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STUDIES ON THE Ca^{2+} TRANSPORT MECHANISM OF HUMAN ERYTHROCYTE INSIDE-OUT PLASMA MEMBRANE VESICLES

V. CHLORTETRACYCLINE FLUORESCENCE *

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The measurement of chlortetracycline fluorescence was employed as a probe for measuring the process to calcium transport by human erythrocyte inside-out vesicles. Chlortetracycline is a divalent metal chelator which increases its fluorrescence when bound to calcium in the presence of a membrane. Addition of calcium and ATP to inside out vesicles in the presence of chlortetracycline increased the chlortetracycline fluorescence as a function of time following an initial delay. Only after a threshold level of calcium had been accumulated did the fluorescence increase. The presence of both ATP and calcium were required. The addition of calmodulin increased the rate and absolute magnitude of the chlortetracycline fluorescence change. Similarly, calmodulin stimulated the rate and extent of ^{45}Ca transport by inside-out vesicles. Moreover, the presence of saponin abolished both chlortetracycline fluorescence change and ^{45}Ca uptake; a non-hydrolyzable ATP analog would not substitute for ATP in either ^{45}Ca transport or chlortetracycline fluorescence experiments. Comparison between the slopes of the linear portions of chlortetracycline fluorescence change and calcium transport time courses at varied free calcium concentrations showed a consistent ratio between the slopes. This suggests that calcium transport change can be calibrated by employing chlortetracycline fluorescence. Based on this data, it is concluded that chlortetracycline fluorescence is a rapid and accurate method for monitoring calcium transport by human erythrocyte inside-out vesicles.

Introduction

Previous studies on the erythrocyte calcium pump have relied on radioisotope techniques, ATP hydrolysis and membrane potential sensitive fluorescent dyes as monitors of calcium transport activity (Refs. 1–4, and unpublished observations). Chlortetracycline fluorescence offers an alternative method to study calcium uptake by inside-out human erythrocyte vesicles. Chlortetracycline complexes with divalent cations in a 1 : 1 ratio; this complex increases its fluorescence when placed in an apolar environment [5,6]. Chlortetracycline fluorescence and calcium transport correlate di-

* This paper is the fifth in a series which includes Refs. 2–4.

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ado *PP*[NH]*P*, adenylyl imidodiphosphate.

rectly in mitochondria, brian microsomes and cardiac muscle sarcoplasmic reticulum [7–10]. Both chlortetracycline fluorescence and calcium transport in mitochondria exhibit similar sensitivity to the ionophore A23187 and respiratory chain uncouplers [7]. However, at higher concentrations, chlortetracycline can alter calcium transport by acting as a calcium ionophore [8].

In the present work, the possible use of chlortetracycline as an indirect monitor of calcium transport in human erythrocyte inside out vesicles was evaluated.

Materials and Methods

Materials

Chlortetracycline was purchased from ICN Biochemical Laboratories. Radioisotopes were purchased from New England Nuclear, Boston, MA. All other chemicals were reagent grade or better and purchased from the Sigma Chemical Co. Calmodulin was prepared according to the method of Wang and Desai [11]. Fluorescence assays were performed on a Perkin Elmer MPF 3 spectrofluorometer kindly provided by Dr. Richard Root and Julie Metcalfe, Department of Internal Medicine, Yale University.

Methods

Inside out vesicles were prepared according to a modification of the procedure of Steck and Kant [2,12]. Radioactively labeled calcium transport was determined by a Millipore filtraton technique as previously described [2–4]. Chlortetracycline fluorescence changes were measured using a MPF 3 Perkin Elmer spectrophotometer at 37°C. The fluorescence assay medium consisted of 20 mM glycylglycine, pH 7.1, 40 mM sodium gluconate, 7.5 mM potassium gluconate, 25 μM chlortetracycline, 0.1–0.3 mg inside-out vesicle protein/ml and varied concentrations of calcium gluconate, magnesium gluconate, EGTA and calmodulin as indicated in the figure legends. Fluorescence changes were monitored at an excitation wavelength of 310 nm and an emission wavelength of 560 nm. Reactions were initiated by the addition of disodium ATP to a final concentration of 0.9 mM. Fluorescence changes are expressed directly as time courses or converted to percentage of the maximal observed linear fluorescence change. The ratio of calcium to EGTA (1 mM EGTA in all assays) was converted to a calculated free calcium concentration based on the stability constants reported by Owen [13]. All data are expressed relative to the calculated from calcium concentration.

Results

Time course

A typical time course of chlortetracycline fluorescence change appears in Fig. 1. Preincubation of the vesicles in the presence of magnesium (3 mM) and calcium (0.15 mM) established a fluorescence baseline. Additon of ATP initiated a time -dependent fluorescence increase. The presence of saturating quantities of calmodulin stimulated the rate and extent of the fluorescence change 3- to 4-fold over a 30-min incubation. Calcium transport exhibited a similar time course under identical conditions, under which calmodulin stimulated calcium transport 3- to 1-fold [2].

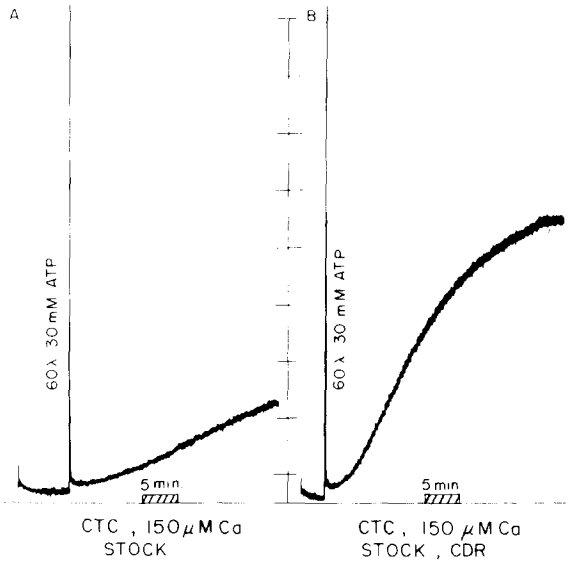

Fig. 1. Chlortetracycline fluorescence of human erythrocyte inside-out vesicles (IOV) under calcium transport conditions: time course. The chlortetracycline fluorescence assay consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 0.15 mM calcium gluconate, 0.1 mg IOV protein/ml and 25 μM chlortetracycline (CTC) in the absence (A) and presence (B) of 0.7 μg calmodulin (CDR)/ml. Following a 5-min preincubation at 37°C to establish the fluorescence baseline, addition of 0.9 mM ATP initiated the reaction.

Calmodulin dependence

The Calmodulin dependence of chlortetracycline fluorescence change is shown in Fig. 2. The K_m (calmodulin) for chlortetracycline fluorescence change was 81.3 ng calmodulin/ml (mean, $n = 3$) calculated by Lineweaver-Burk analysis. Calmodulin stimulated the rate of fluorescence change between 5- and 6-fold (mean, $n = 3$). The K_m (calmodulin) for chlortetracycline fluorescence change did not differ significantly from that of calcium transport (60 ± 22 ng calmodulin/ml) (Table I). However, the extent of calmodulin stimulation was greater for chlortetracycline fluorescence than calcium transport.

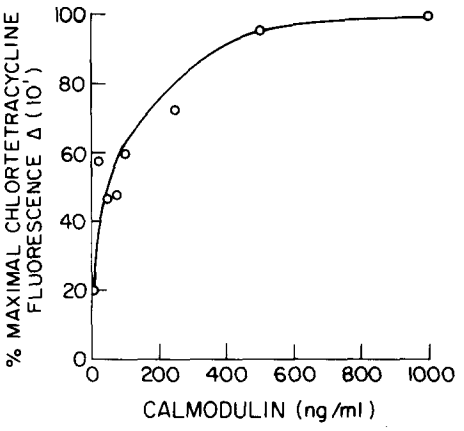

Fig. 2. Chlortetracycline fluorescence of human erythrocyte inside-out vesicles (IOV) under calcium transport conditions: calmodulin dependence. The chlortetracycline fluorescence assay conditions consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 1 mM EGTA, 0.45 calcium gluconate, 0.1 mg IOV protein/ml, 25 μM chlortetracycline and varied concentrations of calmodulin. Following a 5-min preincubation at 37°C to establish the fluorescence baseline, the addition of ATP to a final concentration of 0.9 mM initiated the reaction. The fluorescence change after 10 min was determined and expressed as a percent of the maximal fluorescence change. This experiment is typical of $n = 3$.

Calcium dependence

Calcium/EGTA buffer systems were used to determine the calcium dependence of chlortetracycline fluorescence changes (Fig. 3). The study investigated chlortetracycline fluorescence change over the initial 5 min following ATP addition. The half maximal effect free calcium concentraton was 0.15 μM, corresponding to a calcium/EGTA ratio of 0.24. The optimal calculated free calcium concentratoin, where the maximum fluorescence change was observed, was 0.4 μM. These values are identical to those of calcium transport under the same assay conditions, i.e., in the absence of permeant anions (Table I) (Ref. 2, and unpublished observations). At the higher calculated free calcium concentrations, the extent of chlortetracycline fluorescence change decreased. Likewise, calcium transport fell at the higher calculated free calcium concentrations but not to the same degree.

TABLE I

COMPARISON OF CALCIUM TRANSPORT AND CHLORTETRACYCLINE FLUORESCENCE

The calcium transport data are derived from ref. 2. Values are reported as the mean ±S.D.; The number of experiments is presented in parentheses. The studies were conducted in the absence of permeant anions.

	Calcium transport	Chlortetracycline fluorescence changes
Ca^{2+}/EGTA buffer		
K_m(free calcium) (μM)	0.15 (5)	0.15 (2)
Peak (free calcium) (μM)	0.40 (6)	0.40 (2)
Calmodulin titration		
K_m (calmodulin)(ng/ml)	60±22 (4)	81.0 (3)
Stimulation (x-fold)	3.1±0.9 (11)	5.5 (3)
Magnesium titratoin		
K_m (magnesium) (mM)	0.85 (3)	2.6 (3)
Magnesium dependent	Yes	Yes
ATP dependent	Yes	Yes

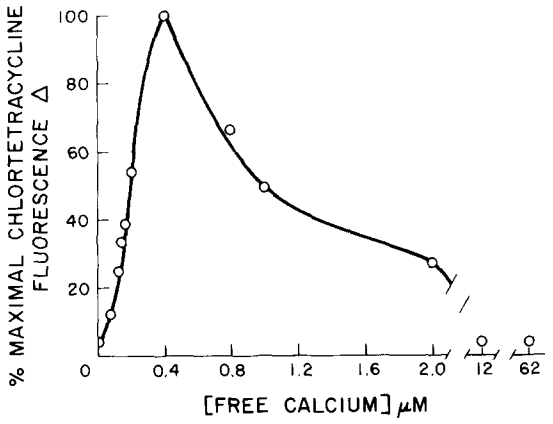

Fig. 3. Chlortetracycline fluorescence of human erythrocyte inside-out vesicles (IOV) under calcium transport conditions: calcium dependence in buffered calcium solutions. The chlortetracycline fluorescence change assay consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 1 mM EGTA, 0.7 μM calmodulin/ml, 0.1 mg IOV protein/ml, 25 μM chlortetracycline and varied concentrations of calcium gluconate. Following a 5-min incubation at 37°C to establish the fluorescence baseline, the addition of ATP to a final concentration of 0.9 mM initiated the reaction. The fluorescent change after a five minute incubation was determined and converted to a percentage of the maximal fluorescence change. This experiment is typical of $n=2$. The data are expressed relative to the calculated free calcium ion concentration.

Complete 30-min time courses of chlortetracycline fluorescence and calcium transport were compared at varied calculated free calcium concentratoins (Fig. 4). Calcium transport increased linearly following the additon of ATP. However, the chlortetracycline fluorescence increase was delayed between 2 to 8 min, resulting in a sigmoidal shaped curve. Only after a threshold level of calcium transport had occurred (23.6 ± 2.4 nmol ^{45}Ca/unit $AChE_{IOV}$ (mean, $n=5$)) did chlortetracycline fluorescence begin to increase linearly*. The slopes of chlortetracycline fluorescence change and calcium transport, could be determined over the linear portion of the time course. Comparison of the rate of fluorescence change to rate of calcium transport at each calculated free calcium concentration yielded a ratio of 0.70 ± 0.11 change in fluorescence (arbitrary units) versus nmol ^{45}Ca/min per $AChE_{IOV}$ unit (mean, $n=5$).

Magnesium dependence

Chlortetracycline fluorescence change exhibited magnesium dependence. The K_m (magnesium) for chlortetracycline fluorescence was determined by Lineweaver-Burk analysis to be 2.6 mM (mean, $n=3$). This value exceeded the K_m (magnesium) determined for calcium transport under identical conditions, 0.85 mM (Table I).

Dependence of chlortetracycline fluorescence on ATP and on the intactness of vesicles

The possibility exists that the change in chlortetracycline fluorescence monitors ATP binding by the $(Ca^{2+}+Mg^{2+})$-ATPase instead of calcium transport. However, the non-hydrolyzable ATP analog, adenylyl imidodiphosphate, failed to initiate the chlortetracycline fluorescence increase (Fig. 5). Thus, the fluorescence change depends on ATP hydrolysis, not ATP binding. Moreover, experiments employing the detergent saponin demonstrated that chlortetracycline fluorescence did not reflect the conformational change of the $(Ca^{2+}+Mg^{2+})$-ATPase during ATP hydrolysis (Fig. 6). Hanahan and Ekholm found that erythrocyte membranes express maximal $(Ca^{2+}+Mg^{2+})$-ATPase activity in the presence of 0.1% saponin; personal observations confirm this [14]. Nevertheless, in the presence of saponin, addition of ATP did not lead to an increase in chlortetracycline fluorescence; intact, impermeant vesicles were necessary.

At high concentratoins, chlortetracycline has been reported to act as a calcium ionophore, altering the transport properties of the system under investigatoin [8]. In the present system, chlortetracycline ionophoretic activity would have manifested itself as an inhibition of calcium transport. However, calcium transport was the same in the presence or absence of 25 μM chlortetracycline in the human erythrocyte inside out vesicle.

* The units of $AChE_{IOV}$ (acetylcholinesterase (IOV)) can be converted to mg protein. For a 100% inside-out preparation of inside-out vesicles, there are 1.74 ± 0.71 (S.D., $n=47$) AChE units/mg protein. Since our preparations were not 100% inside-out vesicles (average 75–80%), it was more convenient to work in $AChE_{IOV}$ units rather than mg inside-out vesicles protein.

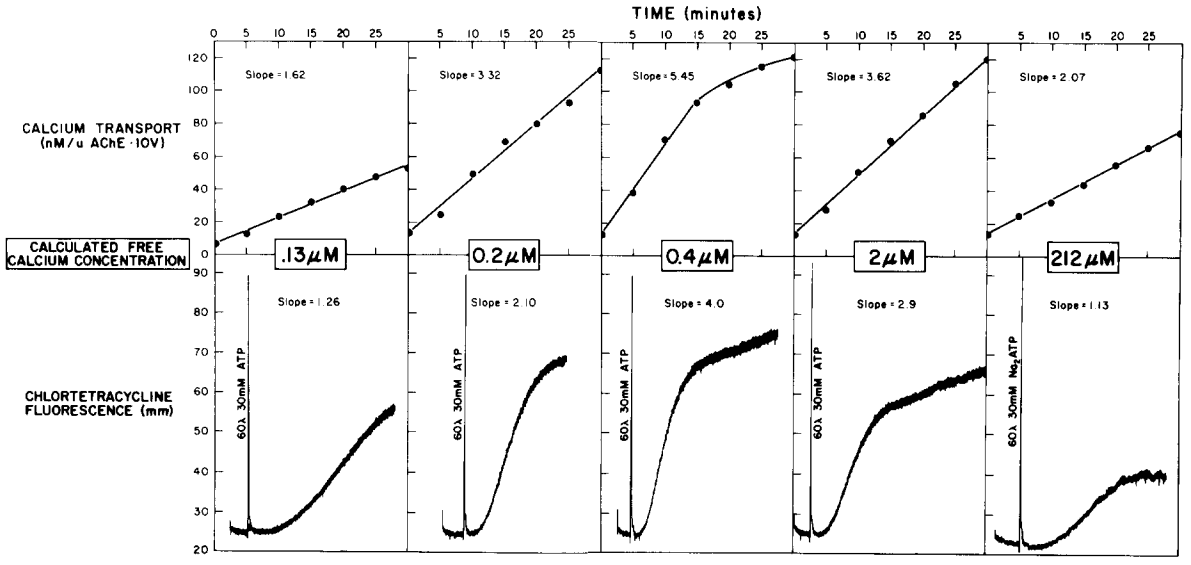

Fig. 4. Chlortetracycline fluorescence and calcium transport by human erythrocyte inside-out vesicles (IOV) at different free calcium concentrations. The assay consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 1 mM EGTA, 1.45 μg calmodulin/ml and the following concentrations of calcium gluconate (corresponding calculated from calcium concentrations reported).

Calcium gluconate (mM)	0.15	0.275	0.45	0.80	1.20
Free calcium (μM)	0.13	0.2	0.4	2	212

In the chlortetracycline fluorescence assay, the medium contained 25 μM chlortetracycline. Addition of ATP, final concentration 0.9 mM, initiated the reaction. Assays were conducted at 37°C. The slope calculated over the linear portion of the curves in nmol ^{45}Ca/min per $AChE_{IOV}$ unit or fluorescence change (mm) per min is recorded.

Discussion

Chlorotetracycline, a fluorescent divalent cation chelator, has been successfully used to monitor calcium transport in mitochondria, microsomes and sarcoplasmic reticulum [7–10]. The present study indicates that chlortetracycline fluorescence can also serve to measure calcium transport into inside-out human erythrocyte vesicles. A number of similarities exist between the properties of calcium transport and chlortetracycline fluorescence. These are summarized in Table I. In particular, these correlations are:

(1) Chlortetracycline fluorscence changes, like calcium transport, are ATP dependent; the non-hydrolyzable ATP analog, adenylyl imidodiphosphate, does not substitute for ATP in either assay (Fig. 5, unpublished data).

(2) Calmodulin stimulates both calcium transport and chlortetracycline fluorescence changes; the respective K_m (calmodulin) values are 60 and 81.3 ng calmodulin per ml (Table I).

(3) Chlortetracycline fluorescence changes and calcium transport exhibit similar calcium dependence (Fig. 3, Table I). The K_m (calculated free calcium concentration) is 0.15 μM for both processes in the presence of calmodulin. Likewise, maximal fluorescence changes and calcium transport occurs at 0.3 μM calculated free calcium. However, at the higher free calcium concentrations, chlortetracycline fluorescence changes over the initial 5 to 8 min following ATP addition fall to 5% of the maximal fluorescence change. Under identical conditions, calcium transport also decreases relative to the maximum rate but to a lesser extent (50–60% of maximum) (unpublished data). This discrepancy may be explained in part by a careful evaluation of the time course of chlortetracycline fluorescence at varied calculated free calcium concentrations (Fig. 4). While calcium transport increases linearly following the addition of ATP, there is anywhere from 2- to 8-min delay

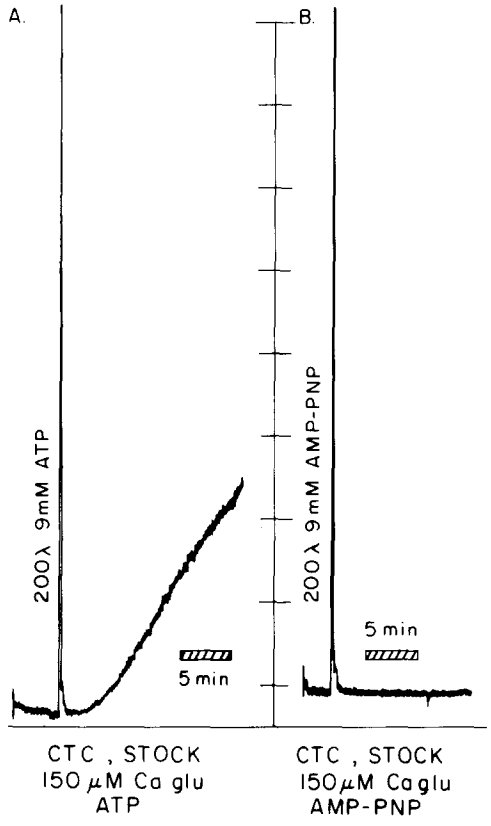

Fig. 5. Chlortetracycline fluorescence of human erythrocyte inside-out vesicles (IOV) under calcium transport conditions: effects of a non-hydrolyzable ATP analogue. Assay conditions consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 25 μM chlortetracycline (CTC), 0.15 mM calcium gluconate (Caglu) 0.7 μg calmodulin/ml and 0.1 mg IOV protein/ ml. After a 5-min preincubation at 37°C to establish the fluorescence baseline, addition of ATP (A) or adenylyl imidodiphosphate (AMP-PNP) (B) to a final concentration of 0.9 mM initiated the reaction.

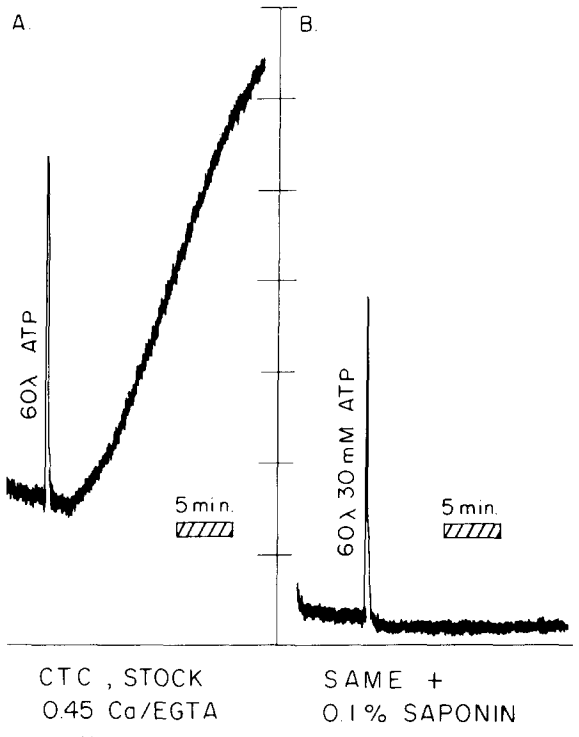

Fig. 6. Chlortetracycline fluorescence of human erythrocyte inside-out vesicles (IOV) under calcium transport conditions: saponin effect. Assay conditions consisted of 40 mM sodium gluconate, 7.5 mM potassium gluconate, 3 mM magnesium gluconate, 20 mM glycylglycine, pH 7.1, 25 μM chlortetracycline (CTC), 1 mM EGTA, 0.45 mM calcium gluconate, 0.7 μg calmodulin/ml, 0.1 mg IOV protein/ml in the absence (A) or presence (B) or 0.1% saponin. After a 5-min preincubation at 37°C to establish the fluorescence baseline, addition of ATP (final concentration, 0.9 mM) initiated the reaction.

before chlortetracycline fluorescence begins to increase linearly. A threshold level of calcium accumulation must occur before chlortetracycline fluorescence will respond to calcium transport. Over a range of free calcium concentrations, 0.1 to 212 μM, the mean threshold level of transported calcium was 24 nmol $^{45}Ca/AChE_{IOV}$ unit. This threshold phenomenon may be accounted for by unoccupied high affinity calcium binding sites on the inner surface of the inside-out vesicle membrane which must be saturated before fluorescent chlortetracycline-calcium complexes can form within the vesicles. After this level of calcium accumulation had occurred, chlortetracycline fluorescence increased in a linear fashion, corresponding to the rate of calcium transport. Direct correlation of the roles of chlortetracycline fluorescence change and calcium transport (slopes calculated over the linear portions of the time course) showed a ratio of 0.70 ± 0.11 fluorescence change (arbitrary units)/min versus mmol ^{45}Ca/min per $AChE_{IOV}$ unit (mean, $n = 5$). Thus, if the threshold level of the system is known, it could be possible to calculate the extent of calcium transport based on chlortetracycline fluorescence.

These parallels suggest that chlortetracycline fluorescence change and calcium transport reflect the same processes. Control experiments dismiss

the possibilities that chlortetracycline fluorescence monitors the conformation of the $(Ca^{2+} + Mg^{2+})$-ATPase or that chlortetracycline acts as a calcium ionophore. One difference does exist between chlortetracycline fluorescence and calcium transport; the K_m (magnesium) for chlortetracycline fluorescence change is 2.6 mM while for calcium transport it is 0.85 mM. An effect of magnesium on chlortetracycline-calcium complex fluorescence may account for this.

In summary, chlortetracycline fluorescence change provides an indirect, non-radioactive method with which to monitor calcium transport in human erythrocyte inside-out vesicles at varied free calcium concentrations.

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References

1 Sarkadi, B. (1980) Biochim. Biophys. Acta 604, 159–190
2 Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 256, 409–414.
3 Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 256, 415–419
4 Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 256, 420–424.
5 Caswell, A.H. and Hutchinson, J.D. (1971) Biochem. Biophys. Res. Commun. 42, 42–49
6 Caswell, A.H. and Hutchinson, J.D. (1971) Biochem. BIophys. Res. Commun. 43, 625–630
7 Luthra, R. and Olson, M.S. (1976) Biochim. Biophys. Acta 440, 744–758
8 Luthra, R. and Olson, M.S. (1978) Arch. Biochem. Biophys. 191, 494–502
9 Schafter, W.T. and Olson, M.S. (1976) J. Neurochem. 27, 1319–1325
10 Fabiato, A. and Fabiato, F. (1979) Nature 281, 146–148
11 Wang, J.H. and Desai, R. (1977) J. Biol. Chem. 252, 4175–4182
12 Steck, T. and Kant, J. (1974) Methods Enzymol. 31, 172–180
13 Owen, J. (1976) Biochim. Biphys. Acta 451, 321–325
14 Hanahan, D.J. and Ekholm, J. (1978) Arch. Biochem. Biophys. 187, 170–179