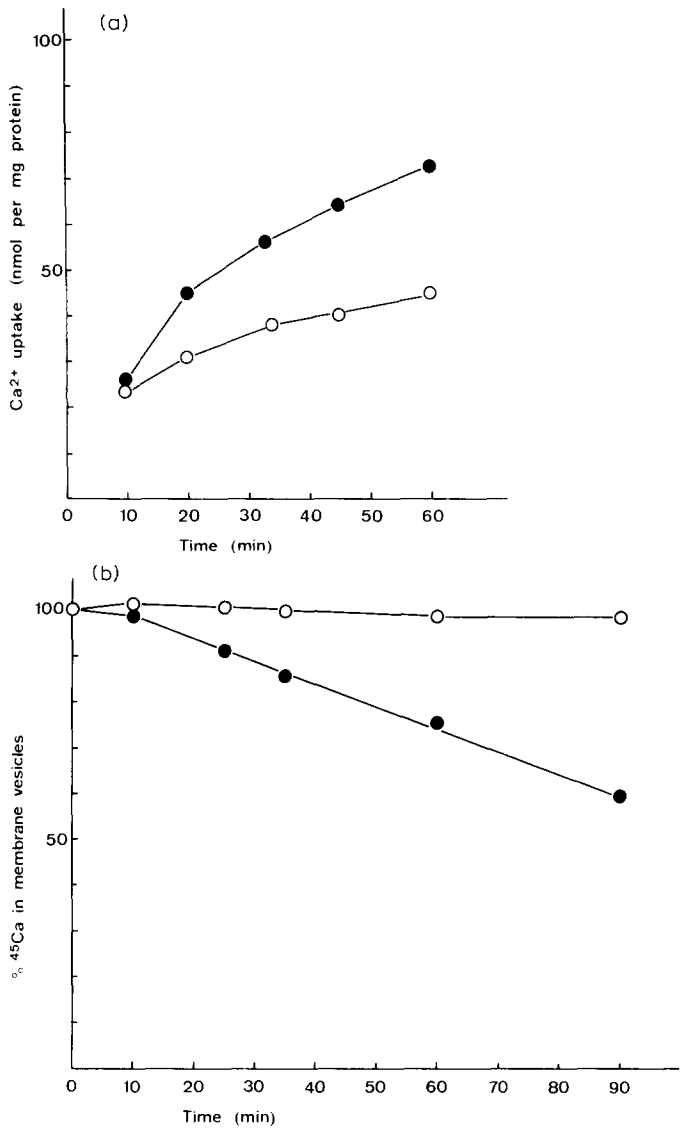

Fig. 5. Effect of lanthanum on calcium uptake and release by platelet membrane vesicles. (a) The 40 000 × *g* sediment was prepared and resuspended in "homogenizing medium". Two 0.4-ml aliquots were then incubated with either 4 µl of "homogenizing medium" alone (●———●) or 4 µl of 50 mM $LaCl_3$ (○———○) for 5 min at room temperature. Then these samples were added to 3.6 ml of "incubation medium" and calcium uptake was measured. (b) The 40 000 × *g* sediment was prepared and incubated with the standard conditions of calcium uptake for 60 min to load the vesicles with ^{45}Ca. Then the suspension was centrifuged at 100 000 × *g* for 60 min and the vesicles were resuspended in "incubation medium" without ATP and calcium and either with (○———○) or without (●———●) 0.5 mM $LaCl_3$. Millipore filtration was performed at intervals, as for calcium uptake. The ^{45}Ca activity of a sample without filtration was designated 100%.

Effect of adenosine 3',5'-cyclic monophosphate on calcium uptake by platelet membranes

The addition of cyclic AMP in a concentration range from 10^{-3} to 10^{-6} M to the 40 000 × *g* sediment, with and without preincubation at room tem-

perature was without effect on calcium uptake.

As shown in Table I, the addition of cyclic AMP together with a cytoplasmic platelet fraction containing protein kinase activity to the 40 000 × *g* sediment had only a minimal effect on calcium uptake. When the sediment was prepared in a "homogenizing medium" devoid of ATP and washed once with this medium, a decrease of uptake was observed, which can be corrected again by the addition of cyclic AMP and protein kinase. Table I shows that this effect was reproducible in a series of seven independent experiments. The uptake values obtained under these conditions always remain below those observed in the original sediment. One reason for this might be the pronounced lability of the isolated membrane vesicles during handling, as already mentioned above.

The time course of the stimulation of calcium uptake by cyclic AMP in the presence of protein kinase is shown in Fig. 6. It is evident that this stimulation is of relatively short duration and is most pronounced in the first samples, tested from 0.5 to 5 min. The initial rate of calcium uptake due to the presence of cyclic AMP and protein kinase calculated from the values listed in Table I is about 2.5 times the control value. Studies on the dependence of this effect

TABLE I

STIMULATION OF CALCIUM UPTAKE IN PLATELET MEMBRANE VESICLES BY CYCLIC AMP AND PROTEIN KINASE

Experiment Nos. 1—6 were done with unwashed, Nos. 7—13 with washed 40 000 × *g* sediments. The unwashed 40 000 × *g* sediment was prepared as indicated in Materials and Methods. The washed 40 000 × *g* sediment was isolated using "homogenizing medium" without ATP and was washed once in this medium at 40 000 × *g* for 60 min. The unwashed and washed membrane vesicles were resuspended in "homogenizing medium" to a final concentration of 0.4% and 0.4-ml aliquots were added to 3.6-ml aliquots of "incubation medium" containing 5 mM ATP, but no Ca^{2+}. To these samples were added: platelet protein kinase (to final concentration 0.54 mg/ml), cyclic AMP (to final concentration $2 \cdot 10^{-6}$ M) or, for control values, the same amounts of buffer. The mixtures were incubated for 20 min at room temperature and then 40 µl of 5 mM $CaCl_2$ (containing ^{45}Ca) were added and calcium uptake was determined. For calculation of the initial Ca^{2+}-uptake rates the 2.5-min values were used. The reported values were obtained in six and seven independent experiments, respectively.

Experiment No.	Ca^{2+} uptake (nmol $Ca^{2+} \cdot mg^{-1} \cdot min^{-1}$)	
	Control	+ cyclic AMP + protein kinase
1	12	12
2	20	20
3	10	14
4	8	10
5	10	11
6	12	13
mean value	12 ± 4.2 *	13 ± 3.8 *
7	2.8	9.2
8	1.6	7.2
9	3.2	8.0
10	2.8	7.2
11	5.6	8.4
12	2.4	5.6
13	1.6	6.2
mean value	2.8 ± 1.3 *	7.4 ± 1.2 *

* Standard deviation.

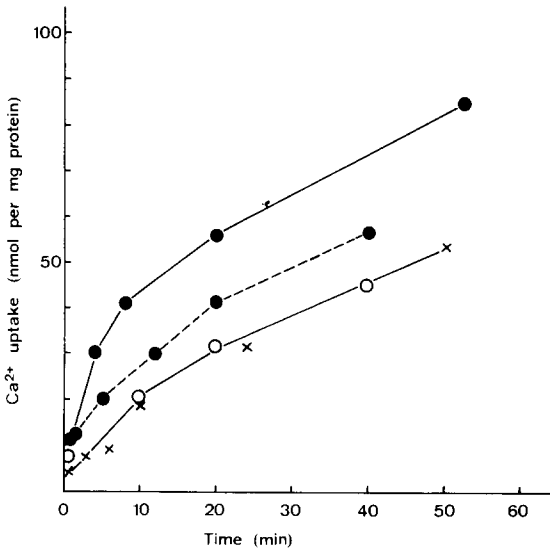

Fig. 6. Stimulation of calcium uptake in platelet membrane vesicles by cyclic AMP and protein kinase. The 40 000 × *g* sediment was isolated using "homogenizing medium" without ATP and after isolation was washed once in this medium at 40 000 × *g* for 60 min. Then the washed membrane vesicles were resuspended in "homogenizing medium" without ATP to a final concentration of 0.4% and 0.4 ml aliquots were added to 3.6-ml aliquots of "incubation medium" without ATP and Ca^{2+}. To these samples were added: platelet protein kinase (to final concentration 0.54 mg/ml), cyclic AMP (to final concentration2 · 10^{-6}M) and ATP (to final concentration 5 mM): ●———●; cyclic AMP and ATP in the same concentrations but without protein kinase: ●- - - - -●. To the control samples were added equivalent amounts of buffer with (○———○) or without (X) ATP. These mixtures were incubated for 20 min at room temperature and then ATP was added to the control sample without ATP, and to all samples were added 40 µl of 5 mM $CaCl_2$ (containing ^{45}Ca) and the calcium uptake was determined.

on the concentrations of the different components of the system are presently in progress.

Discussion

Characterization of calcium accumulation by platelet membrane vesicles

Our experiments demonstrate an active calcium uptake by membrane vesicles from human platelets, confirming and extending earlier studies by Statland et al. [10] and Robblee et al. [11] who used human and calf platelets, respectively. In particular, we have shown that the 100 000 × *g* sediment, although also containing vesicular material, is devoid of calcium-accumulating and Ca^{2+}-stimulated ATPase activities.

The active transport of calcium to the inside of the vesicles of the 40 000 × *g* sediment rather than only passive binding to the vesicle external surface was demonstrated by five criteria: (1) Calcium uptake was increased by oxalate. (2) ATP was required. (3) The platelet fraction with the most active calcium transport correlated with the greatest activity of calcium-activated ATPase. In other membrane systems, where an active calcium pump has been demonstrated, the same correlation between a calcium-activated ATPase and the calcium transport mechanism has been observed. This is the case for red cells [18], skeletal muscle [19], cardiac muscle [20] and smooth muscle [21]. (4)

The release of calcium from the vesicles by the divalent cation ionophore A23187 demonstrates that the calcium is contained within a membrane. A23187 and an ionophore with similar effect, X537A can release calcium which has been concentrated inside skeletal muscle sarcoplasmic reticulum [22,23], and also from intracellular storage sites within intact platelets [2,4.5]. The observed incomplete release of calcium from the platelet membrane vesicles may be due to trapping of calcium either by a calcium-binding protein, analogous to the proteins on the inner surface of sarcoplasmic reticulum vesicles [24], or as calcium oxalate crystals. (5) The competitive inhibition of both calcium uptake and release by lanthanum suggest a specific transport site for calcium in the platelet membrane vesicles similar to red cell membranes and both sarcolemma and sarcoplasmic reticulum from skeletal muscle [22,25—27]. The decreased loss of calcium from vesicles in the presence of lanthanum suggests that calcium is not displaced from external binding sites but rather prevented from crossing the membrane barrier, perhaps due to blockage of a transmembrane carrier system [26].

Stimulation of calcium uptake in platelet membrane vesicles by cyclic AMP and protein kinase

For over 10 years [28] evidence has accumulated that cyclic AMP has a role in the regulation of platelet functions since, when added to the suspension medium, it inhibits platelet aggregation and release reaction caused by ADP, thrombin, collagen and epinephrine [7]. Furthermore any measures taken to increase intracellular cyclic AMP, be it by stimulation of adenylate cyclase or by inhibition of phosphodiesterase will also interfere with platelet activity [7,29].

The question then arises, how the cytoplasmic cyclic AMP level is related to platelet activity. Contractile activity, which is required for clot retraction, for the spontaneous contraction of platelet aggregates, and most likely also for shape change, is calcium dependent. Increasing evidence also points to the importance of cytoplasmic Ca^{2+} for the release reaction [3,5]. Most of these calcium-dependent activities are reversible (cf. ref. 6) and this implies that the cell must be able to reduce its cytoplasmic calcium concentration again to the very low values which are observed in the resting state.

It seems tempting to assume that in the platelet, as in many other cells, cyclic AMP exerts an influence on the level of cytoplasmic Ca^{2+}. In fact, this hypothesis has been postulated several times [4,5,29,31,32], but to our knowledge has never been confirmed experimentally. We have demonstrated that there is indeed a significant increase in Ca^{2+} uptake by a fraction of membrane vesicles prepared by ultrasonic homogenization of platelets followed by differential centrifugation of the homogenate. This increase was only observed in the presence of both cyclic AMP and a protein kinase preparation from platelets. Another prerequisite was that the membrane preparation was thoroughly washed in the absence of ATP in order to decrease endogenous constituents of the calcium-accumulating system. If this step is omitted, then the observed uptake is barely influenced by the addition of cyclic AMP and protein kinase.

Interestingly enough, the observed stimulation of calcium uptake by cyclic

AMP and protein kinase is of relatively short duration. Studies which are presently in progress should show, whether this is due to the exhaustion of an essential component in the system or whether there are other reasons. Thus, the question arises, whether the observed effect could also be explained by the binding of Ca^{2+} to newly formed protein-bound phosphate groups. The work of Le Breton et al. [30], which describes the release, upon induction of rapid shape change, of Ca^{2+} from a membrane-bound form, could point in this direction. On the other hand, in this paper we have provided further evidence for the existence of a defined, calcium-accumulating vesicular structure in platelets, which quite clearly depends on an active pumping mechanism and perhaps corresponds to the dense tubular system described by White [38]. It is of interest that this system appears to be particularly rich in adenylate cyclase [37] and this again suggests a functional role for cyclic AMP in the removal of cytoplasmic calcium.

Finally, it must be mentioned that the existence of a cyclic AMP-dependent mechanism of calcium accumulation is well established for other contractile cells, such as those of cardiac and skeletal muscle [20,33—35], as well as smooth muscle [36]. It therefore would not be unexpected to find a comparable mechanism in the blood platelet, another cell with pronounced contractile activity.

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