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The Role of Calcium in Drug Action

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SECTION EDITOR

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PREFACE

The free cytosolic Ca^{2+} ion concentration controls many cellular functions, and an abnormality in the concentration of intracellular Ca^{2+} can lead to profound metabolic effects. Take, for example, the anaesthetic complication malignant hyperpyrexia. The clinical features of malignant hyperpyrexia are diverse and include muscular rigidity, a rapid and sustained rise in body temperature and metabolic acidosis. These all result from an elevated myoplasmic Ca^{2+} concentration which develops when an abnormal muscle cell membrane is exposed to an anaesthetic such as halothane. The chance observation that a drug, dantrolene sodium, lowers myoplasmic Ca^{2+} concentration, led to the use of dantrolene sodium as a specific and effective treatment for malignant hyperpyrexia. Dantrolene sodium is but one example of a drug which acts by its effect on the cytoplasmic Ca^{2+} concentration, and in this volume the actions of drugs on cytosolic Ca^{2+} concentrations in relation to a variety of other human cells and clinical problems are reviewed. Disorders of red blood cells, platelets, the heart, bone, hypertension and asthma may be linked by a common factor—disturbance of intracellular calcium regulation.

The level of cytoplasmic Ca^{2+} concentration depends on the amount of Ca^{2+} released from intracellular storage sites and on the amount of Ca^{2+} entering the cell through channels in the surface membrane. As our knowledge increases about these two mechanisms as a result of detailed physiological and biochemical investigations, it seems very likely that the range of pharmacological agents which can alter the mechanisms will expand, and have a significant role in preventing and treating a wide variety of human diseases.

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CHAPTER 1

CALCIUM IONS, DRUG ACTION AND THE RED CELL MEMBRANE

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1. INTRODUCTION

Many unique properties of the mammalian erythrocyte make this cell among the most specialized in the body. High concentrations of hemoglobin for the carriage of oxygen, a total dependence on glycolytic metabolism and an unusual biconcave shape and flexibility have long been known. More recent studies have indicated that the ionic permeability of the erythrocyte membrane is also unique. Red cell fluxes of monovalent cations, Na^+ and K^+ are far lower than observed in nucleated cells while divalent cation fluxes are even lower. Analysis of intracellular calcium by atomic absorption spectroscopy has yielded values which are barely measurable and this low value reflects the few Ca^{2+}-accumulating organelles (i.e. mitochondria, endoplasmic reticulum) in the mature erythrocyte. Indeed, the ionized (free) Ca^{2+} concentration in the human red cell has recently been reported to be only 0.2 μM measured by a null-point method with a Ca^{2+}-sensitive electrode (Simons, 1981). Considerably higher values for total red cell Ca^{2+} have been found, and most studies which have used the dry-ashing technique give a Ca^{2+} content of about 15 nmol/ml cells (Harrison and Long, 1968; Lichtman and Weed, 1972). Trace contamination by Ca^{2+} is widespread in reagents from glass bottles while even plastics may contribute Ca^{2+} to an ambient solution, presumably from the plasticizer used to impart flexibility to the material. When rigorous precautions were taken to remove Ca^{2+} from washing solutions and containers, analysis of total red cell Ca^{2+} has yielded values of only 5 nmol/ml cells (Bookchin and Lew, 1981; Wiley and Shaller, 1977). Whatever value is taken for the total red cell Ca^{2+}, it is clear that 95% or more of this cation is bound to some ligand(s). The cytoplasmic protein calmodulin, inorganic and organic phosphates and membrane phospholipids, especially di- and tri-phosphoinositides have all been shown to bind Ca^{2+} with varying affinity. When red cell Ca^{2+} is raised by addition of cells to a Ca^{2+}-medium containing the divalent cation ionophore, A23187, a significant fraction of cell Ca^{2+} (50–80%) behaves as the bound (nonionized) species even when cell Ca^{2+} is raised to the vicinity of 1 mM (Ferriera and Lew, 1976). Again the identity of this low affinity Ca-buffer of high capacity is not certain, although organic phosphates such as 2,3-diphosphoglycerate (2,3 DPG) and adenosine triphosphate (ATP) seem likely candidates.

The majority of Ca^{2+} within the erythrocyte normally appears to be membrane-bound since it separates with the stroma on centrifugation of hemolysates (Harrison and Long, 1968; Weed *et al.*, 1969; Wiley and Gill, 1976). A recent paper has demonstrated that much of this stroma-associated Ca^{2+} is associated with intracellular vesicles with an ATP-dependent capacity to accumulate this cation (Lew *et al.*, 1985). A direct function of membrane Ca^{2+} in maintaining low permeability is suggested by the action of Ca^{2+} in reversing the large increase in cation permeability which can be induced in human

erythrocytes by exposure to nonelectrolyte solutions such as lactose (Bolingbroke and Maizels, 1959). Moreover, extracellular Ca^{2+} does have small but significant effects on both Na^+ and K^+ influxes into human red cells which are reduced by 20–40% in the presence of 10 mM $CaCl_2$ (Ellory *et al.*, 1980). However, perhaps the most important function of membrane Ca^{2+} is to influence the physical state of the cytoskeleton which, in turn, determines the deformability of the cell (see Section 4.4). The role of Ca^{2+} in red cell physiology has been the subject of several recent reviews (Ferreira and Lew, 1977; Parker 1981; Roufogalis, 1979; Vincenzi and Larsen, 1980).

2. CALCIUM PUMP

Many cells utilize Ca^{2+}-sequestering organelles such as mitochondria or endoplasmic reticulum to minimize cytoplasmic free Ca^{2+}, although a plasma membrane Ca^{2+}-pump is still necessary to maintain low cell Ca^{2+} levels. However, erythrocytes depend only on an active Ca^{2+}-pump to maintain cytoplasmic Ca^{2+} at $< 1\ \mu M$. This pump is localized in the membrane and displays a Mg^{2+}-dependent Ca^{2+}-activated ATPase activity (EC 3.6.1.3). The initial studies of Schatzmann and Vincenzi (1969) demonstrating outward net transport of Ca^{2+} from Ca^{2+}-loaded ghosts have been amply confirmed (Lee and Shin, 1969; Olsen and Cazort, 1969). Calcium extrusion was strongly temperature dependent ($Q_{10} = 3.5$) and had a pH optimum between 6.9 and 8.5. Some dependence of pumping on external cations can be demonstrated with a rank order $K^+ > Na^+ =$ choline $> Mg^{2+}$ for stimulation of both Ca^{2+} extrusion and ($Ca^{2+} + Mg^{2+}$) ATPase of ghosts (Romero, 1981; Schatzmann and Rossi, 1971; Wierichs and Bader, 1980). The Ca^{2+}-pump is capable of extruding this cation against a very large concentration gradient. Cells loaded with 0.6 mM Ca^{2+} will extrude this cation into a 110 mM $CaCl_2$ (isotonic) medium, although the pumping rate is only half the maximal value observed with no Ca^{2+} externally (Fig. 1). No selective inhibitors of the Ca^{2+} pump have been discovered to date. However, cation analogs such as ruthenium red, vanadate, Sr^{2+} or La^{3+} do inhibit Ca^{2+} transport in a competitive fashion (Allan and Thomas, 1981b; Rossi *et al.*, 1981; Szasz *et al.*, 1978). Intracellular Mg^{2+} is a cofactor for Ca^{2+} pumping, although Mg^{2+} is not itself transported by the pump. Many studies support the view that the red cell membrane ($Ca^{2+} + Mg^{2+}$) ATPase and the active outward transport of Ca^{2+} are two manifestations of the same enzymatic process (Cha, *et al.*, 1977; Lee and Shin 1969; Olsen and Cazort, 1969).

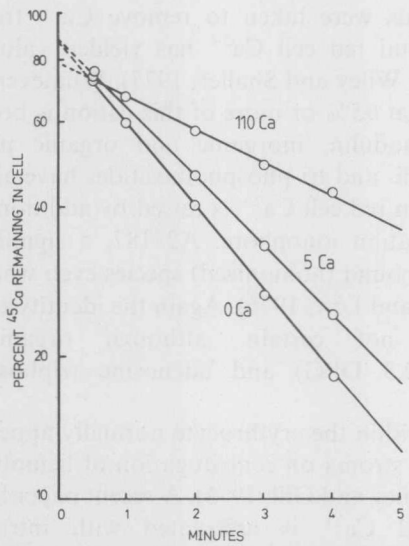

FIG. 1. Calcium extrusion from fresh human erythrocytes. Cells were preloaded with Ca^{2+} at 0°C in a salicylate medium (see Wiley and Gill, 1976), washed and reincubated at 37°C in media containing 0, 5 or 110 mM $CaCl_2$ plus NaCl to maintain isotonicity. Initial cell Ca^{2+} was 0.6 μmol/ml cells.

2.1 Kinetics and Stoichiometry of the Pump

A wide range of kinetic data and stoichiometries have been reported for the red cell Ca^{2+}-pump, much of which may result from differences in methodology. The majority of studies have utilized resealed ghosts or inside-out vesicles (IOV) rather than intact red cells because concentrations of substrates and cofactors can be more easily controlled. However, the kinetic properties of these systems may not necessarily be identical to those in the intact red cell. For example, sonication of ghosts in isosmotic imidazole buffer results in a large loss of (Ca^{2+} + Mg^{2+}) ATPase activity, while repeated freeze thawing has been shown to both increase and decrease (Ca^{2+} + Mg^{2+}) ATPase activity (Farrance and Vincenzi, 1977a; Meltzer and Kassir, 1981). Also, several reports suggest that exposure of IOV to dextran T-70 (or T-110) gradients results in a loss in both Ca^{2+} pumping and (Ca^{2+} + Mg^{2+}) ATPase activity (Quist and Roufogalis, 1977; Vincenzi and Larsen, 1980). The use of high ionic strength buffers, such as 310 mM (isosmotic) imidazole to prepare IOV and resealed ghosts can also cause an increase in the (Ca^{2+} + Mg^{2+}) ATPase activity. Possibly the extent of calmodulin (CaM) binding to the membrane during its preparation (see Section 2.3) could account for some of the differences in the (Ca^{2+} + Mg^{2+}) ATPase activities noted below (Farrance and Vincenzi, 1977a,b).

The results of many kinetic analyses are listed in Table 1 which shows both high and low affinities of the Ca^{2+}-pump towards both Ca^{2+} and ATP. However, the data cannot be taken as evidence for two separate Ca^{2+}-transport mechanisms, since interconvertibility of the high and low affinity transport systems has been demonstrated. Human erythrocyte IOV, devoid of CaM, demonstrate a low Ca^{2+} affinity transport component ($K_m > 100\ \mu M\ Ca^{2+}$). Binding of CaM to the IOV converted the Ca^{2+}-pump to high Ca^{2+} affinity kinetics (K_m 2.5 μM Ca^{2+}; Larsen *et al.*, 1981a). In a different preparation (fragmented membranes), it has also been demonstrated that the binding of CaM alters the (Ca^{2+} + Mg^{2+}) ATPase from a low to a high Ca^{2+} affinity state (Table 1; Foder and Scharff, 1981). Clearly binding of CaM to the membrane alters the microenvironment of the Ca^{2+}-pump, although it is not certain whether the interactions of the cytoskeleton with the membrane are also affected.

Variable stoichiometry of the red cell Ca^{2+}-pump has been reported. Initial studies of Ca^{2+} extrusion by resealed red cell ghosts reported 0.8 Ca^{2+} ions/ATP hydrolyzed, while subsequent work has supported a 1:1 stoichiometry for the red cell Ca^{2+}-pump, both in resealed ghosts and intact cells (Larsen *et al.*, 1978; Mualem and Karlish, 1979; Romero, 1981; Schatzmann and Vincenzi, 1969). In two of these studies, Ca^{2+} pumping was analyzed by multiple techniques: Ca^{2+} selective glass electrodes, $^{45}Ca^{2+}$ efflux and flame analysis by atomic absorption spectroscopy, each of which was compared to ATP splitting measured in the presence of ouabain to remove the contribution of the (Na^+ + K^+)

Table 1. *Reported Affinities for Ca^{2+} Transport and (Ca^{2+} + Mg^{2+}) ATPase*

Assay	Preparation	Ca^{2+} K_m (μM)	Mg^{2+}-ATP K_m (μM)	Reference
Transport	IOV	3.4	100 1000	Mollmann and Pleasure (1980)
Transport	IOV	19	—	MacIntyre and Gunn (1981)
Transport	Intact cells	0.7–1.0	—	Ferreira and Lew (1976)
ATPase	Resealed ghost	0.9	50	Wolf (1972)
Transport and ATPase	Resealed ghost	—	1–2 180	Mualem and Karlish (1979)
ATPase	Membranes	—	1–2	Schatzmann (1977) Rega and Garrahan (1975)
ATPase	Membranes	1.1 32	—	Foder and Scharff (1981)
Transport	IOV	2.5 100	—	Larsen *et al.* (1981a)
Transport	Resealed ghost	3 250	—	Romero (1981)

pump. Control cells under each condition included 250 μM La^{3+} to block Ca^{2+} pumping, which allowed correction for ATP splitting independent of the Ca^{2+}-pump. However, not all investigators agree on the stoichiometry of the Ca^{2+}-pump and a ratio of 2 Ca^{2+} ions/ATP hydrolyzed has been reported by Sarkadi *et al.* (1977) and Quist and Roufogalis (1975). However, these latter studies utilized lower concentrations (100 μM) of La^{3+}, and it is possible that variations in Ca-independent ATP hydrolysis or leakiness of reconstituted ghosts to internal Ca^{2+} may account for the different stoichiometric ratios which have been reported. More recent evidence suggests that the ratio of Ca^{2+} pumped:ATP hydrolyzed can vary with the level of intracellular Ca^{2+}. In an IOV preparation, a stoichiometry of 2 Ca^{2+} pumped:1 ATP hydrolyzed was found with low ambient Ca^{2+} (<20 μM), while with high Ca^{2+} (>100 μM), the ratio became 1:1 (Larsen *et al.*, 1981a).

2.2. Energy Requirements

The addition of Ca^{2+} to a suspension of ATP-replete erythrocytes had no effect on ATP levels and increased ATP breakdown by less than 20 nmol/ml cells/hr (Ferreira and Lew, 1975). This value is less than 1% of normal glycolytic ATP production whereas, in contrast, the sodium pump consumes 10–15% of red cell glycolytic energy. Artificial induction of Ca^{2+} leakiness by ionophore A23187 greatly stimulates the Ca^{2+}-pump and leads to a progressive depletion of ATP levels (Allan *et al.*, 1976; Kirkpatrick *et al.*, 1975; Lang *et al.*, 1977; Plishker and Gitelman, 1977). Various nucleotide triphosphates, other than ATP, can act as substrates for the (Ca^{2+} + Mg^{2+}) ATPase in resealed ghosts, with reported efficacy in the order ATP = CTP = UTP > GTP > ITP (Lee and Shin, 1969). Whether these triphosphates act as phosphate donors for the pump or as substrates for nucleoside diphosphokinase (thereby maintaining ATP levels) is not established.

2.3. Calmodulin (CaM) Stimulation

In 1973, Bond and Clough reported that the red blood cell (Ca^{2+} + Mg^{2+}) ATPase was activated up to 2.5-fold by crude hemolysate, due to an activator protein distinct from hemoglobin. This important discovery has led to the characterization of calmodulin which is a small, heat stable, acidic protein with a ubiquitous distribution in eukaryotic cells. Calmodulin has an important role in activating many Ca^{2+}-dependent enzymes, such as cyclic adenosine monophosphate phosphodiesterase, myosin kinase, adenylate cyclase and phosphorylase kinase, as well as the red cell calcium pump (Gopinath and Vincenzi, 1977; Jarrett and Kyte, 1979; Jarrett and Penniston, 1977; Luthra *et al.*, 1976; MacIntyre and Green, 1978). Molecular sizing by sodium dodecyl sulfate gel electrophoresis indicates that CaM is approximately 18,000 daltons and is present at a concentration of 1–3 μM in the erythrocyte cytosol of many different species (Jarrett and Penniston, 1977; Vincenzi, 1981). It is believed that CaM has four Ca^{2+} binding sites per molecule, two of which are of high affinity and two of lower affinity for binding Ca^{2+} ions (Walsh and Stevens, 1978). Direct binding of CaM to the (Ca^{2+} + Mg^{2+}) ATPase has been reported with a 1:1 stoichiometry both in human ghosts and IOV using photoaffinity labelling with azido-^{125}I-CaM (Hinds and Andreasen, 1981). The major effect of CaM is to increase the maximum velocity (V_{max}) of both the Ca^{2+}-pump and (Ca^{2+} + Mg^{2+}) ATPase, and stimulations ranging from 2 to 10-fold have been reported. Moreover, some studies suggest that CaM also increases the apparent affinity of the (Ca^{2+} + Mg^{2+}) ATPase for Ca^{2+} ions (Al-jabore and Roufogalis, 1981; Gopinath and Vincenzi, 1977; Meltzer and Kassir, 1981; Vincenzi and Larsen, 1980).

CaM is believed to act through a two-step process:

(1) $CaM_{inactive} + nCa^{2+} \rightleftharpoons (CaM^*.\ Ca_n^{2+})_{active}$

(2) $(CaM.\ Ca_n^{2+})_{active} + enzyme_{less\ active} \rightleftharpoons [enzyme^*.\ (CaM.\ Ca_n^{2+})]_{active}$

where * represents a new conformation (Lynch and Cheung, 1979). Thus the interactions of Ca^{2+} with CaM may represent an important regulatory step in the activity of several Ca^{2+} dependent reactions.