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KINETIC CHARACTERIZATION AND SUBSTRATE REQUIREMENT FOR THE Ca^{2+} UPTAKE SYSTEM IN PLATELET MEMBRANE

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An ATP-dependent mechanism for Ca^{2+} uptake in human platelet membrane fractions has been identified and characterized. Ca^{2+} uptake into a membrane fraction is shown to be stimulated at low concentrations of ATP and Ca^{2+} and to require magnesium ions. Initial rate kinetics, using Eadie-Scatchard analysis, indicated a single class of calcium uptake sites in the presence of ATP, with a K_d for free [Ca^{2+}] of 0.145 μM. Ca^{2+} uptake in the presence of several ATP concentrations demonstrates that ATP binds to at least two sites, representing high and low affinities of 3.21 and 80.1 μM, respectively. The neuroleptic drug fluphenazine inhibited ATP-stimulated calcium uptake ($IC_{50} = 55$ μM), suggesting this ATP-dependent Ca^{2+} uptake system may provide a useful ion-transport model with which to study neuroleptic therapy in humans.

Introduction

The mechanisms by which cells regulate calcium transport is complex. Intracellular free calcium is generally estimated to be at least 4 orders of magnitude lower than extracellular Ca^{2+}. The influx of Ca^{2+} occurs down a steep electrochemical gradient. This movement is offset by an energy-dependent process which extrudes Ca^{2+} against this electrochemical gradient. Of the intracellular Ca^{2+} buffering systems involved, one uses ATP directly, in the form of a ($Ca^{2+} + Mg^{2+}$)-ATPase, catalyzing ATP-dependent Ca^{2+} uptake. This mechanism has been well established in red cells [1], nerve [2], liver [3], lymphocytes [4], muscle [5] and adipose tissue [6].

Control of this transmembrane calcium gradient may play a role in the coupling of transmitters and hormones to intracellular Ca^{2+} signals [7,8]. The ability of extracellular signals to regulate calcium gradients would have profound influences on intracellular metabolism. In recent years, the platelet has been studied with regard to its utility as a peripheral nerve model [9,10]. Many aspects of nervous system function are found in the platelet, such as transmitter receptors, voltage-dependent secretion of transmitters, cyclic nucleotide metabolism, uptake and storage of transmitters [11]. In addition, many functions requiring Ca^{2+}, such as Ca^{2+}-dependent transmitter release and protein phosphorylation, have been documented.

Use of the platelet as a peripheral nerve model has prompted us to investigate, in more detail, mechanisms for control of calcium in the platelet. Under resting conditions, platelet intracellular free calcium concentration is maintained in the 10^{-7} M range, and plasma calcium concentration is three-to-four orders of magnitude higher [12]. Several

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid; EGTA, ehtyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis (2-(5-phenyloxazolyl)) benzene.

stimuli can produce a large increase in the intracellular free calcium concentration ($[Ca_f^{2+}]$). The calcium is either released from intracellular stores or moves into the platelet from outside the cell. This increase in intracellular $[Ca_f^{2+}]$ initiates several biochemical processes, which result in platelet activation [11]. The platelet must contain an internal store(s) of calcium which can be mobilized by suitable stimuli to trigger storage granule secretion, platelet activation and the resulting platelet shape changes which precede aggregation. Platelet activation and shape change do not require the presence of external calcium, but platelet aggregation does [13].

The sources of intracellular calcium and the mechanism for its release are not currently known. The dense tubular system, the surface membrane (including the open canicular system) and the mitochondria are possible candidates for significant roles in calcium regulation [14]. Several investigators have reported energy-driven calcium uptake mechanisms in platelets. Statland et al. [15] described an ATP-stimulated calcium sequestering system that was inhibited by amytal and ADP, and suggested that this 'platelet relaxing factor' was a $(Ca^{2+} + Mg^{2+})$-ATPase bound to the microsomes which pumped calcium ion from the surrounding medium into the vesicular components. Robblee et al. [16] reported a platelet subcellular fraction consisting primarily of membrane vesicles which took up calcium in the presence of ATP and oxalate. This system is similar to the one reported in brain synaptic membranes [2,17]. More recently, Kaser-Glanzmann et al. [18] characterized the regulation of free calcium levels in platelets by reporting the existence of an active, ATP-dependent and cAMP-stimulated transport system in a membrane preparation from a platelet sonicate. Further experiments indicate that the dense tubular system or inside-out membrane vesicles are responsible for the calcium accumulating activity [19]. A similar calcium sequestration has been shown to exist in red cell [34] and rat brain microsomes [35] and to be modulated by the endogenous protein calmodulin.

We report in this paper the influence of $[Ca_f^{2+}]$, ATP, Mg^{2+} and fluphenazine concentration on uptake of calcium by a $40000 \times g$ human platelet fraction.

Materials and Methods

NaCl, KCl, $NaHCO_3$, sodium citrate and toluene were purchased from MCB Manufacturing Chemists, Inc., Cincinnati, OH. Prostaglandin E_1 was purchased from Upjohn Diagnostics, Kalamazoo, MI. Dextrose was purchased from Fisher Scientific, Fair Lawn, NJ. EGTA, Hepes and $MgCl_2$ were purchased from Sigma Chemical Co., St. Louis, MO. PPO, POPOP and $^{45}CaCl_2$ (4–10 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Absolute ethanol was purchased from U.S. Industrial Chemicals, Louisville, KY. Fluphenazine dihydrochloride was the gift of E.R. Squibb and Sons, Princeton, NJ.

Platelet membrane preparation

Platelets were isolated from whole blood donated on the morning of the experiments by students in their twenties. Approx. 500 ml whole blood was collected, centrifuged at room temperature at $640 \times g$ for 6 min and the platelet-rich plasma was collected. Approx. 10 μg of prostaglandin E_1 was added to the platelet-rich plasma to prevent aggregation of the platelets. The platelet-rich plasma was then centrifuged at $150 \times g$ in a Beckman Model TJ-6 Centrifuge at 9–4°C for 20 min in nalgene polycarbonate centrifuge tubes. The supernatant was removed and saved. The pellet was resuspended in 5 ml of Ringer's citrate-dextrose buffer containing 110 mM sodium chloride, 3.75 mM potassium chloride, 1.67 mM sodium bicarbonate, 21.2 mM sodium citrate and 0.5% (w/v) dextrose (pH 6.5 in 1 M HCl). Gentle mixing with a Fisher brand polyethylene standard transfer pipette was used for the resuspension. An extra 20 to 30 ml of Ringer's citrate-membrane was added and the solution was centrifuged at $150 \times g$ at 0–4°C for 20 min. The two supernatants were combined and the resulting solution was centrifuged at $550 \times g$ at 0–4°C for 30 min. The supernantant was discarded and the pellet resuspended in Ringer's citrate-dextrose, as described above, to a volume of 30 ml.

The preparation of the platelet membranes was essentially that of Kaser-Glanzmann et al. [18]. Briefly, the platelet suspension was sonicated at 40% maximum for 45 s using an Ultrasonic Cell Disruptor, Model W-220F. The sonicated suspen-

sion was centrifuged at $19000 \times g_{av}$ for 25 min at 0–4°C and the pellet discarded. The supernatant was centrifuged at $40000 \times g_{av}$ at 0–4°C for 60 min. The final pellet was resuspended in Ringer's citrate-dextrose to a concentration of approx. 0.3 mg/ml, as measured by the BioRad protein-dye binding assay, using bovine serum albumin as a standard. 500 ml of whole blood usually yielded about 2 to 4 mg of platelet membrane protein.

Analysis of ATP-dependent and -independent Ca^{2+} uptake in platelet membranes

Calcium uptake by lysed human platelet membranes was measured according to the general outline of Javors et al. [2]. Human platelet membrane were prepared as described above and incubated at 37°C in a 2 ml system with 100 mM KCl, 20 mM Hepes buffer (pH 7.1), 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ouabain, approx. 20 μg platelet membrane protein and about 0.6 μCi $^{45}CaCl_2$ ($1.32 \cdot 10^6$ CPM). In the experiments where ATP was varied, $[Ca_f^{2+}]$ was 5.11 μM, calculated according Portzehl et al. [20]. In the experiments where $[Ca_f^{2+}]$ or $[Mg^{2+}]$ was varied, ATP concentration was 250 μM. The reaction was initiated by the addition of protein and the assay mixture was vortexed once during the incubation period, which was 30 or 60 s. The reaction was terminated by filtration of the assay medium through 0.45 μm HA Millipore filters. The protein retained on the filter was washed with three 3 ml washes containing 100 mM KCl, 20 mM Hepes (pH 7.1), 1 mM $MgCl_2$ and 0.1 mM EGTA. The filters were dried and counted in 9 ml of a scintillation cocktail containing 50 mg/l POPOP, 4 g/l PPO, 150 ml/l absolute ethanol and 850 ml/l toluene. Calcium uptake was determined as nmol Ca^{2+}/min per mg platelet membrane protein.

Results

In order to fully characterize the Ca^{2+} uptake system in human platelet membranes, we investigated the kinetic requirements of the system for each of the substrates. Changes in Ca^{2+} uptake velocity were measured as a function of free Ca^{2+} concentration and are presented in Fig. 1. ATP-dependent and independent Ca^{2+} uptake was measured in the presence of various concentrations of

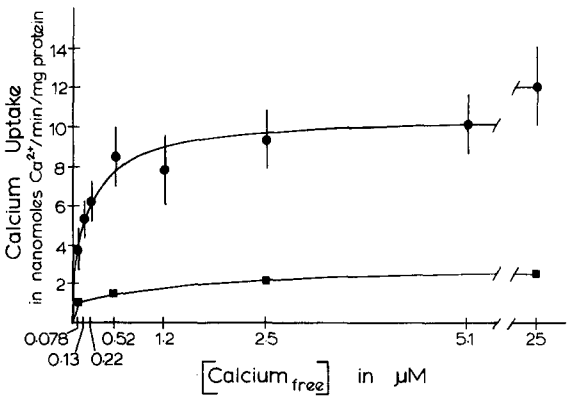

Fig. 1. Effect of $[Ca_f^{2+}]$on calcium uptake by platelet membranes. Calcium uptake was measured at 37°C in a 2 ml solution containing 20 μM Hepes (pH 7.1), 110 mM KCl, 0.1 mM EGTA, 0.1 mM ouabain, 1 mM $MgCl_2$, approx. 0.6 μCi $^{45}CaCl_2$, with or without 250 μM ATP, approx. 30 μg platelet membrane protein per assay and various concentrations of calcium. The $[Ca_f^{2+}]$ values along the abscissa were calculated according to Portzehl et al. [20]. Each point represents the mean ±S.E. for six trials. ■———■, Calcium uptake minus ATP; ●———●, calcium uptake plus ATP.

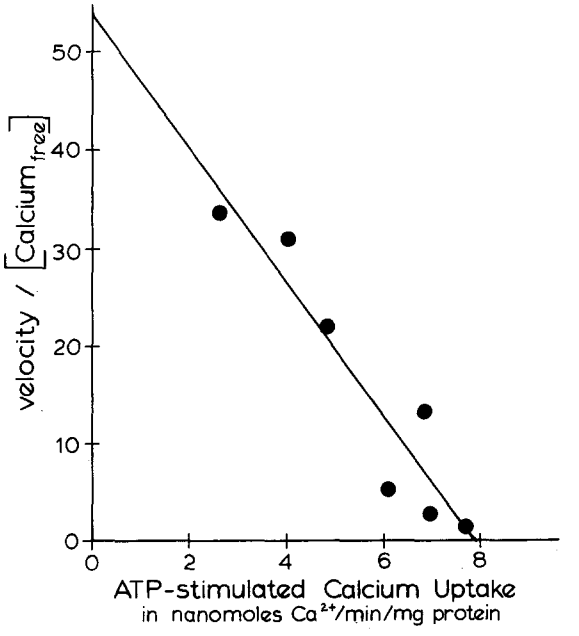

Fig. 2. Eadie-Scatchard plot of velocity of ATP-stimulated calcium uptake versus velocity/$[Ca_f^{2+}]$. The data in this graph were taken from Fig. 1. Calcium uptake activity without ATP was subtracted from the activity with ATP to give the ATP-stimulated calcium uptake. The ordinate is expressed as nmol Ca^{2+}/min per mg protein per μM $[Ca_f^{2+}]$ and is the velocity of ATP-stimulated calcium uptake divided by its respective $[Ca_f^{2+}]$.

Ca_f^{2+}. The addition of ATP (250 μM) produced a 4-fold increase in the velocity of Ca^{2+} uptake at saturating concentrations of Ca_f^{2+} (5.1 μM).

To determine whether more than one class of Ca^{2+} binding sites was present in the tissue, the Ca^{2+} uptake minus ATP was subtracted from the Ca^{2+} uptake plus ATP and the resulting ATP-stimulated Ca^{2+} uptake velocities were plotted according to the method of Eadie-Scatchard (Fig. 2). The linear regression analysis of velocity/substrate vs. velocity yields a correlation coefficient of -0.94. This kinetic treatment indicates that one Ca^{2+} uptake process is present in this platelet membrane fraction. The affinity of Ca^{2+} for this calcium uptake is 0.145 μM and the V_{max} for the ATP-stimulated calcium uptake is 7.9 nmol/min per mg protein at an ATP concentration of 250 μM.

Previous reports have identified multiple nucleotide binding sites for this Ca^{2+} transport process in widely divergent tissues. Thus, high and low affinity sites for ATP have been reported for lymphocytes [6], erythrocytes [1,21] and synaptic plasma membranes [2]. To more completely characterize the system in platelets, we varied the concentration of ATP holding Ca_f^{2+} at a fixed saturating concentration of 5.1 μM. Fig. 3 shows the results of experiments varying ATP over a 250-fold range. Increasing concentrations of ATP increase the initial rate of Ca^{2+} uptake in a linear fashion up to 40 μM. However, the rate of Ca^{2+} uptake saturates from 80 to 640 μM ATP. At least two affinities for ATP are apparent by Eadie-Scatchard analysis of the ATP-dependent Ca^{2+} uptake (Fig. 4). The linear regressions were drawn through the points which appeared linear to the eye assuming only two affinities for ATP. The ATP affinities calculated from the intercepts of the

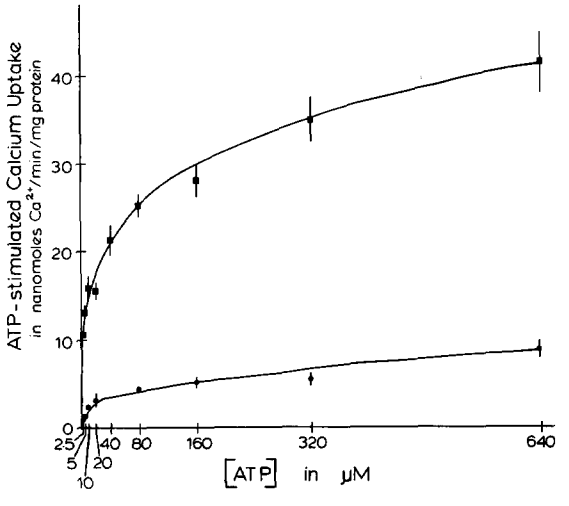

Fig. 3. Effect of increasing ATP concentration on calcium uptake by human platelet membranes. Calcium uptake was measured at 37°C in a 2 ml incubation solution containing 20 mM Hepes (pH 7.1), 100 mM KCl, 0.1 mM EGTA, 0.1 mM ouabain, 1 mM $MgCl_2$, approx. 0.6 μCi $^{45}CaCl_2$, 97.5 μM $CaCl_2$ ($[Ca_f^{2+}]$ equals 5.1 μM), approx. 30 μg platelet membrane protein and various concentrations of ATP. Each point on each curve represents the mean of four trials ±S.E. Each curve represents one experiment with one tissue preparation.

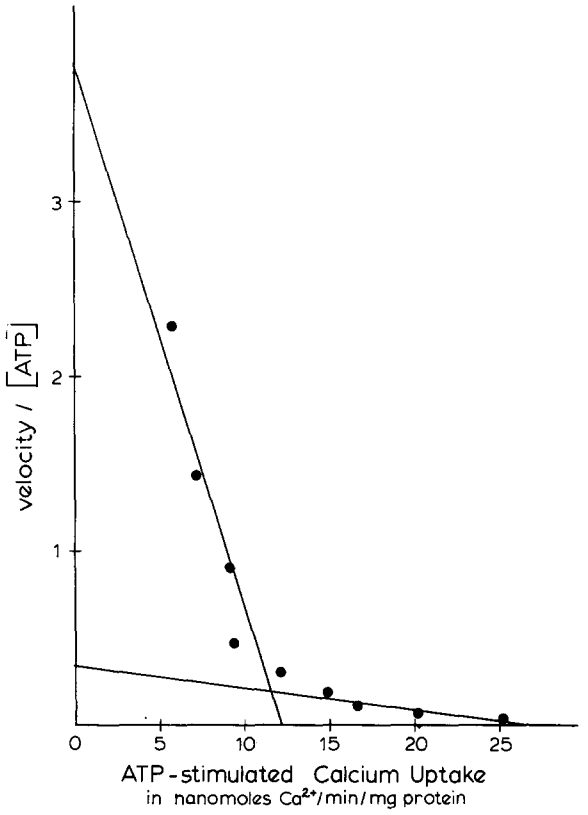

Fig. 4. Eadie-Scatchard plot of velocity of ATP-stimulated calcium uptake versus velocity/[ATP]. The data in this graph were replotted from Fig. 3. Each point is the average of the two corresponding velocities for each respective ATP concentration. The ordinate is expressed as nmol Ca^{2+}/min per mg protein per μM [ATP]. The linear regressions were drawn through points which appeared visually linear assuming two affinities.

two linear regressions were 3.21 μM and 80.1 μM. The difference in rate constants varies 25-fold, suggesting two separate nucleotide binding sites for stimulation of Ca^{2+} uptake, one active site with two or more reaction pathways or two sites with negative cooperativity.

Mg^{2+} requirements for the Ca^{2+} uptake system were evaluated by performing Mg^{2+} concentration-dependent velocity studies at fixed Ca_f^{2+} (5.1 μM) and ATP (250 μM) concentrations. Fig. 5 illustrates the effects of varying the Mg^{2+} over a 10-fold range of concentrations. The influence on calcium uptake minus ATP, calcium uptake plus ATP and ATP-stimulated Ca^{2+} uptake is presented in Fig. 5. Mg^{2+} stimulation reaches a saturation at 250 μM. As reported by other investigators, however, there appears to be appreciable Ca^{2+} uptake acting in the absence of Mg^{2+} from the incubation (Refs. 1 and 22, and Javors, M.A. and Ross, D.H., unpublished data). Inclusion of CDTA, an Mg^{2+} chelator, in the assay at zero Mg^{2+} concentration failed to alter the activity (data not shown), indicating that endogenous Mg^{2+} had been removed from the tissue fracton during its preparation.

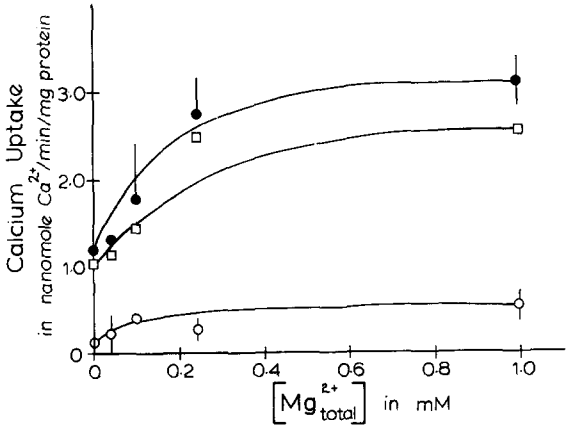

Fig. 5. Effect of increasing magnesium concentration on calcium uptake. Calcium uptake was measured at 37°C in a 2 ml incubation solution containing 20 mM Hepes (pH 7.1), 100 mM KCl, 0.1 mM EGTA, 0.1 mM ouabain, approx. 0.6 μCi $^{45}CaCl_2$, 97.5 μM $CaCl_2$, 5.1 μM [Ca_f^{2+}], 250 μM ATP, approx. 30 μg platelet membrane protein and various concentrations of $MgCl_2$ from 0 to 1 mM. Error bars are standard errors of the means. O——O, Calcium uptake minus ATP; ●——●, calcium uptake plus ATP; □——□, ATP-stimulated calcium uptake.

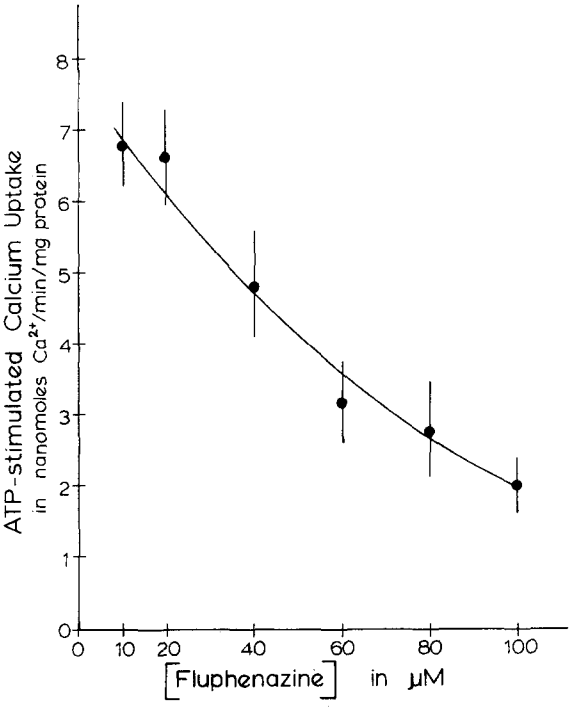

Fig. 6. Effect of fluphenazine concentration of ATP-stimulated calcium uptake by human platelet membranes. Calcium uptake was measured at 37°C in a 2 ml incubation containing 20 mM Hepes (pH 7.1), 100 mM KCl, 1 mM $MgCl_2$, 97.5 μM $CaCl_2$ (5.1 μM [Ca_f^{2+}]), approx. 30 μg platelet membrane protein and various concentrations of fluphenazine. Each point represents the mean ±S.E. for eight trials.

To extend these studies on kinetic characterization of the Ca^{2+} uptake system, we have included a pharmacological treatment of the uptake system that has altered the Ca^{2+} pump activity in other cells [23,24]. Calmodulin has been reported to stimulate ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum and synaptic membranes treated with EGTA [25,26]. Phenothiazines have been demonstrated to alter many calmodulin-dependent reactions by binding to calmodulin itself [27–29]. Therefore, it seemed appropriate to test a potent drug of this class, fluphenazine, on the Ca^{2+} transport ability of platelet membranes. Results of this study are shown in Fig. 6. Using substrate concentrations which maximally stimulate ATP-dependent Ca^{2+} uptake, membrane preparations were incubated with varying concentrations of fluphenazine. The resulting concentration response curve indicates fluphenazine has an IC_{50} of 55 μM

for ATP-stimulated Ca^{2+} uptake. This value is similar to neuroleptic inhibition constants reported for Ca^{2+} transport by other investigators [24] and for neuroleptic binding affinities to calmodulin and inhibition of calmodulin-dependent phosphodiesterase [27].

Discussion

A high affinity plasma membrane Ca^{2+} pump should be expected to exhibit a number of characteristics in relation to its function, i.e. the pump should retain a high affinity for Ca^{2+}, should saturate over a concentration range equivalent to concentrations found intracellularly and should be stimulated by low concentrations of ATP.

Data reported here characterize a Ca^{2+} pump in human platelet membrane in the form of ATP-dependent Ca^{2+} uptake into a $40000 \times g$ fraction of the type reported by Robblee et al. [16] and Kaser-Glanzmann et al. [19]. In these papers [16,19] electron micrographs of the membrane vesicles were presented. It is not clear whether these structures are plasma membrane vesicles or intracellular structures. By using initial rate kinetics, we have characterized the Ca^{2+} uptake (ATP-dependent) mechanism as having a $[Ca_f^{2+}]$ affinity of 0.145 μM in the presence of 250 μM ATP. This process appears relatively selective with a single class of uptake sites (Fig. 2).

Data from ATP-dependent experiments, however, suggests two nucleotide binding sites with affinities of 3.21 and 80.1 μM. Also, Mg^{2+} uptake stimulation of ATP-dependent Ca^{2+} uptake with a $K_m = 80$ μM. There was a Ca^{2+} uptake process which did not appear to require Mg^{2+} since the inclusion of 25 μM CDTA and Mg^{2+} chelator, in the assay did not completely abolish ATP-stimulated Ca^{2+} uptake. Graf and Penniston [1] have recently reported the possibility that Ca^{2+}–ATP alone may serve as a substrate for the Ca^{2+} pump. However, in their system, Mg^{2+} significantly enhances velocity and degree of calmodulin stimulation. We have not exhaustively characterized this aspect of Ca^{2+} transport here.

The results presented here are in agreement with previous reports characterizing the Ca^{2+} pump in other systems [1–6]. Additionally, our data agree, in a general sense, with previous reports on Ca^{2+} uptake in platelets [16,18,19]. Robblee et al. [16] demonstrated the subcellular location of the calcium uptake and the oxalate-enhanced ATP requirement. They also showed a concomitant inorganic phosphate release which associated an ATPase activity with the calcium transport. In a series of three papers, Kaser-Glanzmann et al. [18,19,33] further characterized this calcium accumulating system. In a tissue fraction similar to the one Robblee et al. [16] used, ATP was required for the calcium uptake into membrane vesicles and a cAMP-stimulated, protein kinase mediated phosphorylation appeared to be the regulatory mechanism. In this paper, we have used EGTA as a free calcium concentration buffer to examine more closely the kinetic effects of free calcium concentration on the calcium transport sytem and we have done the experiments without the inclusion of oxalate in the assay. Also, to our knowledge, this is the first report of multiple ATP affinities for the ATP-stimulated calcium transport in platelet membranes vesicles.

These data support our original aims of defining a Ca^{2+} system in human platelet membrane which operates in a fashion similar to that found in synaptic membranes [2]. The common requirements for Ca^{2+} exhibited by both platelets and synaptosomes in many transmitter-meditated processes, such as receptor binding, transmitter release, phosphorylation and the high affinity Ca^{2+} pump capacity, strongly support the use of the platelet for a peripheral model system in which to study Ca^{2+}-dependent reaction mechanisms common to synaptic membranes.

The ability of the neuroleptic fluphenazine to inhibit the ATP-stimulated calcium uptake studied here addresses the possibility of calmodulin involvement in the calcium transport. Either fluphenazine directly affects the $(Ca^2 + Mg^{2+})$–ATPase that drives the transport system or it indirectly affects the transport system by binding to calmodulin and reversing the calmodulin stimulation of the calcium transport. Direct calmodulin stimulation of a calcium transport has been shown in red cell [34] and lysed synaptosome preparations [35]. However, in our platelet membrane preparation we have been unable to demonstrate a direct calmodulin stimulation of the calcium transport by removal of endogenous calmodulin with

EGTA treatment and exogenous calmodulin replacement (data not shown). Recently, Chiesi and Carafoli [36] have reported trifluoperazine inhibition of a calcium transport in sarcoplasmic reticulum was due to binding of the drug to a 53000 glycoprotein. It is possible that the fluphenazine effect seen here: (a) may be mediated by an effect on calmodulin that cannot be removed with EGTA treatment, (b) may be a direct effect on the (Ca^{2+} + Mg^{2+})-ATPase or (c) may be due to an effect at an allosteric site, as another protein. Work is in progress in our laboratory to examine these posibilities in platelet membranes.

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