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THE Ca^{2+} UPTAKE AND THE HYDROLYSIS OF VARIOUS NUCLEOTIDE TRIPHOSPHATES BY HUMAN PLATELET MEMBRANES

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Several nucleotide triphosphates (NTPs) were tested as energy source for the Ca^{2+} uptake by human platelet membrane vesicles. The Ca^{2+} uptake by these membranes was driven by ATP, GTP, ITP, UTP and CTP. The steady-state level of accumulated Ca^{2+} was equal with the different NTPs. The highest uptake velocity was found with ATP, but about 40–80% of the velocity with ATP could be accomplished with the other nucleotides. The highest affinity was also found with ATP (K_m apparent = 15 μM). The liberation of P_i from the various NTPs was measured simultaneously with the Ca^{2+} uptake. The coupling ratio (moles of Ca^{2+} taken up/moles of P_i liberated) varied from 0.4 for ATP to 2.3 for UTP and was almost independent of the NTP concentration. The enzyme activity with ATP as substrate is strongly dependent on the Ca^{2+} concentration in contrast to the activity with GTP, ITP, UTP or CTP.

The regulation of the intracellular Ca^{2+} concentration plays a key role in the activation process of blood platelets [1–4]. Several microsomal membrane fractions from human blood platelets have been described which accumulate calcium ions at the expense of a Mg^{2+}-dependent hydrolysis of ATP [5–7]. The membrane fraction consists of intracellular membranes but plasma membranes can also be demonstrated [5,6].

It is believed that the Ca^{2+} uptake and the $(Ca^{2+} + Mg^{2+})$-ATPase activity is to a large part effected by membranes from intracellular origin, i.e., the dense tubular system [5]. These membranes resemble the sarcoplasmic reticulum of muscle [8] and the $(Ca^{2+} + Mg^{2+})$-ATPase in these membranes cross reacts with antibodies raised against the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum of skeletal muscle [9]. However, it cannot be assumed that the similarity between platelet and muscle membranes bears on all aspects. For instance the role of cAMP-dependent protein kinase [6] and acidic phospholipids [10] might differ in the two systems. As the enzyme from sarcoplasmic reticulum [11] is not only active with ATP but also with other natural NTPs, we decided to investigate the substrate specificity of the enzyme from platelet membranes. The Mg^{2+}-dependent hydrolysis of the various NTPs and the Ca^{2+} uptake were measured simultaneously.

For our studies we used a platelet membrane fraction, which was prepared using a modification of the method of Käser-Glanzmann et al. [5] as described by Le Peuch et al. [6]. The fraction was characterized by Le Peuch et al. [6] and by Enouf and Lévy-Tolédano [12]. The membranes were suspended in 100 mM KCl, 20 mM Hepes-KOH (pH

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Abbreviations: NTP, unspecific nucleotide triphosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetracetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

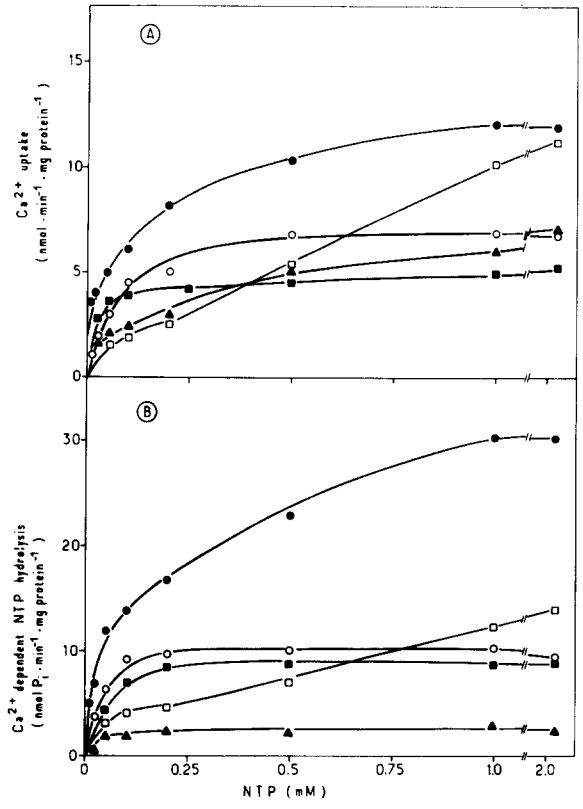

Fig. 1. The Ca^{2+}-uptake (A) and the $(Ca^{2+} + Mg^{2+})$-NTPase activity (B) in the presence of ATP (●), ITP (○), GTP (■), CTP (□) or UTP (△). The platelet membranes (0.5 mg of protein, Ref. 18) were tested at 30 °C in 2 ml 100 mM KCl, 20 mM Hepes-KOH (pH 8.0), 5 mM oxalate, 10 mM $MgCl_2$, 10 μM $^{45}CaCl_2$ (1300–1500 dpm·nmol^{-1}, Radiochemical Centre, Amersham) and NTPs (Sigma St-Louis) as indicated. Ca^{2+}-uptake experiments were performed using the Millipore filtration technique (pore size 0.45 μm Type HA) as described by Le Peuch et al. [6]. The amount of P_i liberated was measured by adding 2 ml of malachite green reagent, which is used for the colorimetric determination of P_i [19], to 0.2 ml incubation mixture. The Ca^{2+}-dependent NTP hydrolysis was calculated by subtracting the values obtained in the presence of 0.5 mM EGTA without adding $CaCl_2$ from the values obtained in the presence of $CaCl_2$.

8.0), stored at 4 °C and used within 48 to 72 h. The Ca^{2+} uptake and the hydrolysis of various NTPs are shown in Fig. 1. Both activities can be demonstrated with all of the NTPs used. When the data from Fig. 1 are plotted according to Lineweaver-Burk, at least with ATP and CTP two different K_m values can be extrapolated. The highest apparent K_m value is found with ATP (K_m apparent = 15 μM). It is not likely that the activity with GTP, ITP, CTP and UTP is due to residual ATP or ADP in the platelet membrane fraction as the concentration of ATP plus ADP was less than 10^{-8} M in this fraction as measured by the luciferin-luciferase assay [13]. Furthermore it is unlikely that the activity with different NTPs is effected by different enzymes as the activity in the presence of saturating amounts of one NTP (2 mM) never increased by adding suboptimal concentrations of another NTP (data not shown). On the other hand, the activity with a suboptimal concentration of one NTP always increased by adding another NTP in suboptimal concentrations. Hence we conclude that all the NTPs can serve as substrate for a single $(Ca^{2+} + Mg^{2+})$-NTPase, which is driving the Ca^{2+} transport.

Whereas ATP is the most potent substrate for the hydrolysis and the Ca^{2+} uptake activity, the relative velocities with the NTPs are different for both activities (Fig. 1). The coupling ratio (moles of Ca^{2+} taken up/moles of P_i liberated) is however rather constant over a wide range of NTP concentrations. With ATP 12 nmol Ca^{2+}/min per

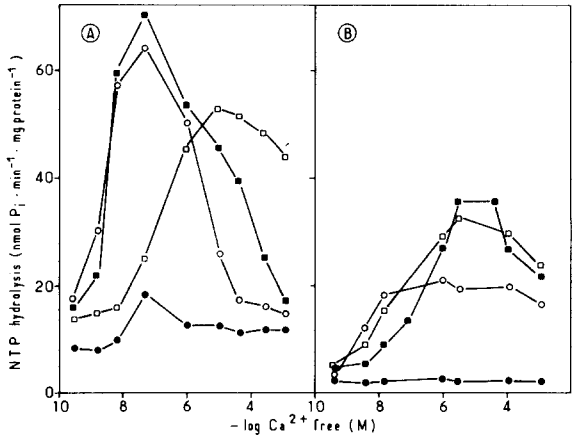

Fig. 2. The Ca^{2+}-dependence of the hydrolysis of ATP (A) and ITP (B). 0.5 mM ATP or ITP was added to the reaction mixtures containing 0 (●), 0.2 (○), 2 (■) or 20 mM $MgCl_2$ (□). Different amounts of $CaCl_2$ were added to 0.2 ml reaction mixtures containing 100 mM KCl, 20 mM Hepes-KOH (pH 8.0) and 0.5 mM EGTA. The free Ca^{2+} concentration was calculated according to Lotersztajn et al. [20], using an apparent association constant of $6.03 \cdot 10^8$ for Ca-EGTA. The hydrolysis of ATP and ITP was measured as described in the legend to Fig. 1.

mg protein can be accumulated whereas 30 nmol P_i/min per mg protein is liberated (a coupling ratio of 0.4). For the sarcoplasmic reticulum of muscle a coupling ratio of 2 is reported [11]. The best coupling ratio with platelet membranes could be effected with UTP (2.3), suggesting a low leakiness of the membranes. The reason for the different coupling ratio with the various NTPs is not known. We do not think that a different ratio between the efflux and influx of calcium ions with the various NTPs might play a role as the steady-state level of accumulated calcium ions in the membrane vesicles was equal with the various NTPs.

A possible explanation might be found in the effect of Ca^{2+} on the enzyme activity with the various NTPs. Mg^{2+}-ATP is probably the true substrate for the $(Ca^{2+} + Mg^{2+})$-ATPase from the sarcoplasmic reticulum of muscle [14]. High Ca^{2+} concentrations might inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase activity, especially at low Mg^{2+} concentrations, because the Ca^{2+}-ATP concentration increases at the expense of the substrate Mg^{2+}-ATP. This has been demonstrated with the $(Ca^{2+} + Mg^{2+})$-ATPase from muscle [14] and similar results are obtained with the enzyme from platelets. It is shown (Fig. 2A) that Ca^{2+} stimulates the ATPase activity. However, concentrations of 10^{-7} M were already suboptimal when 0.2 mM $MgCl_2$ was present. The inhibition beyond the maximum could be antagonized by increasing the $MgCl_2$ concentration.

The inhibition by Ca^{2+} was almost absent when ITP, CTP, GTP or UTP was used as substrate. This is shown for the $(Ca^{2+} + Mg^{2+})$-NTPase activity with ITP (fig. 2B). No inhibition by Ca^{2+} was observed at concentrations lower than 10^{-4} M. Similar results are given by Vianna [14] for the $(Ca^{2+} + Mg^{2+})$-NTPase from muscle with ITP. Martonosi and Feretos [15] have demonstrated with the enzyme from muscle that inhibition by Ca^{2+} was less pronounced when measuring the Ca^{2+} uptake instead of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. This is not to be expected if the inhibition is only due to competition between Ca^{2+}-ATP and Mg^{2+}-ATP. However, this effect was measured and could be reproduced with platelet enzyme (not shown), when oxalate is present in the reaction mixture. Therefore the difference between the inhibition by Ca^{2+} of ATPase and uptake might be related with the precipitation of Ca oxalate in the membrane vesicles.

The results of Robblee et al. [16] who also tested the NTP requirement of the Ca^{2+} uptake by a platelet subcellular fraction, are quite different from our results. They concluded that the NTP requirement appears to be specific for ATP, although a slight Ca^{2+} uptake was found with UTP, CTP and GTP. We think that this discrepancy is due to the completely different homogenization procedure used by Robblee et al. Furthermore the ATP specificity might be an indication for the presence of a $(Ca^{2+} + Mg^{2+})$-ATPase from plasma membranes, as the $(Ca^{2+} + Mg^{2+})$-ATPase activity from plasma membranes seems to be much more specific for ATP than similar enzymes from intracellular origin [17]. We have found that various NTPs can serve as substrate for the Ca^{2+}-uptake system in our platelet membrane preparations. This supports its intracellular origin. A contamination with $(Ca^{2+} + Mg^{2+})$-ATPase from platelet plasma membranes, whose existence has not yet been proven, can, however, not be excluded. The search for such a plasma membrane enzyme might be facilitated by using different NTPs as substrate in membrane subfractions with $(Ca^{2+} + Mg^{2+})$-ATPase activity.

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