The effects of cadmium (Cd\(^{2+}\)) on transmembrane Ca\(^{2+}\) transport and on the membrane permeability for Ca\(^{2+}\) were studied in human erythrocytes. The erythrocyte Ca\(^{2+}\) pump is inhibited competitively by Cd\(^{2+}\) via interaction with the Ca\(^{2+}\) transport site of the carrier and not via interaction with its activator calmodulin. The affinity of the Ca\(^{2+}\) pump for Cd\(^{2+}\) is extremely high (\(K_d = 2.0 \times 10^{-16}\) M) does not alter the membrane permeability for Ca\(^{2+}\). We conclude that the pivotal mechanism in the toxic action of Cd\(^{2+}\) is the inhibition of Ca\(^{2+}\)-ATPase mediated Ca\(^{2+}\) extrusion. As a result Cd\(^{2+}\) disturbs intracellular Ca\(^{2+}\) homeostasis and may increase cytosolic Ca\(^{2+}\) (Ca\(^{2+}\_i\)) to toxic levels.

Cadmium, reputed for its toxicity, has become widely distributed in the environment as an industrial waste. The mechanism of cadmium toxicity is incompletely understood although a specific interaction with the cellular calcium metabolism has been indicated. Several cellular Ca\(^{2+}\) acceptors are Cd\(^{2+}\) targets as well. The similar behavior of Ca\(^{2+}\) and Cd\(^{2+}\) with regard to, for example, calmodulin (1–4) has been attributed to a comparable charge and ionic radius of Ca\(^{2+}\) and Cd\(^{2+}\) (5). However, Cd\(^{2+}\) blocks Ca\(^{2+}\) channels (6, 7), inhibits Na\(^+\)/Ca\(^{2+}\) exchange processes (8), and impedes the plasma membrane Ca\(^{2+}\) pump (9–11).

In vivo, Cd\(^{2+}\) poisoning results in anemia as a result of increased erythrocyte clearance (12). An important indicator for Cd\(^{2+}\) poisoning is an increase in Ca\(^{2+}\). Incubation of erythrocytes with Cd\(^{2+}\) (0.5 mM for 1 h) accelerates age-related changes of the cells, such as increased cell density, loss of discoidal shape, and decreased filterability. The same changes can be induced by calcium loading of the cells (13). Scott et al. (10) have shown that Cd\(^{2+}\) exposure increases 45Ca\(^{2+}\) accumulation by lymphocytes, but not as a result of enhanced Ca\(^{2+}\) influx. The authors related the increased accumulation of 45Ca\(^{2+}\) to an inhibition of the Ca\(^{2+}\) pump by Cd\(^{2+}\). Accumulation of Cd\(^{2+}\) in human platelets resulted in increased protein phosphorylation (14) which may be ascribed to an increase in Ca\(^{2+}\) (15).

Cd\(^{2+}\) may increase Ca\(^{2+}\), by enhancing the Ca\(^{2+}\) permeability of the cell membrane (15) or by inhibiting the Ca\(^{2+}\) pump (10). At least two mechanisms of Ca\(^{2+}\) pump inhibition by Cd\(^{2+}\) may be anticipated, viz., by interaction with the Ca\(^{2+}\) binding site of the ATPase or by interaction with the Ca\(^{2+}\) pump (1). This study was supported by a grant from the Foundation for Fundamental Biological Research (BION), which is subsidized by the Dutch Organization for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Materials and Methods**

Preparation of Ghosts—Ghosts were prepared according to Schatzmann (26), with minor modifications; the medium for hemolysis contained 15 mM KCl, 4 mM MgCl\(_2\), 10 mM sucrose, 10 mM Tris-HCl, pH 7.4. 1 mM EGTA was added to this hemolyzing medium if the ghosts had to be loaded with EGTA. The osmolality of the hemolyzing medium was 69 mosm.

The membrane orientation was determined according to Steck and Kant (27). The ghost preparation was 8.7 ± 3.0% inside-out and 87.3 ± 2.8% right-side-out (n = 9). Preparation of ghosts in the presence of Cd\(^{2+}\)-EGTA had no effect on the membrane resealing or orientation: 65.3 ± 3.8% was inside-out and 88.2 ± 2.8% was right-side-out (n = 3).

Preparation of Vesicles—Resealed IOV were prepared from freshly collected human blood, according to Sarkadi et al. (28). Sodium-heparin was used as an anticoagulant (100 IU/ml). The vesicle preparation used in these studies was 52.3 ± 10.2% IOV and 39.0 ± 12.3% (n = 7) right-side-out vesicles.

**Ca\(^{2+}\) Accumulation in Ghosts**—Before the 45Ca\(^{2+}\) accumulation experiments, ghosts were washed and resuspended in incubation medium (126.5 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO\(_4\), 20 mM HEPES/Tris, pH 7.4) to a protein concentration of 3.15 ± 0.67 corresponding to 25.7 ± 4.5% hemocrit (n = 14). After prewarming this suspension to 37 °C, 45Ca\(^{2+}\) uptake was started by adding 45Ca\(^{2+}\) (1 × 10\(^6\) Bq⋅ml\(^{-1}\)). CaCl\(_2\) (final concentration 0.42 mM), and Cd(NO\(_3\))\(_2\) as required. Sequential 100-μl samples were taken and mixed with 2 ml of ice-cold stop buffer containing 150 mM NaCl, 20 mM Tris, pH 7.2, and 1 mM LaCl\(_3\). After centrifugation (10 min, 1200 × g) the supernatant was removed by suction. Variation in the amount of protein per sample collected by centrifugation was constant (deviation 6.2 ± 1.5%, n = 28). The pellet was lysed in 300 μl of water and transferred to a counting vial.

**Ca\(^{2+}\) Transport Assay**—Vesicles were resuspended in 150 mM KCl, 1.5 mM MgCl\(_2\), 20 mM Hepes/Tris, pH 7.4. Ca\(^{2+}\) transport into membrane vesicles was determined using a rapid filtration technique (29). The content of membrane vesicles in suspension was determined on the basis of protein concentration, determined with a Coomassie Blue kit with bovine serum albumin as standard (Bio-Rad). Media and assay conditions are described in detail in Ref. 31. Free Ca\(^{2+}\), Cd\(^{2+}\), and Mg\(^{2+}\) concentrations were calculated with a matrix computer program (29) taking into account the first and second protonations of the respective ligands (ATP, EGTA, HEEDTA, NTA).

The abbreviations used are: EGTA, [ethylenebis(oxy-ethylene<nitritolo)]tetraacetic acid; HEEDTA, N-(2-hydroxyethyl)-ethylene-nitridine-N\(^\prime\),N\(^\prime\)′-triacetic acid; NTA, nitrilotriacetic acid; IOV, inside-out vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
binding constants were taken from Sillen and Martell (30) except the one for Cd-ATP (Kd = 5.43 M⁻¹) which was determined in our laboratory (31). ATP-driven Ca²⁺ transport in plasma membrane vesicles was determined as the difference of Ca²⁺ retained in the presence and in the absence of ATP. In all kinetic studies initial velocities were determined from 1-min incubations. Under all conditions Ca²⁺ transport followed Michaelis-Menten kinetics. Kinetic parameters were derived from best fits of the curves using a nonlinear regression data analysis program (32).

RESULTS

Ca²⁺ accumulation in ATP-depleted, erythrocyte ghosts plateaued within 5 min at around 15 nmol of Ca²⁺ (mg protein)⁻¹ (Fig. 1). Loading with EGTA does not affect Ca²⁺ accumulation. Permeation of the ghosts with the Ca²⁺ ionophore A23187 (10 µg·ml⁻¹) increases the amount of ⁴⁵Ca²⁺ accumulated, showing that a Ca²⁺ gradient (high outside, low inside) is conserved during the experimental period of 60 min. Cd²⁺ levels up to 0.1 mM have no effect on the Ca²⁺ accumulation, and 1.0 mM Cd²⁺ significantly inhibited the accumulation.

The rate of ATP-dependent Ca²⁺ transport was constant for at least 2 min. At 15 min, 95 nmol of Ca²⁺ (mg protein)⁻¹ had accumulated. Addition of A23187 (10 µg·ml⁻¹) induced release of ⁴⁵Ca²⁺ accumulated in the membrane vesicles by the ATP-driven process (inset, Fig. 2).

The maximum transport velocity of the Ca²⁺ transporter (Vₘₐₓ) was 18.8 ± 1.2 nmol of Ca²⁺ min⁻¹ (mg protein)⁻¹ (or 35.9 nmol of Ca²⁺ min⁻¹ (mg IOV protein)⁻¹), and an affinity (Kₘ) for Ca²⁺ of 0.48 ± 0.10 µM was observed.

The IOV isolation method used (28) yields calmodulin-depleted membrane vesicles. Calmodulin repletion (10 µg·ml⁻¹) increased the Vₘₐₓ by 140% but did not effect the Kₘ for Ca²⁺. Thus, calmodulin exclusively stimulates the maximal transport velocity of the Ca²⁺ pump, an observation in line with that of other researchers using Ca-EGTA buffers (16).

In a medium in which Ca²⁺ is buffered to 1 µM Ca²⁺, Ca²⁺ transport is half-maximally inhibited at 6.06 ± 1.72 nM Cd²⁺ (Fig. 3). At all Cd²⁺ concentrations above 1 nM (no effect level) Ca²⁺ transport was significantly inhibited.

Cd²⁺ did not effect the Vₘₐₓ of Ca²⁺ transport but significantly increased the Kₘ for Ca²⁺, both in the absence and in the presence of calmodulin (Fig. 4). This increase was linear from 1 to 5 nM Cd²⁺, which defines the inhibition as competitive.

In Fig. 5 the apparent Kₘ for Ca²⁺ is plotted versus medium Cd²⁺ concentration. From this plot a Kᵢ value of 2.0 nM Cd²⁺ was derived (33).

**DISCUSSION**

Erythrocyte, ATP-driven transmembrane movement of Ca²⁺ proved extremely sensitive to Cd²⁺. The Kᵢ for Cd²⁺ is 100 times as high as the Kᵢ for Ca²⁺. This Kᵢ indicates the involvement of thiol groups in Ca²⁺ transport. Thiol groups are known to have a high affinity for Cd²⁺ (pKᵢ ~17; Ref. 17). Indeed, the DNA sequence of the Ca²⁺-ATPase from rabbit muscle sarcoplasmic reticulum (18) predicts that the Ca²⁺ binding site contains a SH group. Hepatocyte microsomal Ca²⁺ sequestration (which is Ca²⁺-ATPase-mediated) is critically dependent on protein sulphydryl groups, and modification of protein thiols may be an important mechanism for the
inhibition of microsomal Ca$^{2+}$ sequestration by a variety of toxic agents (19). Although the Ca$^{2+}$ pumps of the plasma membrane and of the endoplasmic or sarcoplasmic reticulum are immunologically distinct (21), we postulate that a conserved Ca$^{2+}$ binding region makes these systems equally sensitive to Cd$^{2+}$ inhibition. Indeed, we observed that Ca$^{2+}$ sequestration in endoplasmic reticulum and Golgi apparatus of rat duodenal cells (a process dependent on a similar Ca$^{2+}$-ATPase as found in plasma membranes of rat duodenum (20)) is as sensitive to Cd$^{2+}$ as the plasma membrane Ca$^{2+}$ pump (11). We conclude that for Cd$^{2+}$ intoxication membrane Ca$^{2+}$ pumps are the most sensitive membrane transport system described thus far.

One could argue that Cd$^{2+}$ affects the membrane integrity instead of inhibiting Ca$^{2+}$ transport in membrane vesicles. High concentrations of Cd$^{2+}$ may increase the permeability to Ca$^{2+}$ of plasma membranes of erythrocytes and hepatocytes (15, 22). However, from our experiments such an effect appears unlikely: the membrane permeability for Ca$^{2+}$ in ghosts is not influenced by up to 0.1 mM Cd$^{2+}$. Moreover, at 1.0 mM Cd$^{2+}$, 45Ca$^{2+}$ accumulation was inhibited rather than enhanced as one may predict when Cd$^{2+}$ increases the membrane permeability for Ca$^{2+}$. Competition of Cd$^{2+}$ with Ca$^{2+}$ pumps may be responsible for this observation.

We have at least three reasons to conclude that the inhibition is not through calmodulin. First, although Cd$^{2+}$ activates calmodulin (1–3, 5) and may stimulate Ca$^{2+}$- and calmodulin-dependent enzymes (23), such an interaction seems unlikely on the erythrocyte Ca$^{2+}$ pump. As the affinity of calmodulin for Ca$^{2+}$ and Cd$^{2+}$ in vitro is in the micromolar range (4, 23) and the Cd$^{2+}$ concentration in our media is at least a 100 times lower than the Ca$^{2+}$ concentration, the formation of Cd-calmodulin complexes will be insignificant. Second, indirect inhibition of the Ca$^{2+}$ pump via Cd$^{2+}$ binding to calmodulin is unlikely since the inhibition is competitive, and stimulatory effects of Cd$^{2+}$ on calmodulin would have affected the $V_{\text{max}}$. Third, at 5 nM Cd$^{2+}$ (inhibiting Ca$^{2+}$ transport in calmodulin-depleted membrane vesicles by 40–50%) calmodulin repletion has stimulatory effects on IOV Ca$^{2+}$ transport. The same $V_{\text{max}}$ is observed for calmodulin-stimulated Ca$^{2+}$ transport whether Cd$^{2+}$ is present or not. Thus, a binding site for Cd$^{2+}$ other than on calmodulin is involved. We therefore conclude that Cd$^{2+}$ inhibits Ca$^{2+}$ transport by competing with Ca$^{2+}$ for the Ca$^{2+}$ binding site of the enzyme.

In several studies (4, 9, 23, 24) it was concluded that Cd$^{2+}$ exerts its toxic action by upsetting calmodulin dependent enzyme systems by binding to calmodulin. Micromolar concentrations Cd$^{2+}$ are required in vitro to obtain significant activation of calmodulin by Cd$^{2+}$ (e.g. induction of tyrosine fluorescence, stimulation of phosphodiesterase). Obviously, the unprecedented 1000-fold higher affinity for Cd$^{2+}$ of the membrane Ca$^{2+}$ pump makes this enzyme the "most sensitive Cd$^{2+}$ target" in a cell exposed to Cd$^{2+}$.

The conclusion that the Cd$^{2+}$ inhibition of the Ca$^{2+}$ pump is calmodulin-independent has important consequences for the extrapolation to in vivo conditions. At the resting Ca$^{2+}$, (≤0.1 μM), most of the calmodulin is dissociated from the erythrocyte Ca$^{2+}$ pump (25). As we have shown here, the pump may still be inhibited by Cd$^{2+}$ at this Ca$^{2+}$. Yet, a subsequent calmodulin stimulation, resulting from a rise in Ca$^{2+}$, may give a partial restoration of low Ca$^{2+}$, as inhibition by Cd$^{2+}$ of the enzyme activity does not preclude activation of the enzyme by calmodulin. For a full understanding of these processes it is necessary to monitor Ca$^{2+}$, during cadmium exposure. However, this research has proved difficult since Cd$^{2+}$ interacts with the well known Ca$^{2+}$ probes quin2, fura-2, and indo-1.2,3

REFERENCES
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