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The role of calmodulin on Ca^{2+}-dependent K^+ transport regulation in the human red cell

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Several lipophilic calmodulin antagonists (phenotiazines, butyrophenones and diphenylbutylpiperidines) inhibited Ca^{2+}-induced loss of KCl from human red cells. However, the K_i values for this effect did not bear good correlation with the K_i values reported for well-known calmodulin-dependent systems. In addition, the inhibition was strongly dependent on the haematocrit and valinomycin-induced KCl fluxes were also affected. Added calmodulin did not have any effect on Ca^{2+}-dependent ^{86}Rb uptake by inside-out vesicles derived from red cell membranes whereas stimulation of Ca^{2+}-dependent ATPase was apparent. Lipophilic anticalmodulins at high doses had all kinds of effects on ^{86}Rb uptake by inside-out vesicles: increase, decrease or no change of the fraction of activated vesicles reached at submaximal Ca^{2+} concentrations, with or without modification of the relative rate of ^{86}Rb uptake. The hydrophylic compound 48/80 decreased the fraction of activated vesicles reached at submaximal Ca^{2+} concentrations without affecting the relative rate of ^{86}Rb uptake, but this effect took place only at concentrations 10-fold higher than the reported K_i for calmodulin-dependent systems. These results suggest that Ca^{2+}-dependent K^+ channels of red cells are not regulated by calmodulin.

Introduction

The existence of K^+-selective channels activated by intracellular Ca^{2+} has been documented in many animal cells [1,2]. The human red cell possesses such channels and, although their functional role remains unknown, it has been widely used as a technically convenient model for the study of Ca^{2+}-dependent K^+ transport [3]. The published information on the effects of calmodulin on Ca^{2+}-dependent K^+ channels is contradictory. Lackington and Orrego [4] reported that several calmodulin antagonists inhibited the net loss of K^+ induced by the divalent cation ionophore A23187 + Ca^{2+} from human red cells incubated in low-K medium. On the contrary, Plishker [5] reported that calmodulin antagonists facilitate the activation of Ca^{2+}-dependent K^+ transport in the human erythrocyte, an effect he attributed to the inhibition of the Ca^{2+} pump. With subcellular preparations the reports have been equally conflicting. Sarkadi et al. [6] reported that a cytoplasmic protein extract was able to restore Ca^{2+}-dependent ^{86}Rb transport by inside-out vesicles from human erythrocytes which were otherwise insensitive to Ca^{2+}. These results could not be reproduced by other investigators (unpublished results in our laboratory and Ref. 7). Pape and Kristensen [8] have also reported an activatory action of purified calmodulin on Ca^{2+}-dependent K^+ transport by inside-out vesicles. This contrasts with our previous failure to evidence such an effect [9].

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In the present work the effects of calmodulin on Ca^{2+}-dependent K^+ transport by human erythrocytes have been investigated using two different approaches: (1) The effects of several calmodulin antagonists have been studied in both, intact cells and inside-out vesicles. The antagonists used included the lipid-soluble phenotiazines, butyrophenones and diphenylbutylpiperidines [10] as well as the hydro-soluble compound 48/80 [11]. Since some of these drugs were shown before to act on K^+-fluxes not related to Ca^{2+}-dependent K^+ channels [12], their action on valinomycin-induced fluxes has been systematically checked in parallel experiments. (2) The effect of added calmodulin on Ca^{2+}-dependent ^{86}Rb transport by inside-out vesicles has been examined. In parallel experiments the effect on the Ca^{2+}-activated ATPase, a well known calmodulin-sensitive system [13], was also studied to allow direct comparison under the same experimental conditions.

Materials and Methods

Red cells were obtained from fresh blood drawn into heparin. The cells were washed twice with a washing solution containing (mM): NaCl, 75; KCl, 75; Na-EGTA, 0.1; K-Hepes, 10 (pH 7.5). Then they were packed by centrifugation at 3000 rpm during 10 min.

The activity of the Ca^{2+}-dependent K^+ channel was estimated from the loss of KCl observed on incubation in low-K medium after the increase of the cell Ca^{2+} levels by the addition of Ca^{2+} and the ionophore A23187 to the incubation medium. For these experiments the cells were first washed twice with a solution containing 150 mM NaCl, 0.5 mM $CaCl_2$ and 10 mM K-Hepes (pH 7.5) and resuspended in the same medium at 1–15% haematocrit. Just before the experiment 2 ml of this cell suspension were mixed with 1 ml of solution containing 150 mM NaSCN, 0.5 mM $CaCl_2$ and 10 mM K-Hepes (pH 7.5) in a spectrophotometre cuvette. The addition of SCN^- to the medium speeds up the loss of K^+ since the electrogenic permeability of the membrane to this anion is so large that it does not limit the K^+ loss after maximal activation of Ca^{2+}-dependent K^+ transport (García-Sancho and Lew, in preparation). The activation of the K^+ channels was accomplished by the addition of the ionophore A23187 to the cell suspension to give a final concentration of 0.5 to 2 μM. In all the cases the concentration of A23187 used gave maximal response. The rate and extent of the KCl loss was estimated by following the cell volume changes by measurements of transmittance at 650 nm. In a few experiments (Table III) the loss of K^+ was started by the addition of valinomycin (final concentration 1–2 μM) instead of A23187.

The A23187-induced uptake of Ca^{2+} was measured by adding $^{45}CaCl$ (final specific activity about 10^6 cpm/μmol) to cell suspensions similar to those used in the scattering measurements. The experiments were started by the addition of the ionophore A23187 to give a final concentration of 1 μM in the cell suspension. After different periods (1–5 min), the incubation was terminated by mixing 80-μl aliquots of the cell suspension with 1 ml of ice-cold washing solution placed on top of 0.4 ml of dibutylphthalate oil in a 1.5 ml Eppendorf tube. The tube was immediately centrifuged at $12000 \times g$ during 15 s. The supernatant medium and the oil were removed by aspiration, the walls of the tube were wipped off with cotton swabs and the cell pellet was extracted with 6% trichloroacetic acid and counted for radioactivity by liquid scintillation counting [14]. Data were referred to the volume of original cells, which was estimated from haemoglobin measurements in aliquots of the cell suspensions [15]. All the experiments were performed at room temperature.

One-step inside-out vesicles [7,16] were prepared as described previously [9]. The activity of the Ca^{2+}-dependent K^+ channels was assessed from measurements of the uptake of ^{86}Rb, which behaves similarly to K^+ for this transport system [17]. For these experiments an aliquot of vesicles suspension was first mixed with the incubation medium containing the desired amount of Ca^{2+}, chelators and the calmodulin antagonist in each case. The equivalent haematocrit [16] of this suspension was about 20%. At $t = 0$ tracer amounts of ^{86}Rb were added (final activity $(1–5) \cdot 10^6$ cpm/ml) and samples (usually 0.1 ml) were taken after different incubation periods (15 s to 60 min). Extravesicular ^{86}Rb was removed by filtration through Dowex-50 columns as described before [9] and the ^{86}Rb contents of the vesicles was de-

termined by Cerenkov counting. The incubation medium for these experiments contained (mM): KCl, 18; Tris-EGTA, 0.4; $CaCl_2$, 0 to 0.5; K-Hepes, 16.5 (pH 7.5). Note that the relative K^+, Cl^- and Hepes concentrations within the vesicles were about the same as that in the incubation medium, so that the experiments were performed near equilibrium-exchange conditions. The concentrations of Ca^{2+} were calculated using the constants measured previously for EGTA [18]. In a few experiments (see Results) the incubation medium used was the same as that used for Ca^{2+}-ATPase measurements (see below). All the experiments were performed at room temperature.

Measurements of the Ca^{2+}-ATPase activity of the one-step inside-out vesicles were performed using the same incubation medium described above for the experiments of ^{86}Rb uptake except that EDTA replaced EGTA and 3 mM $MgCl_2$ and 3 mM Mg-ATP were added. Ca^{2+} concentrations in this solution were estimated using the following values for the dissociation constants of EDTA [19]: K_1 $6.76 \cdot 10^{-11}$, K_2 $7.76 \cdot 10^{-7}$, K_{Ca} $2.45 \cdot 10^{-11}$, K_{Mg} $1.48 \cdot 10^{-9}$. After the adequate incubation period at 37°C, 0.4-ml samples of the incubation mixture were mixed with 0.7 ml of a solution containing 0.25 M H_2SO_4, 0.5% ammonium heptamolybdate and 2% sodium dodecyl sulphate (SDS). To this mixture 20 μl of a solution containing (w/v) 1.2% of each sodium sulphite and sodium metabisulphite and 0.2% 1-amino-2-naphthol-4-sulphonic acid were added. After 30 min at room temperature absorbance at 650 nm was measured and compared with standards containing known amounts of inorganic phosphate [20].

Calmodulin was partly purified from human erythrocyte lysates by ion-exchange chromatography in DEAE-cellulose as described by Jarret and Penniston [20]. After concentration with a CX-10 Millipore immersible and dialysis against 10 mM imidazole-HCl buffer (pH 7) this partly purified fraction was heated at 100°C for 5 min and centrifuged at $100000 \times g$ for 60 min. The supernatant of this centrifugation was used in the assays. The calmodulin content of this preparation amounted about 13% of the protein according to its ability to activate Ca^{2+}-ATPase activity in calmodulin-depleted erythrocyte membranes [21].

$^{86}RbCl$ and $^{45}CaCl_2$ were purchased from Amersham International. A23187 was purchased from Boehringer Mannheim. Bay-K-8644 and nifedipine were a generous gift of Professor A. García, Department of Pharmacology, The University of Alicante, Spain. Chlorpromazine, trifluoperazine, clozapine and haloperidol were generous gifts of Rhodia Ibérica, Smith, Kline & French, Sandoz S.A.E., Sandoz A.G. Ltd. and Laboratorios Latino, respectively. Medazepam and chlorprothixene were a generous gift of Roche S.A. Pimozide and penfluoridol were a generous gift of Laboratorios Esteve. Other chemicals were purchased from E. Merck Darmstadt, BDH Chemicals Ltd. or Sigma London Chem. Co. Ltd.

Results

Effects of calmodulin antagonists on Ca^{2+}-dependent K^+ transport in intact cells

Fig. 1 shows the effects of three calmodulin inhibitors on the shrinkage induced by A23187 + Ca^{2+} in cells incubated in low-K SCN-containing medium. The three inhibitors decreased the rate of shrinkage, the effect increasing with the concentration of the drug. If the experiment was prolonged for enough time the same final value of transmittance (T_∞) was reached with and without inhibitors. The time-course of the decrease of transmittance (T) could be linearized by plotting $\log(T - T_\infty)$ vs. time (Fig. 2A). From the slopes of

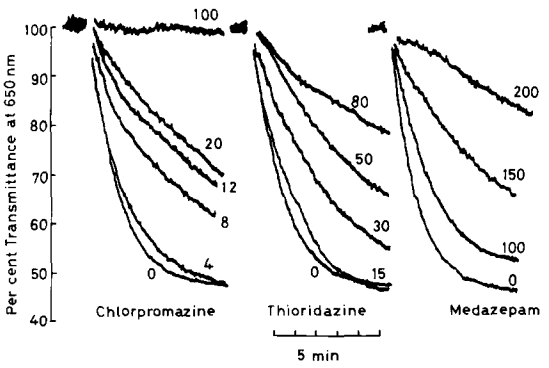

Fig. 1. Effects of several calmodulin inhibitors on Ca^{2+}-dependent K^+ transport in intact erythrocytes. The changes of transmittance at 650 nm were measured as described in Methods. The haematocrit of the cell suspension was 6.7%. Figures on the traces denote the concentration of the inhibitor in μM.

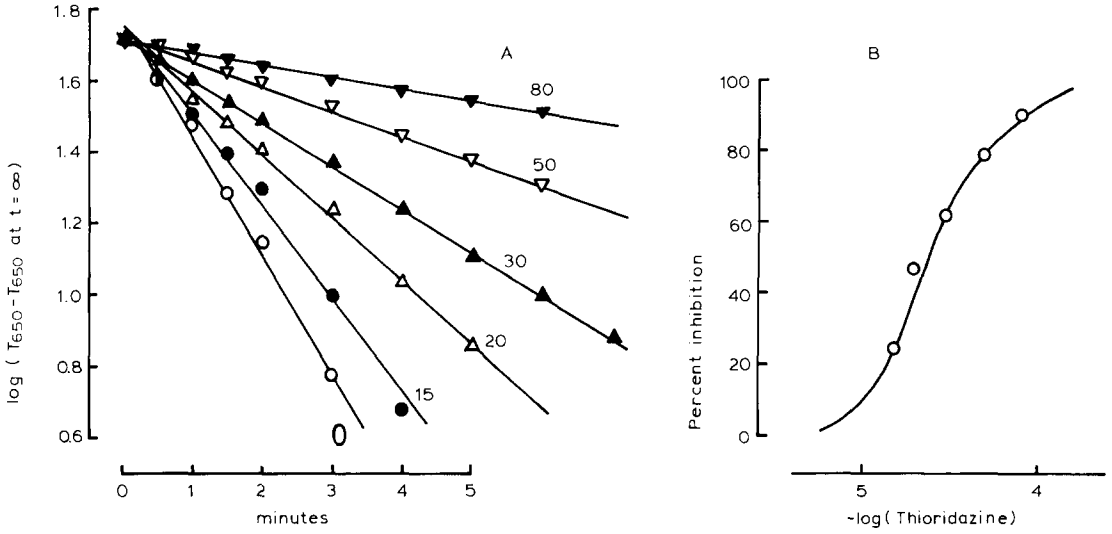

Fig. 2. Inhibition of Ca^{2+}-dependent K^+ transport in intact erythrocytes by thioridacine. Panel A shows a logarithmic transformation of the results of an experiment similar to that of Fig. 1. Figures on the lines denote the concentration of the drug (μM). Panel B shows the same results plotted as % inhibition vs. the concentration of the drug (in M, logarithmic scale).

TABLE I

INHIBITORY EFFECT OF SEVERAL ANTICALMODULINS ON Ca^{2+}-DEPENDENT K^+ TRANSPORT IN INTACT ERYTHROCYTES

The values of K_i listed in the first two columns where calculated from experiments similar to those of Fig. 1, performed at two different haematocrit (Htc) values. The third column lists the K_i values reported by Lackington and Orrego [4] for the inhibition of the loss of KCl from intact erythrocytes treated with A23187 + Ca^{2+}. The haematocrit used in those experiments was 2.5%. The fourth column list the K_i values reported for the inhibition of calmodulin-dependent phosphodiesterase activity [10]. All the values are given in μM.

Drug	K_i			K_i for phosphodiesterase [10]
	6.7% Htc	2.5% Htc	Ref. 4	
Chlorpromazine	5–8	2	35	42
Thioridazine	13–20	4	–	18
Trifluoperazine	30	11	–	10
Pimozide	30	10	6	7
Penfluoridol	15	6	2	–
Haloperidol	–	140	–	60
Chlorprothixene	–	60	–	16
Clozapine	200	87	–	80
Medazepam	130	25	–	150
Bay K-8644	10 [a]	–		–
Nifedipine	10 [a]	5		–

[a] Experiments performed at 5% haematocrit.

these lines the value of the rate constant for shrinkage in each condition could be calculated. Finally by plotting the percent inhibition of the rate of shrinkage vs. the concentration of the drug, the value of K_i (the concentration producing 50% inhibition) could be estimated, as illustrated in Fig. 2B for thioridazine.

Table I shows the values of K_i for several calmodulin antagonists at two different haematocrits obtained by the procedure described above. The third and forth columns of this table list the K_i values obtained previously by Lackington and Orrego [4] for inhibition of Ca^{2+}-dependent K^+ transport and the K_i values for inhibition of phosphodiesterase, a well-known calmodulin-dependent enzyme [10], respectively. Our values do not bear a good correlation with those previously reported for phosphodiesterase inhibition. They also differ from those reported by Lackington and Orrego [4] for the inhibition of Ca^{2+}-dependent K^+ efflux. This difference is particularly striking for chlorpromazine, which is, in our hands, a much more powerful inhibitor.

In all the cases the value estimated for K_i was strongly dependent on the haematocrit at which the assay was made, as documented in Table I for two different haematocrits (2.5 and 6.7%). Fig. 3 shows the correlation between the K_i value esti-

mated for perfluoridol and the haematocrit (six different values) at which the experiment was performed. Table I shows also the effects of two lipophilic dihydropyridines, nifedipine and Bay-K-8644, which act as an antagonist and an agonist of Ca^{2+} channels, respectively [22]. Nifedipine has also been reported recently to act as a calmodulin antagonist [23]. Both drugs seemed to be potent inhibitors of the shrinkage induced by A23187 + Ca^{2+} in low-K SCN-containing medium, their K_i being again dependent on the haematocrit used in the measurements (Table I). The effect of all the drugs listed on Table I on the uptake of ^{45}Ca under the same conditions in which the shrinkage measurements were made were also studied. The inhibitory effect on shrinkage could not be attributed to a decrease of ^{45}Ca uptake by the cells in any case since all the drugs increased it (Table II). The hydrophylic anticalmodulin compound 48/80

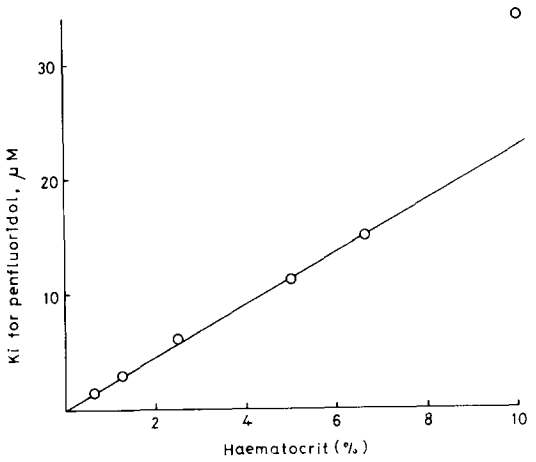

Fig. 3. Effects of haematocrit on the K_i value estimated for the inhibition of Ca^{2+}-dependent K^+ transport in intact erythrocytes by penfluoridol.

TABLE II

EFFECTS OF SEVERAL ANTICALMODULINS ON THE UPTAKE OF ^{45}Ca INDUCED BY A23187 IN HUMAN RED CELLS

Fresh cells were suspended at 6.7% haematocrit in a solution containing (mM): NaCl, 100; NaSCN, 50; $^{45}CaCl$, 0.5; K-Hepes, 10 (pH 7.5). At $t = 0$ A23187 was added to give a final concentration of 1 μM in the cell suspension and samples were taken at 1, 2 and 5 min for determination of ^{45}Ca uptake. The incubation was terminated by dilution with 13 vol. of ice cold-medium and centrifugation through dibutylphthalate oil, as described before [14]. The figures given for the controls are mean ± S.D. of three experimental values. All the other figures correspond to single determinations. The equilibrium value for ^{45}Ca uptake, obtained at 10 μM ionophore was about 2600 μmol/l cells.

Added drug, μM	Ca uptake, (μmol/l cells)		
	1 min	2 min	5 min
None	58 ± 2	91 ± 12	164 ± 14
Chlorpromazine, 8	162	375	631
Trifluoperazine, 30	84	158	196
Pimocide, 30	110	308	844
Penfluoridol, 15	109	241	684
Haloperidol, 300	72	140	304
Chlorprothixene, 150	483	940	1835
Clozapine, 200	238	479	1006
Medazepam, 150	227	564	892
Bay K-8644, 10	83	266	472
Nifedipine, 10	85	133	249

TABLE III

EFFECTS OF SEVERAL ANTICALMODULINS ON THE RATE OF K^+ LOSS INDUCED BY VALINOMYCIN IN INTACT ERYTHROCYTES

The rate of K^+ loss was estimated from the rate of shrinkage measured in experiments of similar design to those of Fig. 1, except that they were started by the addition of valinomycin (1 or 2 μM, (1) and (2), respectively) instead of A23187. Values are given as percent of the rate in the control, to which no drugs were added. Two haematocrits (Htc) were used.

Drug	Concn. (μM)	Rate of KCl loss, percent of control	
		2.5% Htc	6.7% Htc
Chlorpromazine (2)	5	211	–
Chlorpromazine (2)	50	323	–
Chlorpromazine (2)	150	458	–
Thioridazine (1)	16	199	–
Trifluoperazine (1)	30	309	–
Pimozide (1)	20	126	–
Penfluoridol (2)	5	–	134
Penfluoridol (2)	10	32	–
Penfluoridol (1)	10	53	–
Penfluoridol (2)	15	–	13
Penfluoridol (2)	40	–	3
Haloperidol (1)	150	160	–
Chlorprothixene (1)	120	309	–
Clozapine (1)	133	160	–
Medazepam (1)	50	177	–
Medazepam (1)	200	323	–

added to the external medium at concentrations up to 5 mg/l did not significantly modify the rate of shrinkage induced by A23187 + Ca^{2+} in low-K SCN-containing medium (data not shown).

We have shown previously that chlorpromazine stimulates the rate of K^+ loss induced by valinomycin from red cells incubated in low-K medium under conditions in which the electrogenic permeability to Cl^- was the limiting step for salt loss [12]. Other lipophilic drugs have been reported to modify the rate of net loss of cations by acting either on membrane fluidity or on the permeability to anions. In order to test this kind of actions we studied the effects of the different anticalmodulins on the cell shrinkage induced by valinomycin. The experimental conditions were as in the experiments of Fig. 1, including the presence of the permeant anion SCN^- into the incubation medium. The results of these experiments are summarized in Table III. Only penfluoridol decreased the rate of shrinkage induced by valinomycin at the same range of concentrations at which inhibited the (A23187 + Ca^{2+})-induced shrinkage. All the other drugs tested increased the rate of valinomycin-induced shrinkage under these conditions.

Effects of calmodulin on Ca^{2+}-dependent ^{86}Rb uptake in one-step inside-out vesicles

The effects of partially purified calmodulin (see Methods) on Ca^{2+}-dependent ^{86}Rb uptake and Ca^{2+}-dependent ATPase activity were studied using the same batch of inside-out vesicles and the same incubation medium for the measurements of transport and enzyme activities. Since the presence of Mg^{2+} is required for ATPase activity this cation was included in the incubation medium to give a final concentration of 3 mM. Under these conditions the affinity of the K^+ channel for Ca^{2+} is decreased, the half-activating Ca^{2+} concentration being in the 10^{-5} M range [9]. The concentrations of Ca^{2+} and Mg^{2+} were fixed in these ex-

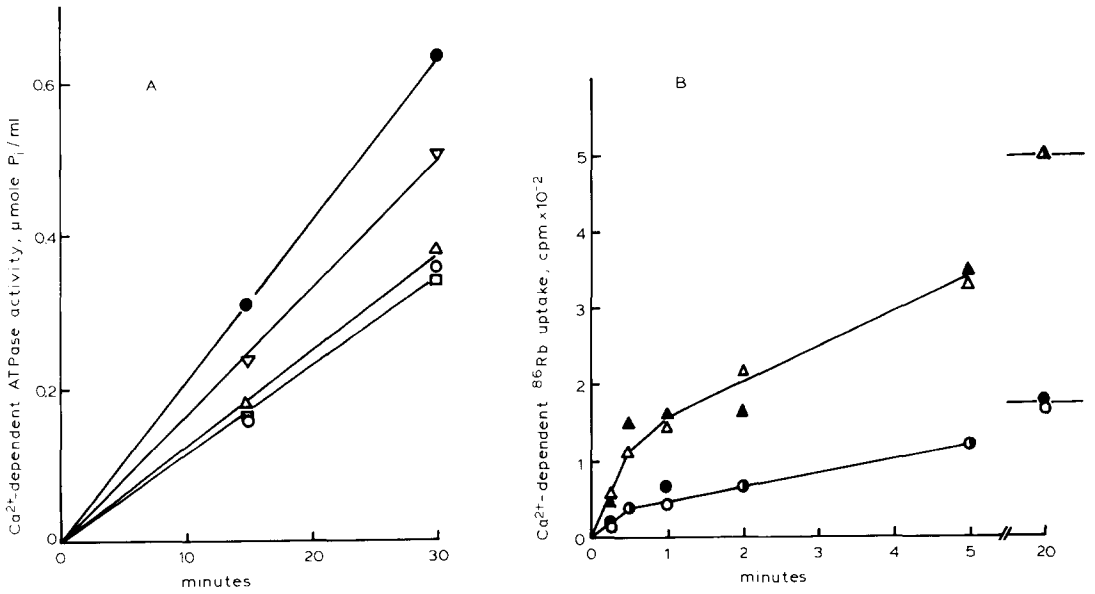

Fig. 4. Effects of calmodulin on the Ca^{2+}-dependent ATPase activity (Panel A) or Ca^{2+}-dependent K^+ transport by one-step inside-out vesicles (Panel B). The same batch of vesicles was used for both experiments. In panel A symbols represent: without added calmodulin (○); with 3 µg/ml of calmodulin (●); with calmodulin and the following inhibitors: 50 µM chlorpromazine (▽); 50 µM trifluoperazine (△); 10 µg/ml compound 48/80 (□). Ca^{2+}-dependent ATPase activity was measured in the presence of 10 µM Ca^{2+}. The activity without Ca^{2+} (0.4 mM EGTA) has been subtracted from all the values. In panel B Ca^{2+}-dependent ^{86}Rb uptake in the presence of either 100 µM (triangles) or 20 µM Ca^{2+} (circles) is represented. Measurements were performed in the absence of calmodulin (open symbols) or with 4.5 µg/ml calmodulin (closed symbols). The uptake obtained in the absence of Ca^{2+} (0.4 mM EGTA) was substracted from all the values. The Ca^{2+}-dependent ATPase was measured at 37 °C and the ^{86}Rb uptake at 20 °C.

periments by using Ca/Mg/EDTA buffers (see Methods). The inside-out vesicles were prepared as described in Methods, but they were stored overnight at 2–4°C in the EGTA-containing washing solution. In this way a larger calmodulin depletion is achieved (Lew, V.L., personal communication). The vesicles were sedimented by centrifugation and resuspended again in EGTA-containing washing solution just prior to the experiments.

The results of a representative experiment are shown in Fig. 4. Calmodulin increased the Ca^{2+}-dependent ATPase activity of the vesicles to about twice the value obtained without calmodulin. Trifluoperazine at 50 μM or compound 48/80 at 10 μM, concentrations well above the K_i of these drugs acting as anticalmodulins [8,10], prevented completely the effect of calmodulin. Chlorpromazine at 50 μM, a concentration close to the K_i of this drug acting as anticalmodulin [10], inhibited the calmodulin-induced enhancement of Ca^{2+}-dependent ATPase activity by 48% (Fig. 4A). Panel B of Fig. 4 illustrates the effects of calmodulin on the Ca^{2+}-dependent ^{86}Rb uptake by the same batch of inside-out vesicles. Two different concentrations of Ca^{2+} were tested, 20 μM, which produced 45% activation, and 100 μM, which gave full activation. Calmodulin did not modify either the amount of ^{86}Rb taken up at equilibrium (20 min in Fig. 4B), nor the time-course of the uptake in any case.

Effects of calmodulin antagonists on the Ca^{2+}-dependent ^{86}Rb uptake by one-step inside-out vesicles

The effects of calmodulin antagonists on Ca^{2+}-dependent ^{86}Rb uptake by inside-out vesicles were quantified using two different transport parameters: (1) The Ca^{2+}-dependent uptake of ^{86}Rb at the steady state, from which the fraction of activated vesicles can be estimated as described previously [9]. (2) The half-equilibration time, estimated graphically as the time required to take up ^{86}Rb to a level which is one half that taken up at the steady state. Although the Ca^{2+}-induced

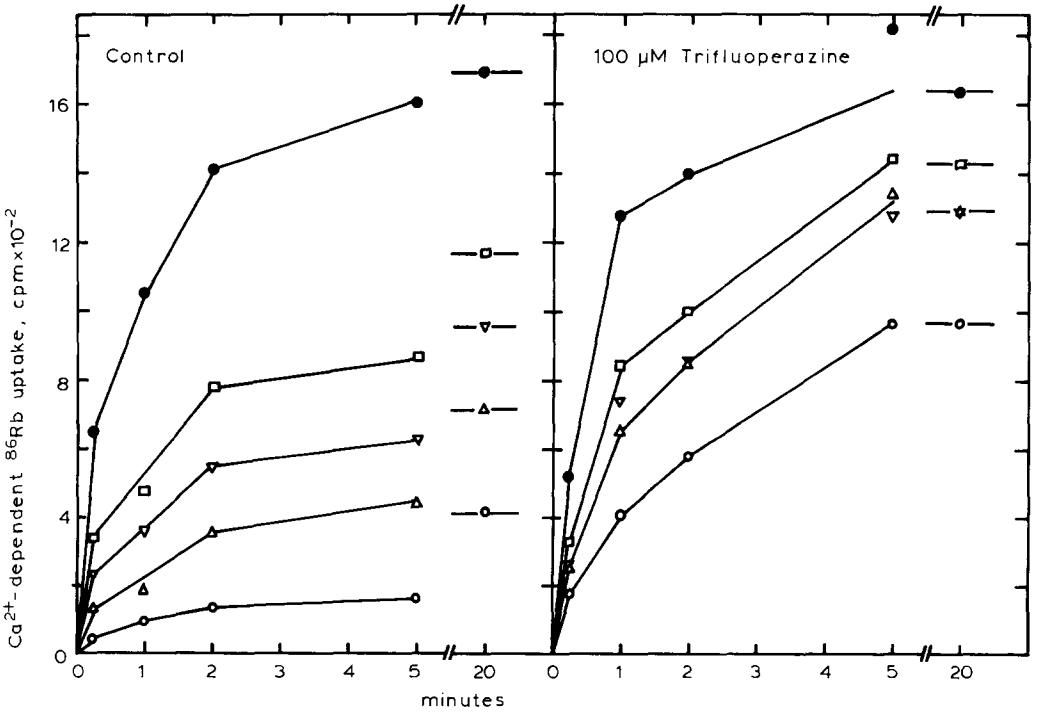

Fig. 5. Effects of trifluoperazine, 100 μM, on the Ca^{2+}-dependent ^{86}Rb uptake by inside-out vesicles. The following Ca^{2+} concentrations were used (in p*Ca*): 7.6 (○); 7.4 (△); 7.2 (▽); 7.0 (□) and 6.0 (●). In all the cases the uptake obtained in the absence of Ca^{2+} (0.4 mM EGTA) has been substracted.

^{86}Rb uptake does not follow single exponentials [9], the half-equilibration time still allows us to compare the rates of uptake by the same population of vesicles under different conditions.

The effects of the different anticalmodulins on the fraction of activated vesicles were not homogeneous. Chlorpromazine, thioridazine and penfluoridol did not have significant effects at concentrations which were able to inhibit almost completely the Ca^{2+}-induced shrinkage in intact cells. Only very high concentrations of these drugs (> 150 μM) decreased significantly the fraction of activated vesicles (data not shown). Fig. 5 shows the effects of 100 μM trifluoperazine. This drug, when used at high concentrations (> 50 μM), was able to increase the fraction of activated vesicles obtained at submaximal Ca^{2+} concentrations without modifying the maximal activation value obtained at saturating Ca^{2+} concentrations (10^{-6} M in Fig. 5).

The effects of compound 48/80 are shown in Fig. 6. Compound 48/80 had little or no effect at concentrations below 10 mg/l (not shown in the figure). At 20 mg/l, a concentration about 20-times larger than its reported K_i value as anticalmodulin [11], it decreased the fraction of activated vesicles obtained at submaximal Ca^{2+} concentrations without modifying the maximal effect (10^{-4} M Ca^{2+} in Fig. 6). This corresponds to a decrease

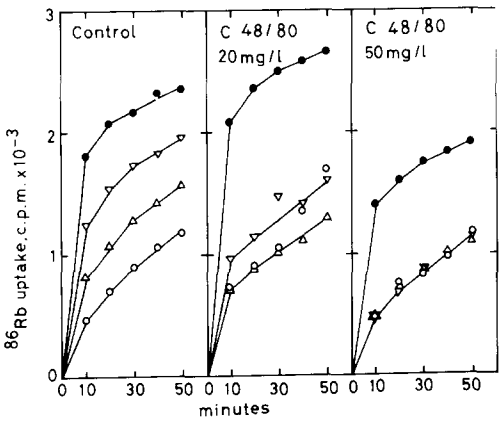

Fig. 6. Effects of compound 48/80 on the uptake of ^{86}Rb by inside-out vesicles. The control experiment, without the inhibitor, is shown in the left panel; the middle and right panels refer to 20 and 50 mg/l of compound 48/80, respectively. Symbols denote the following Ca^{2+} concentrations: No Ca^{2+} (0.4 mM EGTA) (○); p*Ca* 7.2 (△); p*Ca* 6.8 (▽); p*Ca* 4 (●).

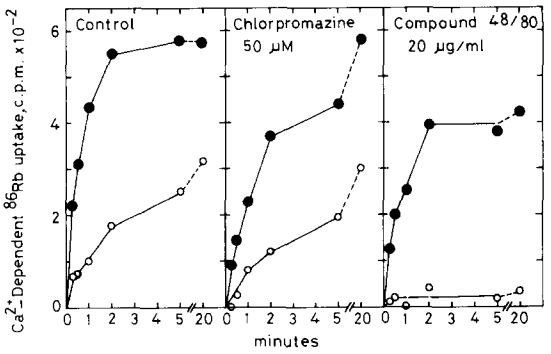

Fig. 7. Effects of 50 μM chlorpromazine and 20 mg/l compound 48/80 on the Ca^{2+}-dependent uptake of ^{86}Rb by inside-out vesicles. Panels refer, from left to right, to the control with no inhibitors, chlorpromazine and compound 48/80, respectively. Symbols represent the following Ca^{2+} concentrations: open circles, p*Ca* 7.2; closed circles, p*Ca* 6.0. The uptake obtained in the absence of Ca^{2+} (0.4 mM EGTA) has been substracted in all the cases.

of the apparent affinity [9] to about 1/5 of the control without inhibitors. At 50 mg/l compound 48/80 decreased also the fraction of activated vesicles obtained at 10^{-4} M Ca^{2+} (Fig. 6). In another experiment it was found that the maximal fraction of activated vesicles obtained in the presence of compound 48/80 at 50 mg/l was about 70% of the maximal value in the control and independent of the Ca^{2+} concentration between 10^{-6} and $5 \cdot 10^{-4}$ M (data not shown).

The effects of the different anticalmodulins on the rate of Ca^{2+}-activated ^{86}Rb uptake by inside-out vesicles were inhomogeneous as well. The effects of chlorpromazine and compound 48/80 are shown in Fig. 7. Chlorpromazine reduced the rate of ^{86}Rb uptake, both at submaximal and maximal Ca^{2+} concentrations. The estimated half-equilibration times at p*Ca* values of 6 and 7.2 were 87 and 195 s, respectively, in the presence of 50 μM chlorpromazine compared with 27 and 102 s for the controls. Similar effects were found for thioridazine and penfluoridol at concentrations of 50 μM (data not shown). On the contrary, compound 48/80 at 20 mg/l did not significantly modify the rate of uptake of ^{86}Rb at 10^{-6} M Ca^{2+} (the half-equilibration time was 33 s compared with 27 s for the control), even though the fraction of activated vesicles was largely decreased at this Ca^{2+} concentration and the uptake of ^{86}Rb at a

p*Ca* of 7.2 was almost completely blocked. The effects of trifluoperazine on the kinetics of Ca^{2+}-induced ^{86}Rb uptake by inside-out vesicles has already been documented without comment in Fig. 5. Even though the drug was able to increase the fraction of activated vesicles at submaximal Ca^{2+} concentrations, the half-equilibration times were not significantly modified when compared with the controls in which a similar fraction of activated vesicles had been attained (see Fig. 5 legend for values). The addition of calmodulin to the incubation medium did not modify the effects of the calmodulin antagonists on Ca^{2+}-dependent ^{86}Rb uptake (results not shown).

Discussion

The strongest support for the participation of calmodulin in the control of the Ca^{2+}-dependent K^+ transport is the reported inhibitory effect of several calmodulin antagonists in intact red cells [4]. Although the anticalmodulin effect of these drugs is well documented [10], this is not their only pharmacological action. Some of these drugs have been reported to affect redox processes [24], cholinergic [25] and dopaminergic [26] synaptic transmission, calcium currents and exocytosis [27]. On the other hand, most of these drugs are strongly lipid-soluble and could conceivably have non-specific effects on transport processes by modification of the fluidity or the thickness of the membrane. It has been well documented, for example, that chlorpromazine increases the fluidity of the erythrocyte membrane [28]. In our hands, the K_i values of the lipophilic anticalmodulins acting on Ca^{2+}-dependent K^+ transport bear a poor correlation with those reported previously for its action on calmodulin-dependent processes (Table I). On the other hand, the estimated K_i depended linearly on the haematocrit at which the experiments were performed. This suggests that the inhibitory effect could be due to the accumulation of the drugs within the cell membrane with modifications of its physical properties. The observation that the lipophilic anticalmodulins did also modify the valinomycin-induced K^+ loss (Table III) is consistent with the last view. Neither chlorpromazine nor trifluoperazine inhibited the loss of K^+ induced by A23187 + Ca^{2+} in the Ehrlich ascites-tumor cell (Valdeolmillos, M., unpublished results). Since it has been shown previously that this cell possesses Ca^{2+}-dependent K^+ channels very similar to those of the erythrocyte [29], the differences in the action of the anticalmodulins could be attributed to differences in membrane composition between both cells. In any case they indicate that the inhibition by anticalmodulins is not an essential property of the Ca^{2+}-dependent K^+ channels.

The previous reports dealing with the effects of calmodulin on Ca^{2+}-dependent ^{86}Rb transport in inside-out vesicles [8] are subjected to several criticisms as commented in the Introduction. Under the conditions in which the vesicles were prepared most of the activity of the Ca^{2+}-dependent transport is lost [30] and the behaviour of the channels could be atypical [31]. In our hands, the addition of purified calmodulin had no effects on Ca^{2+}-dependent K^+ transport under conditions in which the Ca^{2+}-dependent ATPase present in the same vesicles was activated. Still it could be argued that calmodulin is strongly bound to the K^+ channels and, owing to this reason, it is not lost during the preparation and the subsequent treatment of the vesicles. Some examples of enzymes belonging to this class are known [32]. However, an inhibitory effect of the calmodulin antagonists should still be expected in this case, and this was not observed.

Three patterns of behaviour were observed with calmodulin antagonists acting on Ca^{2+}-dependent ^{86}Rb transport in inside-out vesicles: (1) Chlorpromazine, thioridazine and penfluoridol had no effect on the fraction of activated vesicles, except at very high concentrations, whereas they decreased the rate of uptake of ^{86}Rb. The last action could be related to the inhibitory effect of these compounds on the Ca^{2+}-dependent K^+ transport observed in intact cells. (2) Compound 48/80 at 20 μg/ml decreased the fraction of activated vesicles at submaximal Ca^{2+} concentrations without modifying the rate of uptake of ^{86}Rb. It should be kept in mind, however, that this concentration of compound 48/80 is well above its K_i acting as calmodulin antagonist, and at this concentration range effects on calmodulin-independent systems have been reported [11]. (3) Trifluoperazine increased the fraction of activated

vesicles at submaximal Ca^{2+} concentrations without modifying the rate of uptake of ^{86}Rb. This effect is reminiscent of that reported previously for electron donors, which are able to increase the fraction of activated vesicles at submaximal Ca^{2+} concentrations [18]. The electron donors, however, increase the rate of uptake of ^{86}Rb (unpublished observations). These results show clearly that both parameters, the fraction of activated vesicles and the rate of uptake, can be modified independently. These parameters could reflect two different aspects of the channel function, namely the threshold and the mean open time of individual channel units. The first one would be responsible of the 'all or nothing' behaviour of the channels [7]. The second one, which has been thoroughly studied in patch-clamp experiments [33,34], would influence the rate of uptake observed in intact cells where many channels are acting jointly. Regarding to its significance to the participation of calmodulin in the control of Ca^{2+}-dependent ^{86}Rb transport in inside-out vesicles our results are negative since the effects were different for the different calmodulin antagonists and not related with its potency as anticalmodulins.

As a whole, the evidence presented in this work is inconsistent with the view that the Ca^{2+}-dependent K^+ channel of the human erythrocyte is a calmodulin-dependent system. Other alternative means of modulating the Ca^{2+}-sensitivity of these channels have, however, been proposed [18].

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