The role of band III in calcium transport across the human erythrocyte membrane

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1. INTRODUCTION

The ATP-dependent Ca^{2+} pump of the human erythrocyte maintains a low intracellular Ca^{2+} concentration ($\leq 10^{-7}$ M) against a large extracellular concentration (10^{-3} M) [1–3]. Two models have been presented to explain the mechanism of Ca^{2+} translocation in erythrocyte plasma membranes. The first model [4,5] was developed from studies of Ca^{2+} transport into inside-out membrane vesicles (IOV), from which it was observed that the active transport of Ca^{2+} into IOV was stimulated by anions such as phosphate, chloride, acetate and sulfate, and that the stimulation of calcium transport by these anions was due to an active, calcium-dependent uptake of the anions into IOV. Since both Ca^{2+} and anion transport were blocked by agents that inhibit the membrane anion transport protein, band III, it was proposed that Ca^{2+} transport was electrogenic; i.e., the transport of Ca^{2+} into IOV was associated with the development of a positive membrane potential. This, in turn, drove the transport of anions into the IOV through band III. In later work, direct evidence was obtained for an increase in membrane potential as a consequence of the ATP-dependent transport of calcium [12]. The second model of Ca^{2+} transport was based on studies of the purified Ca^{2+} pump reconstituted into liposomes [6]. According to this model, the Ca^{2+} pump operates as an electroneutral Ca^{2+}-H^{+} antiporter.

Recently Niggli et al. [7] presented evidence that band III inhibitors block the Ca^{2+}-ATPase directly. They call into question our previous results [4] and suggest that band III does not play a role in Ca^{2+} transport.

In order to define more precisely the role of anion transport in Ca^{2+} translocation, further investigation of the effects of band III inhibitors on calcium transport and Ca^{2+}-ATPase activity were conducted.

2. MATERIALS AND METHODS

The human erythrocyte membranes were prepared at 0–4°C as follows. Blood was drawn from healthy donors into heparinized tubes, centrifuged at 5000 × *g* for 10 min in an SS-34 fixed-angle rotor of a RC-2B Sorvall refrigerated centrifuge. The serum was aspirated, and the packed cells were washed three times with 2 vol. of 150 mM NaCl, 5 mM sodium phosphate (pH 8.0), removing any remaining buffy coat at each step. The erythrocytes were lysed in 10 vol. of 5 mM sodium phosphate (pH 8.0), washed three times in the same, then once in distilled deionized water. The resultant membranes were resuspended in one volume of 50 mM Tris (pH 7.4), 10 mM β-mercaptoethanol and subjected to two cycles of freeze–thaw in a dry ice–methanol bath. Membranes were used within 24 h of preparation. IOV were prepared as previously described [4].

Abbreviations: DIDS, 4-4-di-isothiocyano-2,2-stilbene disulfonic acid; SITS, 4-acetamido-4′-isothiocyano-2,2′-stilbene disulfonic acid; IOV, inside-out vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; uACE, unit of acetylcholinesterase activity; Pipes, piperazine-*N,N*-bis[2-ethanesulfonic acid]

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The ATPase assay incubation medium contained (in a final vol. of 0.5 ml) 40 mM Tris (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ouabain, 1 mM β-mercaptoethanol, 0.9 mM ATP (Vanadium free), 100–200 μg/ml of membranes and, when appropriate, 0.1 mM $CaCl_2$, 1 mM EGTA, or 2 μg/ml purified bovine brain calmodulin. All assay tubes were preincubated for 10 minutes at 37°C before addition of ATP to initiate the reaction. At appropriate timed intervals, 0.4 ml aliquots were removed, and added to 0.1 ml of 10% SDS to terminate the reaction. The liberated phosphate was analyzed by the method of Fiske and SubbaRow [8]. The activity at zero time was subtracted from all timed determinations to control for non-enzymatic breakdown of ATP and for the presence of inorganic phosphate in the membrane preparation. Tubes incubated without Ca^{2+} but with EGTA and calmodulin indicated hydrolysis due to the Mg^{2+}-ATPase: This value was subtracted from the activity in the presence of Ca^{2+} and calmodulin to yield activity of the Ca^{2+}-ATPase.

3. RESULTS AND DISCUSSION

DIDS and SITS are two commonly used band III inhibitors which react covalently with this protein (K_i 0.04 and 10 μM, respectively [9,10]). These compounds act to block anion exchange through band III only when added to the exoplasmic face of the plasma membrane [11]. In previous work [4] we found an 80% inhibition of anion-stimulated Ca^{2+} transport and a 77% inhibition of Ca^{2+}-dependent anion transport in IOV prepared from SITS-treated erythrocytes.

Figure 1 presents the results of a new experiment in which 20 μM DIDS was incubated with intact erythrocytes, the erythrocytes then washed free of DIDS, IOV prepared and the transport of Ca^{2+} then measured. In these sealed vesicles, it was found that DIDS treatment led to a 90% inhibition of anion-stimulated Ca^{2+} transport. Furthermore, a similar inhibition of anion transport was observed (data not shown). This dramatic effect suggests that band III mediates Ca^{2+}-dependent anion transport, and that the transport of the anions serves to charge-compensate an electrogenic translocation of Ca^{2+}.

Alternatively, Niggli et al. [7] have provided evidence that DIDS directly inhibits the Ca^{2+}-ATPase, and suggest, therefore, that band III plays no role in Ca^{2+} transport. These studies differ from the studies shown (fig.1). In the experiments of Niggli et al. [7] the inhibitors DIDS and SITS were applied directly to the exposed Ca^{2+}-ATPase. Yet as seen in fig.1, when applied to the external face of intact erythrocytes, DIDS produced inhibition of Ca^{2+}-transport in subsequently prepared IOV; the catalytic portion of the Ca^{2+}-ATPase which is on the endoplasmic face of the membrane should not have been exposed to these compounds. This would make the report of Niggli et al. [7] relevant only if band III inhibitors can penetrate the intact erythrocyte before cells lysis or if the reagents are incompletely removed by repeated washing.

In analyzing this problem, we first repeated the studies of Niggli et al. [7]. As shown in fig.2, when erythrocyte membranes were prepared and then

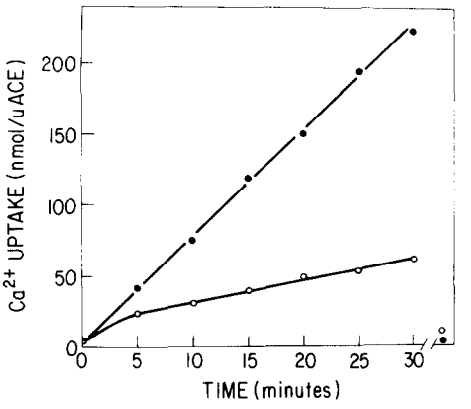

Fig.1. Inhibition of Ca^{2+} transport into IOV by DIDS. Washed erythrocytes were suspended in 155 mM sodium chloride, 5 mM sodium phosphate (PBS) at a 20% hematocrit with (○) or without (●) 20 μM DIDS at 37°C for 30 min. The erythrocytes were washed 3 times in 10 volumes of PBS buffer, and IOV were prepared as in [4]. Ca^{2+} uptake into IOV was measured by millipore filtration [4] in a buffer consisting of 40 mM sodium pipes (pH 7.4), 5 mM magnesium Pipes, 0.9 mM ATP, 0.15 mM calcium Pipes, 2 μg calmodulin/ml and 5 mM sodium phosphate. The points after the break in the time line indicate the amount of calcium in the loaded vesicles 10 min after A23187 was added to the vesicles after 30 min of incubation. Addition of ionophore caused the prompt release of calcium from both types of vesicles.

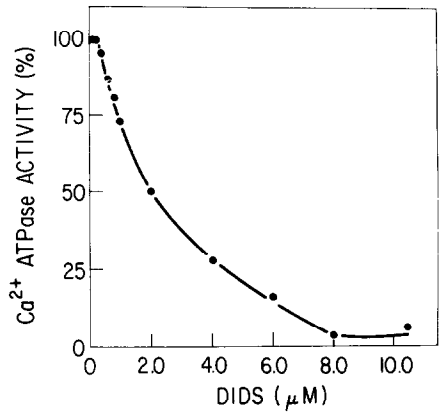

Fig.2. Inhibition of Ca^{2+}-ATPase by DIDS. Erythrocyte membranes were preincubated with differing concentrations of DIDS for 10 min at 37°C. ATP was then added and the reaction was terminated after 40 min, and Ca^{2+}-ATPase activity determined. Activity of 100% corresponds to the activity measured in the absence of DDS (12 mmol P_i . mg protein^{-1} . min^{-1}).

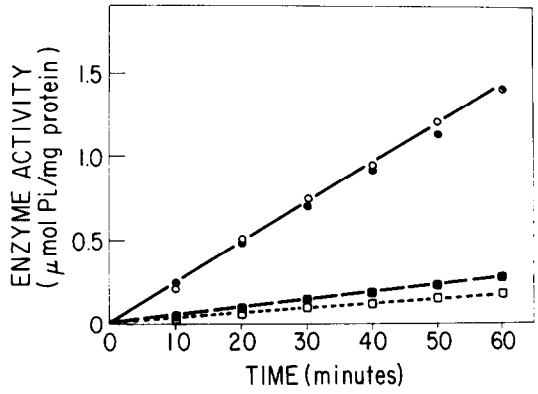

Fig.3. Effects of DIDS preincubation with intact erythrocytes on the Ca^{2+}-ATPase of erythrocyte membranes. Washed erythrocytes were incubated at 37°C for 30 min either with or without (control) 10 μM DDS. The erythrocytes were washed 3 times in 10 vol of 155 mM NaCl, 5 mM sodium phosphate (pH 8.0) and membranes prepared (Materials and Methods). The Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of the membranes were then measured. The Mg^{2+}-ATPase was the same for the DIDS pre-treated (▪) or untreated (□) membrane. Likewise, the Ca^{2+}-ATPase activity was the same in the untreated (○) and DIDS pre-treated (●) membranes. Compare with the results in fig.2 in which DIDS was added directly to the membrane and ATPase activity then measured.

incubated with DIDS under conditions where DIDS had access to the endoplasmic face of the membrane, a profound inhibition of Ca^{2+}-ATPase activity was observed. However, if intact cells were incubated with DIDS for 30 min, then washed and membranes then prepared for measurement of Ca^{2+}-ATPase activity, preincubation with DIDS had no effect upon the Ca^{2+}-ATPase of the subsequently isolated membranes (fig.3).

It is noteworthy that preincubation of erythrocytes with a concentration of DIDS 2500-fold higher than the reported K_i for DIDS action on band III activity had no effect on the Ca^{2+}-ATPase of subsequently isolated membranes (fig.3) even though much lower concentrations of DIDS in the preincubation buffer profoundly inhibited anion-stimulated calcium transport into IOV (fig.1).

The present results clearly distinguish between a high affinity effect of DIDS upon transmembrane transport of calcium (fig.1) and a low affinity effect of DIDS upon the Ca^{2+}-ATPase (fig.2). When DIDS specifically inhibits anion transport via band III, it simultaneously blocks anion-stimulated Ca^{2+} transport. The present results support the postulated role of band III in ATP-dependent Ca^{2+} transport across the erythrocyte membrane [12]. Our results show that band III inhibitors markedly reduce Ca^{2+} translocation across the red cell membrane without inhibiting the Ca^{2+}-ATPase activity of this membrane. These results support our previous conclusion that the energy-dependent transport of Ca^{2+} via the ATPase leads to the development of a positive membrane potential (positive-interior IOV) which acts, in turn, as the driving force for anion uptake through band III. The uptake of these anions cause the collapse of this membrane potential and, thus, promotes the further accumulation of calcium.

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