Characteristics of the Passive $^{45}Ca^{2+}$ Transport by Human Blood Platelets. Their Dependence on the Monovalent Cation Composition of the Suspension Media

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Abstract. In the suspension of washed human platelets (32 °C, up to 30 min) we measured the uptake of $^{45}Ca^{2+}$ in various media. We found that the substitution of Na^+ by K^+ or choline led to the stimulation of the uptake. According to the degree of the Na^+ substitution we observed two phases of stimulation. The first one was observed in K^+-containing media, but not in choline-containing media; the second one was observed in both K^+- and choline-containing media. The transport in both phases was saturatable by Ca^{2+} and sensitive to other divalent cations. The saturating Ca^{2+} concentration and the sensitivity to other divalent cations were different in both phases. Dihydropyridine (nifedipine, nitrendipine) and phenylalkylamine (verapamil) calcium channel blockers were without effect in either medium up to 100 μmol/l. Indomethacin and acetylsalicylic acid were also without effect (100 μmol/l, and 1 mmol/l, respectively). On the other hand, veratridine (up to 75 μmol/l) stimulated the $^{45}Ca^{2+}$ transport in Na^+-rich medium but not in Na^+-free medium. These results are in accordance with a notion that the first phase corresponds to a depolarization-stimulated Ca^{2+} – transport, and the second one to the reversal of the Na/Ca exchange activity.

Key words: Blood platelets — $^{45}Ca^{2+}$ uptake — Membrane depolarization

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Introduction

The metabolism of serotonin in blood platelets is strongly homologous to that of nerve endings in both biochemical and pharmacological aspects (Sneddon 1973; DaPrada et al. 1988). This is certainly true for the transport of serotonin from cell exterior, and its storage in platelet dense granules (Sneddon 1973, Pletscher and DaPrada 1975). As to the platelet serotonin secretion, there is no convincing evidence that there is such a homology with a neurotransmitter release from nerve endings. An important difference between platelet serotonin secretion, and the neurotransmitter release seems to lie in the mechanism of the initiation of the secretion. It was shown that there are two main differences between blood platelets and isolated nerve endings (synaptosomes): First, the platelet secretion (induced by thrombin or other inducers) is dependent on the extracellular Ca^{2+} in pig (Grette 1962), rat (Belville et al. 1979), and rabbit (Sneddon 1973) platelets but not in human platelets (Mürer 1968; Massini and Lüscher 1971). Second, the role of the membrane potential changes (depolarization) as a causative factor of the platelet activation has not been demonstrated in both human (Horne and Simmons 1978; McIntyre and Rink 1982; Varečka and Pogády 1983; Pipili 1985) and pig (Varečka et al. 1978) blood platelets. The membrane potential changes elicited by the inducers of the secretion rather accompany the secretion reaction in blood platelets as a consequence of the Ca^{2+} entry into the platelet cytoplasm.

On the other hand, there are known the observations which show that the substitution of Na^{+} by other monovalent cation does influence platelet functions like aggregation (Born and Cross 1964; Hallam and Rink 1985) or secretion (Sneddon and Williams 1973; Connolly and Limbird 1983; Greenberg-Sepersky and Simons 1984; Alonso et al. 1989) induced by physiological platelet activators. Sodium substitution by some of the monovalent cations which leads to the diminution of the physiological response to the activator seems to be the common feature of their effects. This effect could not be reconciled with the action on any known Ca^{2+} – transporting system in the platelet membrane, and we proposed that the inhibitory action of the sodium withdrawal on the platelet response lies at the level of agonist-receptor interaction.

Because the activation of nerve endings is not a receptor-operated process the effect of thrombin and other activators could be regarded as irrelevant to the problem of homology between platelet and nerve endings. The homology, however, may exist and could be based on the membrane processes and/or transport systems which are present in the platelet membrane but may not participate in the major events of platelet activation. In this paper we attempt to examine this proposal. Presented data suggest that the depolarization-activated Ca^{2+} uptake is present in human blood platelets. Furthermore, we confirmed firmly the presence of the Na/Ca exchanger in the platelet membrane.

Materials and Methods

Blood of healthy both male and female drug-free volunteers was obtained by venipuncture at the day of experiment using chelatonate anticoagulant (5 mmol/l). Blood platelets were isolated by differential centrifugation. Platelet-rich plasma was obtained by the centrifugation for 25 min at $60 \times g_{av}$. Platelets were separated from plasma centrifuging it at $1000 \times g_{av}$ for 15 min and were suspended into medium containing (in mmol/l):Tris-Cl 20, NaCl 135, KCl 5, glucose 10, pH 7.3, and EGTA 0.5 (further referred to as medium containing EGTA),recentrifuged as above and resuspended in the same medium but without EGTA (further referred to as a medium). They were used for experiments within 3 hours being stored as a thick suspension (about $6 \cdot 10^9$ cells/ml) at room temperature. Platelets prepared in such a way aggregated neither spontaneously, nor after additions of Ca^{2+} alone,or with combination with thrombin.

Transport of Ca^{2+} was measured using $^{45}Ca^{2+}$ radionuclide (Institute of Isotopes, Budapest, Hungary), and the centrifugation through hydrophobic phase as a separation method. We modified the method of Brass and Shattil (1982) as follows: The desired medium (0.2 ml) was supplemented by tested substances and/or vehicle(s), and $^{45}Ca^{2+}$ isotope (in final concentration 0.675 mmol/l, 6000–10000 cpm/nmol). Mixture was preincubated at 32 °C. The transport reaction was started by addition of the suspension (about $4 - 8 \times 10^7$ cells in 10 μl), and gentle mixing on the vortex mixer, and the incubation at the same temperature was continued for 30 min if not indicated otherwise in the legends to the Figures. The transport reaction was stopped by the addition of 50 μl of medium containing 20 mmol/l EDTA. The suspension was then immediately layered on 100 μl of dibutylphtalate (Serva, Heidelberg, Germany), or phenylsilicone oil (Lučební závody, Kolín, Czech Republic), and centrifuged down on the microcentrifuge. The upper water layer was aspirated, and the space over the hydrophobic layer was washed two times with 1.25 ml water. Finally, the hydrophobic phase was aspirated carefully not to disturb the pellet. The last was dissolved in solution containing 5% (w/v) SDS, 5% (v/v)Triton X 100, and 5 mmol/l EDTA overnight at 37 °C, and the whole solubilizate was counted by liquid scintillation method.

Alternatively, we separated platelets by means of the membrane filtration technique using either Sartorius nitrocellulose membrane filter (0.65 μm pore), or Whatman GF/A glass microfiber filters. Filters were washed with 3×3.5 ml of medium containing EGTA to remove the extracelularly bound radioactivity. We got essentially the same results as with the separation through the hydrophobic phase. The same procedure was applied to the measurement of the $^{22}Na^+$ uptake. About 10^7 cpm of the radionuclide (The Isotopic Institute of the Academy of Sciences, Moscow, Russia) was added per assay.

Putative inhibitors of the Ca^{2+} transport [verapamil (Knoll, Germany), nifedipin, nitrendipin (Institute for Drug Research, Modra, Slovakia)], and of the proteolytic activity of thrombin [N-benzoyl-phenylalanyl-L- valyl-L-arginine- 4-methylcoumarinyl- 7-amide.HCl (Serva, Germany); Phenylmethyl sulphonyl fluoride (Sigma, USA)] were added from their solutions in dimethylsulphoxide (Lachema, Czech Republic) keeping the concentration of solvent constant in all samples - 0.5% (v/v); D-prolyl- phenylalanyl- arginyl aldehyde (Bajusz et al. 1978; a kind gift of Prof. Dr. L. Kováč, Comenius University, Bratislava), and heparin (Spofa, Prague, Czech Republic) were dissolved in water and added in 1 μl aliquots.

Platelets were counted in the light microscope by means of a haemocytometer.

Results

In our experiments we used human blood platelets isolated from chelatonate-treated blood, and washed in buffered saline solution containing EGTA. All experiments were performed at 32 °C without stirring. These conditions completely eliminated the aggregation after subsequent recalcification, and even after the addition of purified thrombin as checked visually by light microscopy.

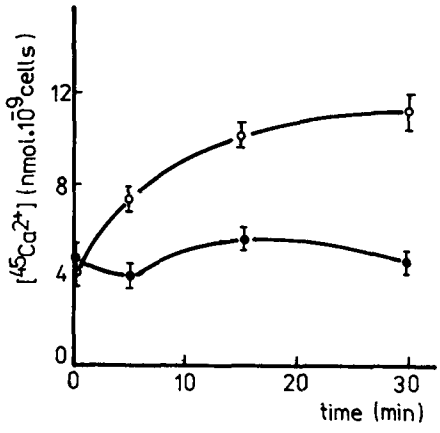

Figure 1. The time course of the Ca^{2+} uptake by human platelets at two temperatures.
The experiment was performed according to the procedure described in Materials and Methods. The suspension was incubated either at 32 °C (open circles), or in ice-water mixture (closed circles). The transport reaction was stopped at the time indicated in parallel aliquots. The values of the uptake are presented as a mean +/− standard error of parallel samples, and are not corrected. One of two experiments is presented.

Basal observations

$^{45}Ca^{2+}$ labels platelets in both time, and temperature-dependent manner. It is shown in the Figure 1 that there is no Ca^{2+} labelling at 0 °C whereas at 32 °C there is a time-dependent increase of labelling which reaches the stationary state after approximately 30 min. Because the incubation with the radionuclide was stopped by the addition of EGTA-containing solution, we supposed that the radioactivity was internalized. The radioactivity contained in platelet pellets at 0 °C, and in "zero-time" samples corresponds probably to the volume of medium trapped in platelet pellets spun down through the hydrophobic phase. This we confirmed by the use of ^{14}C-labelled sucrose instead of Ca^{2+} (not shown). All other experiments shown in this manuscript present the values of uptake which were corrected for this value. In other experiments which we do not present here we observed that the kinetics and the extent of the uptake was not affected by a pre-incubation of platelet suspension for one hour at room temperature with either 0.5 mmol/l EGTA or 0.5 mmol/l $^{40}Ca^{2+}$. This suggests that the Ca^{2+} uptake by platelets is not a consequence of their previous depletion of Ca^{2+} during the treatment by anticoagulant.

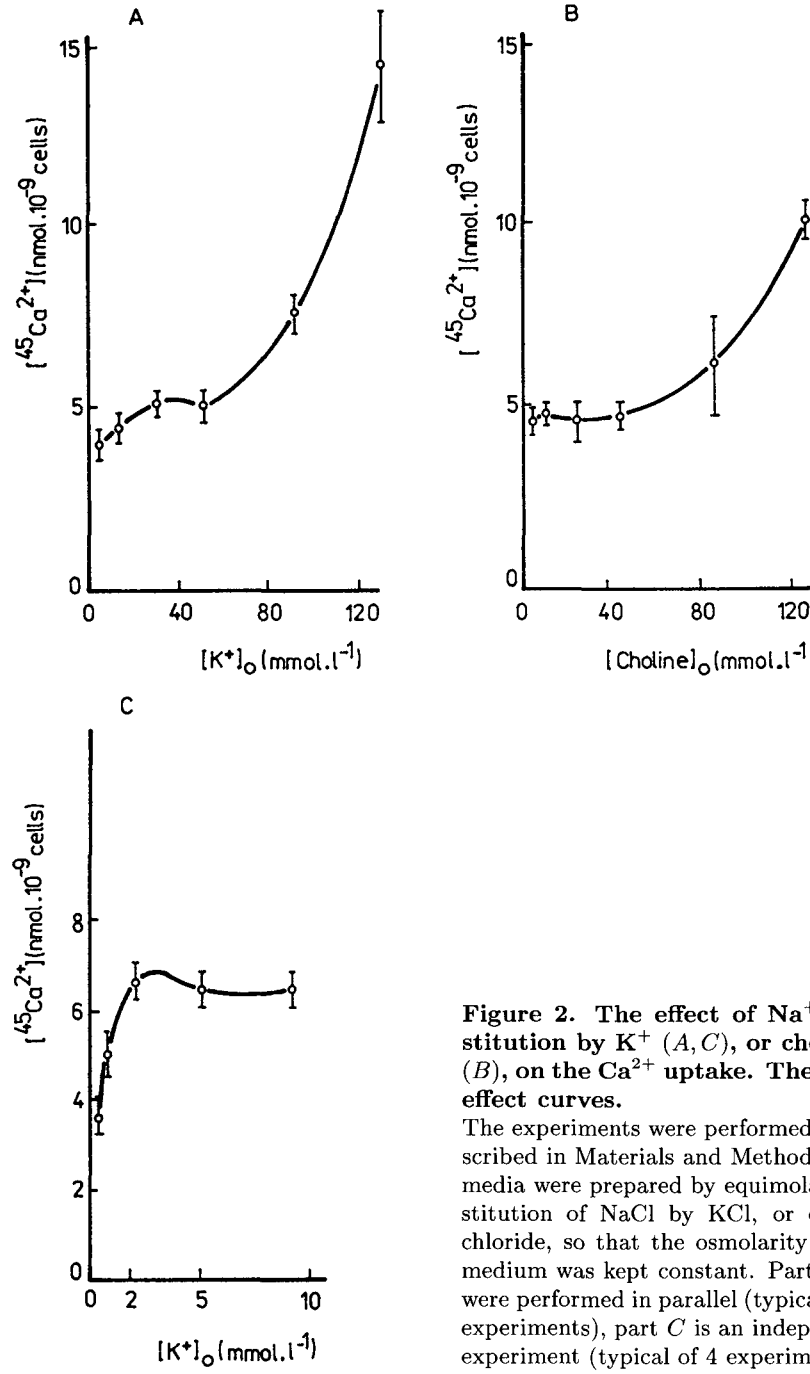

Figure 2. The effect of Na^+ substitution by K^+ (A, C), or choline$^+$ (B), on the Ca^{2+} uptake. The dose-effect curves.

The experiments were performed as described in Materials and Methods. The media were prepared by equimolar substitution of NaCl by KCl, or choline chloride, so that the osmolarity of the medium was kept constant. Parts A, B were performed in parallel (typical of 24 experiments), part C is an independent experiment (typical of 4 experiments).

Effects of the monovalent cation substitution

When Na^+ in the medium were isosmotically replaced by K^+ the stimulation of the Ca^{2+} was observed which depended on the degree of the substitution (Fig. 2*A*). The course of the dose-effect curve was biphasic. The first phase of the stimulation started at the lowest increase of the K^+ concentration, and had an optimum between 10–30 mmol/l K^+. As an extreme we found a bell-shaped dose-effect curve (not shown). This phase was absent in control experiments where choline was used as a substituent of Na^+ (Fig. 2*B*). On the other hand, when K^+ concentration was lowered below 5 mmol/l we always observed the decrease of the uptake to some minimal value (Fig. 2*C*). This may explain results of several experiments where we found no stimulation of the uptake, or even its small decrease upon the increase of the K^+ concentration above 5 mmol/l (not shown). These results could be explained supposing that the optimum stimulation of the transport is approximately at 5 mmol/l K^+ in these cases.

The second phase of the stimulation started at higher degree of the substitution (50–60 mmol/l Na^+), and the stimulation of the uptake started approximately at the same concentration of the substituting cation (Fig. 2*A*, *B*). In most experiments choline stimulated the uptake less than K^+ (Fig. 2*A*, *B*) but in other their potency

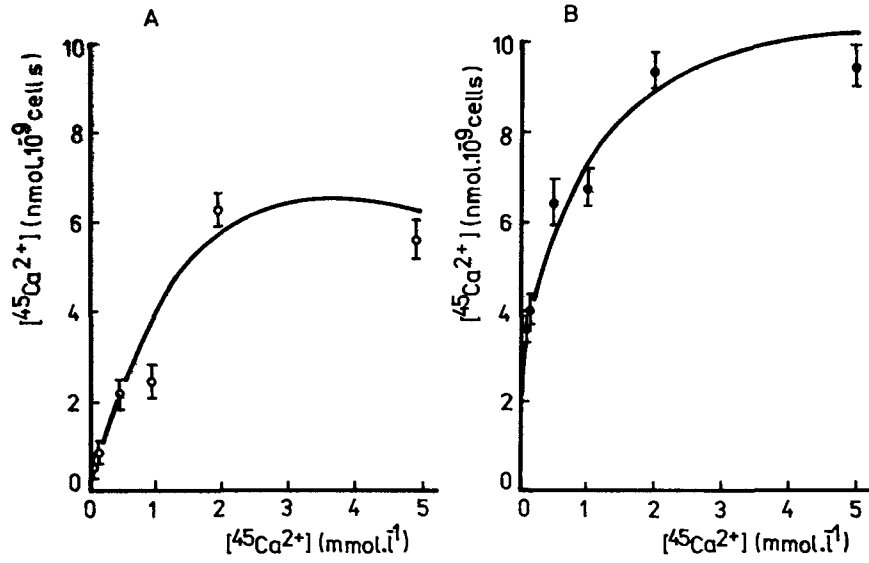

Figure 3. The Ca^{2+} uptake by platelets at various extracellular Ca^{2+} concentration in Na^+-, and K^+-rich media.

The uptake was measured in a standard (*A*) medium, and in medium where 120 mmol/l NaCl was substituted by KCl (*B*). The extracellular Ca^{2+} concentrations are indicated in the Figures. Experiments in *A*, and *B* were performed in parallel. They are typical of 3 similar experiments.

was approximately equal (not shown). The properties of the uptake shown in the Fig. 2 suggest that the uptake is mediated by two transport systems: one stimulated by the increase of K^+ concentration, and the second that is stimulated by the Na^+ withdrawal from the medium. We performed following experiments to verify this explanation.

Transport characteristics of the $^{45}Ca^{2+}$ uptake

In order to show that we observe two distinct transport systems it is indispensable to prove also that the observed uptake obeys the criteria of the carrier-mediated transport. In the Fig. 3 we show that the uptake is saturatable with Ca^{2+} in both Na^+-rich, and Na^+-poor (K^+-rich) media. It is apparent that the uptake in K^+-rich medium is saturatable at the lower Ca^{2+} concentration than that in the Na^+-rich medium. This suggests the affinity toward the substrate of the transport increases upon decreasing the extracellular $[Na^+]$. It must be mentioned at this point again that we did not observe the aggregation even at highest Ca^{2+} concentration we used. Also, the presence of prostaglandin E_1 (10 μmol/l), an inhibitor of the aggregation, did not affect the results (not shown).

The Ca^{2+} uptake was inhibited by other divalent cations which can be regarded as substrate analogues. We tested Sr^{2+}, Mn^{2+} (up to 5 mmol/l),Cd^{2+} (up to 1.5 mmol/l), Cu^{2+} (up to 0.1 mmol/l). It is shown in the Fig. 4 that the first three ions inhibit the transport in a dose-dependent manner. The inhibitory efficiency differs in Na^+-rich and K^+-rich media being slightly higher in K^+-rich media (Fig. 4*A*, *C*, *D*). The effect of Cu^{2+} was of different kind (Fig. 4*B*). Even the lowest concentrations of Cu^{2+} tested (30 μmol/l) caused the stimulation of the uptake. Similar action exerted Ni^{2+} (up to 2 mmol/l) (not shown). The effect of divalent cations (without Cu^{2+} and Ni^{2+}) confirms that the transport observed carrier-mediated, and that it was mediated by distinct carriers. The effects of Cu^{2+} and Ni^{2+} we cannot explain on the basis of the results obtained so far.

In other experiments we tried to find other inhibitors of the passive Ca^{2+} transport. The known calcium blockers of dihydropyridine type, nifedipine and nitrendipine, were ineffective up to 0.3 mmol/l, the phenylalkylamine derivative verapamil was ineffective up to 0.1 mmol/l (not shown). The inhibitors of arachidonate metabolism such as indomethacin (up to 100 μmol/l), or acetylsalicylic acid (up to 1 mmol/l) were also without effect (not shown). The experiments presented so far represent a solid evidence for the existence of two transport systems in the membrane of blood platelets: Na/Ca exchanger, and a carrier which operates apparently without any stimulus, and which behaves as a depolarization-stimulated Ca^{2+} channel. Veratridine which is known to activate the sodium permeability in the nerve cell membrane inducing thereby the membrane depolarization was used as a tool which could confirm or disprove the presence of the depolarization – stimulated Ca^{2+} transport system. It is shown in the Fig. 5 that veratridine in the concentrations up to 100 μmol/l stimulated the Ca^{2+} uptake in Na^+ medium

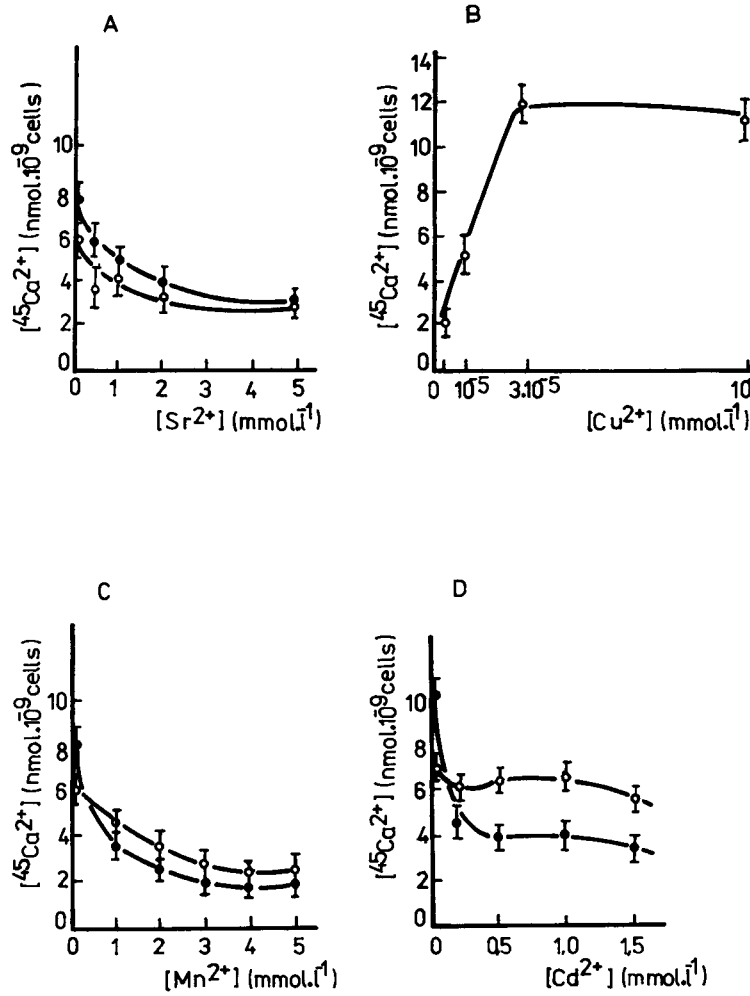

Figure 4. Effects of divalent cations on the Ca^{2+} uptake in Na^+-rich, and K^+-rich media.

Experiments were performed according to Materials and Methods in a standard medium (open circles), or in medium with 120 mmol/l KCl instead of NaCl (closed circles), and in the presence of divalent cations in concentrations indicated in the Figure. *A*: Sr^{2+}, *B*: Cu^{2+}, *C*: Mn^{2+}, *D*: Cd^{2+}. All experiments were independent, presented are typical of at least two experiments.

but not in the Na^+-free medium so that its Na^+-specific effect on the Ca^{2+} transport is present also in the blood platelet membranes. However, we were unable to detect any increase of the $^{22}Na^+$ uptake by veratridine under similar conditions

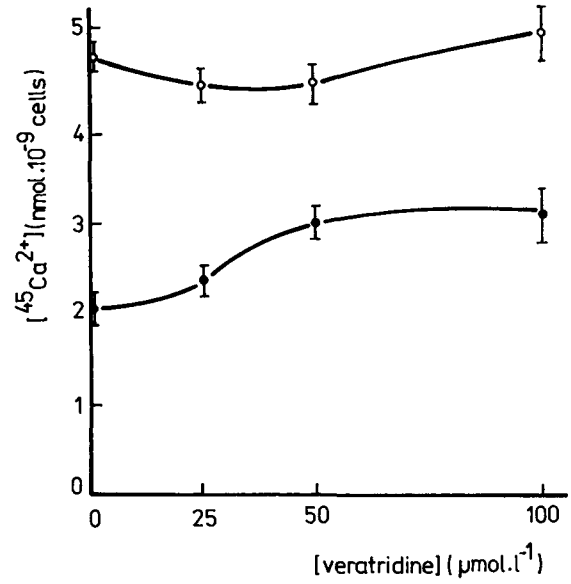

Figure 5. Effect of veratridine on the Ca^{2+}; the dose-effect curve in Na^+-, and K^+-rich media.
The uptake was measured as described in Materials and Methods in a standard medium (closed circles), or in medium with 120 mmol/l K^+ (open circles). Veratridine was added into the medium before the suspension in the same volume of methanolic solution, and methanol (0.5%, v/v) was added to control test tubes. One of three experiments presented.

(not shown). Furthermore, we tested two ionophores with a specificity to Na^+ ions, gramicidin (up to 1 μg/ml) and the neutral Na^+-ionophore Fluka I (up to 1 μg/ml). These substances exerted different effects on the Ca^{2+} transport. Whereas Na^+-ionophore Fluka I was without effect, gramicidin (0.1–1.0 μg/ml) slightly but not reproducible stimulated the initial phases of the uptake. The effect of veratridine quantitatively exceeded the effect of gramicidin, although gramicidin strongly depolarizes the blood platelet membrane (Pipili 1985), and the approximately equal effect could be expected.

In order to test the possibility that the $^{45}Ca^{2+}$ uptake we observed without inducer is due to the generation of thrombin on the platelet surface (Ardlie and Han 1974) and the subsequent activation of the thrombin – activated Ca^{2+} channel (Zschauer et al. 1988) we studied the effect of the inhibitors of thrombin on the platelet $^{45}Ca^{2+}$ uptake. We found that three thrombin inhibitors, N-benzoyl-phenylalanyl-L-valyl-L-arginine-4- methyl coumarinyl-7-amide.HCl (up to 0.14 mmol/l); phenylmethyl sulphonyl fluoride (up to 1 mmol/l), heparin (up to 30 units/ml) stimulated the $^{45}Ca^{2+}$ uptake, whereas only D-prolyl- phenylalanyl-

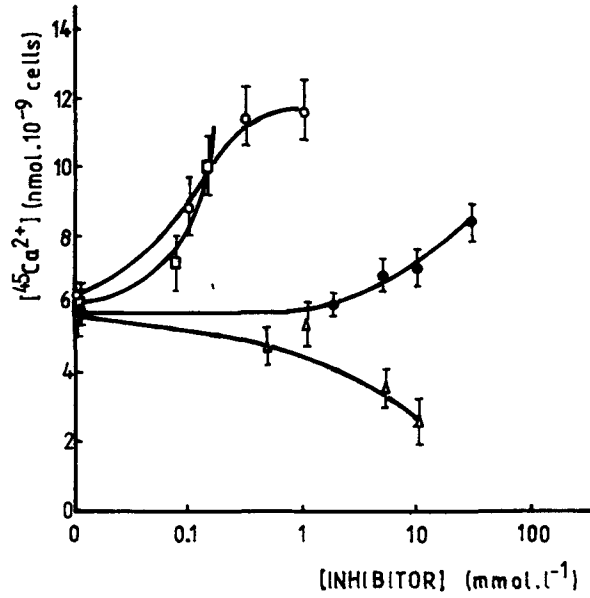

Figure 6. The effect of inhibitors of thrombin on the Ca^{2+} uptake in Na^{+}-rich media.

The uptake was measured as described in Materials and Methods in the presence of indicated concentrations of following thrombin inhibitors: N-benzoyl-phenylalanyl-L- valyl-L-arginine- 4-methylcoumarinyl- 7-amide.HCl (open squares); phenylmethyl sulphonyl fluoride (open circles); D-prolyl- phenylalanyl- arginyl aldehyde (open triangles), and heparin (closed circles). The first two inhibitors were added in dimethylsulphoxide solution, the last two in water. The same concentration of solvents (0.5% v/v) was added to the controls. Presented is a set of three independent sets of experiments. Note that the concentrations are plotted in the logarithmic scale. The concentrations of heparin are expressed in units/ml.

arginyl aldehyde (Bajusz et al. 1978) inhibited the uptake in concentrations above 1 mmol/l (Fig. 6).

Discussion

We studied the uptake of Ca^{2+} by blood platelets using two independent methods with the aim to find out any resemblance to excitable cells and/or nerve endings. We found out that the uptake is present, and that it reaches the extent between about 3–15 nmol/10^9 cells in the steady state which completes in about 30 min at 32°C. Our values are generally higher than those published by other authors (Steiner and Tateishi 1974; Massini and Lüscher 1976; Brass and Shattil 1982;

Brass 1984; Magócsi et al. 1989; Johnston and Heptinstall, 1988;) who used the same method. These differences could be ascribed to the differences in experimental conditions such as extracellular Ca^{2+} concentraction (Steiner and Tateishi 1974; Massini and Lüscher 1976), or temperature (Massini and Lüscher 1976; Connolly and Limbird 1983; Brass 1984), or platelet preparation (Steiner and Tateishi 1974; Connolly and Limbird 1983; Brass 1984) so that we suppose that our values represent a consistent part of the whole spectrum of published values. The uptake exhibited the features of a carrier-mediated process. These are: a) temperature dependence (Fig. 1); b) substrate saturatability (although the linear component of the uptake seems to be present in some experiments as well) (Fig. 3); c) sensitivity to substrate analogues (although the degree of the uptake inhibition did not exceed 60% by any of divalent cations used) (Fig. 4). Further indirect evidence supporting this statement is the behaviour of the uptake which conforms the rules for the depolarization-induced transport observed in excitable cells (Fig. 2). Substitution experiments demonstrated unequivocally the activity of the Na/Ca exchanger in human (but also in pig – not shown) platelet membranes as revealed by the reversal of its transporting activity. This is in agreement with the results of Steiner and Tateishi (1974), Brass (1984) and Johannson and Haynes (1988). The same experiments also supported the presence of the second transport system in the platelet membrane which is depolarization-activated, and has a distinctly lower affinity for Ca^{2+} than the Na/Ca exchanger (Fig. 3). These experiments enlighted also the nature of the "basal" transport observed in standard conditions such as those in the Fig. 1. The decrease of the uptake in media with the very low K^+ concentrations (Fig. 2C) suggests that the membrane is in conditions of our experiment partially depolarized. This depolarization may occur during the isolation and storage of platelets (due to the shift of monovalent cations, or due to the inhibition of Na-pump (McIntyre and Rink 1982) and following activation of the depolarization-stimulated transport. This suggests that the considering the real turnover of Ca^{2+} cycling may, generally, include also this important factor. In our experimental conditions we estimate the real rate of Ca^{2+} uptake lower by a factor about 2 than we observed in the standard medium. The presence of the depolarization-induced Ca^{2+} transport in the platelet membrane is supported also by the effect of veratridine (Fig. 5) which is used as an alternative tool for the membrane depolarization in neurobiology (Narahashi 1974). Its action on the platelet membrane implies that there may exist also the tetrodotoxin-sensitive Na^+ channel. So far, however, we did not test the effect of tetrodotoxin on the Ca^{2+} uptake, or on the Na^+ uptake. On the other hand, in experiments which we do not present here, we were unable to stimulate the transport by ionophores gramicidin or Na-ionophore I (Fluka) in a reproducible and concentration-dependent manner. Therefore the clear relationship between the depolarization effect of potassium and veratridine should be analyzed in the future to the more detail. The demonstration

of the depolarization-induced Ca^{2+} transport in our experiments contradicts to the results of Doyle and Ruegg (1985), or Pannocchia et al. (1987) who did not find the evidence for the presence of this transport on the basis of the quin-2 measurements, or ^{3}H-desmethoxyverapamil binding, respectively. These discrepancies may be explained by the short time of measurement by Doyle and Rüegg (1985) who even did not demonstrate the presence of the Na/Ca exchanger, probably from the same reason. It is also possible that the sequestering activity of intracellular Ca^{2+} stores (Statland et al. 1969; Gerrard et al. 1981), together with the high Ca-buffering capacity of quin-2 prevented the detection of the depolarization induced changes in $[Ca^{2+}]_i$ by the fluorescent chelate technique. The lack of ^{3}H-desmethoxyverapamil binding to the platelet membranes is easier to explain because the Ca^{2+} uptake is not sensitive to this compound (not shown)(Magócsi et al. 1989). Not least, as we already pointed out, we did not find the stimulation of the Ca^{2+} uptake by K^{+} in all experiments. In approximately half experiments we either observed no significant stimulation of the uptake by the depolarization, or the experimental error of the parallel assays was too high to enable us to draw any conclusion. The possible causes of these results were already mentioned (see text to the Fig. 2*C*). We also considered the possibility that the activity of the putative depolarization-induced Ca^{2+} transport system rapidly inactivates upon the platelet storage. However, this possibility we did not confirm experimentally (not shown). Neither we obtained the unequivocal evidence that the $^{45}Ca^{2+}$ uptake we observed is caused by the spontaneous generation of thrombin on the blood platelet surface (Ardlie and Han 1974) which could result in the opening of the thrombin-activated Ca^{2+} channel (Zschauer et al. 1988, Pales et al. 1991). The effect of thrombin inhibitors we studied might be complemented in the future by a method with a direct detection of thrombin on the platelet surface.

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