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Calmodulin antagonists do not inhibit IK_{Ca} channels of human erythrocytes

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Abstract

Patch-clamp recordings were performed to study the effects of three calmodulin (CaM) antagonists on the gating of intermediate calcium-activated K^+ channels (IK_{Ca}) of human erythrocytes. In the cell-attached configuration, both opening frequency and open probability of IK_{Ca} channels were not significantly different in control cells and in those incubated with calmidazolium, trifluoperazine or W7. IK_{Ca} channels in excised membrane patches, were normally activated by the calcium bathing the cytoplasmic side in the presence of CaM antagonists, at calcium concentrations ranging from 10^{-7} to 10^{-3} M. The activity of IK_{Ca} channels, which had been previously up-modulated by an endogenous cAMP-dependent protein kinase, was not inhibited when perfused with CaM antagonists. The results presented in this study demonstrate that calmodulin antagonists do not inhibit the activity of native IK_{Ca} channels of human erythrocytes. These data are in accordance with findings on the cloned IK_{Ca} indicating that calmodulin is constitutively associated with these channels.

Keywords: Erythrocyte; IK_{Ca} channel; Calmodulin

1. Introduction

Calcium-activated K^+ channels (K_{Ca}) transduce fluctuations in intracellular calcium concentration into changes of potassium permeability. They differ in their primary amino acid sequences and pharmacological profiles, they also exhibit different single-channel conductances and are divided in three subfamilies: BK_{Ca}, IK_{Ca} and SK_{Ca}, with large, intermediate and small conductances, respectively [1]. K_{Ca} channels also display different calcium sensitivities. Unlike BK_{Ca}, both IK_{Ca} and SK_{Ca} channels are high-affinity calcium sensors. IK_{Ca} are involved in fundamental cellular responses, such as vasodilatory effects of bradykinin in the kidney [2], activation of brain capillary endothelial cells by endothelin [3], cell dehydration in sickle cell anemia [4] and activation of secondary immune responses [5]. IK_{Ca} channels of human erythrocytes are also called Gardos channels because they mediate the calcium induced K^+ efflux or 'Gardos effect' [6], which probably contributes to the elimination of senescent red cells [7]. The high calcium sensitivity of IK_{Ca} and SK_{Ca} channels is not due to a calcium-binding motif in their α subunits (such as the calcium bowl of BK_{Ca} channels) [8,9] but rather to the association with calmodulin (CaM), acting as calcium sensor. In the α subunit of SK_{Ca} channels a well defined region in the prox-

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imal portion of the C-terminus has been found essential for the expression of Ca^{2+}-induced gating [10]. Site-directed mutagenesis combined with electrophysiology measurements demonstrated that the sensitivity of SK_{Ca} channel gating to Ca^{2+} reflects binding to constitutively SK_{Ca}-associated CaM [10,11]. IK_{Ca} channels display a CaM-binding site to the proximal C-terminus and Ca^{2+}-CaM-induced conformational changes are necessary for the channel to open [12,13]. However, it is not yet clear whether CaM is constitutively associated with the IK_{Ca} channel. On one hand, Fanger et al. [12] concluded that CaM is prebound to the C-tail of the IK_{Ca} channel of T lymphocytes in a Ca^{2+}-independent fashion and mediates its Ca^{2+}-induced gating. On the other hand, Khanna et al. [13] provided evidence that CaM antagonists inhibited IK_{Ca} currents of T lymphocytes, suggesting the possibility of a Ca^{2+}-dependent binding of CaM to the channel. The involvement of CaM in calcium-dependent potassium effluxes in red blood cell is debated [14–18]. Since the experimental conditions may play a critical role both in preserving the Ca^{2+}-dependent K^+ channels of erythrocytes and in identifying the K^+ efflux through the Gardos channels, we performed experiments to study the effects of three calmodulin antagonists on the gating of single IK_{Ca} of human erythrocytes [19–23]. Here we show that in different experimental conditions such as cell-attached and inside-out configurations, as well as in different states of the channel modulation by an endogenous cAMP-dependent protein kinase (PKA), these three main CaM antagonists do not inhibit the activity of these channels.

2. Materials and methods

2.1. Preparation

The experiments were performed on blood samples from healthy volunteers (age 20–55 years). Blood was collected by vein-puncture into heparinized tubes. The samples were centrifuged for 4 min at $1500 \times g$ and resuspended three times in a washing solution containing (mM): NaCl 154, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10, pH adjusted to 7.35 with NaOH. Erythrocytes were stored in the same solution supplemented with glucose 10 mM (physiological solution) at 5°C (hematocrit 2%) and used within 1–2 days. Aliquots of this suspension were diluted at low density in tissue culture Petri dishes (Falcon 3001, Becton Dickinson UK), with a recording solution containing (mM): KCl 120, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, pH adjusted to 7.2 with KOH (high K^+ solution) or in physiological solution. Solutions containing different free calcium concentrations were obtained using 1 mM EGTA (ethyleneglycol-bis[β-aminoethylether]*N*,*N*-tetraacetic acid) following instructions on the MaxC software by C. Patton (Stanford University). Recordings were performed at room temperature (20–24°C) after at least 15 min of incubation in the recording solution. All solutions were filtered with a 0.2 μm Millipore filter.

2.2. Electrophysiology

Single-channel currents were recorded in cell-attached and inside-out configurations [24] with an Axopatch 1 D amplifier (Axon Instruments, CA, USA) and were low-pass filtered at 1 kHz, using a 4-pole Bessel filter. Pipettes were pulled in two stages from disposable capillaries (Blaubrand 7087) and were coated with Sigmacote (Sigma, Milan, Italy). Pipettes were systematically checked for both resistance (10 MΩ) and 'bubble number' [25]. The pipette solution was the high K^+, without $MgCl_2$. Cell-free patches were tested with solutions of different composition applied by a rapid solution changer (Biologic RSC 200, France) with the motor speed set at 100 ms/tube.

2.3. Chemicals

Calmidazolium chloride (compound R24571), trifluoperazine dimaleate and W7-hydrochloride (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl) were purchased from Calbiochem (Inalco, Italy) whilst all other reagents were obtained from Sigma (Milan, Italy).

2.4. Data analysis

Data were analyzed on-line with purposely developed software and simultaneously stored on a modified videotape recorder [26]. Records were digitized off-line at 3 kHz with a Labmaster TL1 interface

(Axon Instruments) and then analyzed with a pClamp (Axon Instruments) software package. Mean patch current, opening frequency, percent of open time, as well as mean open and closed times (for patches containing a single channel) were continuously monitored on-line during the experiments. Transitions between open and closed states were detected using a half-amplitude threshold criterion and a minimum event width of 0.2 ms. The mean patch current was computed by dividing the total ionic charge transported by the duration of the data segment. The mean frequency of events was obtained from the ratio between the number of opening transitions and the total recording time. The number of channels in each membrane patch was determined by the maximum number of channels simultaneously open, observed in records longer than 2 min. The apparent number of channels per patch ranged between one and three. Only records with a single channel were used to study channel kinetics. Mean channel open and closed times were determined by the arithmetic means of dwell time data.

Sigma Plot 2 and Sigma Stat 1 (Jandel Scientific) were used for mathematical transformations and statistical analysis. Results are expressed as means ± standard error of the mean (S.E.M.). Differences between groups were examined with the Mann–Whitney test and a P value of less than 0.05 was taken as significant.

3. Results

3.1. Cell-attached recordings

Single IK_{Ca} channels were identified by their conductance value, inward rectification, voltage independence, kinetics and Ca^{2+} sensitivity [19–23]. Ca^{2+}-activated K^+ channel were studied in the cell-attached configuration (c.a.), while the inner side of the channel molecule faced both the cytosol and erythrocyte cytoskeleton. Opening frequency and open probability were taken as indicators of the channel activity and measured at a membrane potential of −80 mV, in cells bathed for at least 15 min either in high K^+ solution containing 1 mM Ca^{2+}, or in the same solution supplemented with one of the three CaM antagonists: calmidazolium, trifluoperazine or W7. The CaM antagonists concentrations used in most experiments of this study (calmidazolium 0.5 μM, trifluoperazine 10 μM and W7 25 μM) were reported to induce strong inhibition of IK_{Ca} macroscopic currents [13] and to convert erythrocytes into cupped cells, by CaM inhibition [27].

Fig. 1A illustrates typical current records obtained in the four experimental conditions. Both open probability and opening frequency did not significantly differ in the four groups, as shown in Fig. 1B.

The lack of inhibitory effects on channel activity of the three CaM antagonists was not due to the time of their application, since channel activity did not change as a function of time in recordings lasting for about 30 min. On the other hand the three drugs affected the cell shape as illustrated in Fig. 2.

3.2. Inside-out recordings

Our investigations were extended to the study of membrane patches excised from erythrocytes that had been incubated for at least 15 min prior to measurement. The inside-out configuration allowed us to apply the drugs at the given concentrations to the cytoplasmic side of the membrane patches. None of the CaM antagonists inhibited channels activity in this experimental condition. As an example, Fig. 3 illustrates the activity of three IK_{Ca}, first recorded in cell-attached (c.a.) and then in inside-out (i.o.), from a cell preincubated for 40 min with W7 and treated with the CaM-antagonist throughout the experiment. After patch excision, the channel activity value rapidly increased (Fig. 3b), in response to the higher calcium concentration in the bath solution (1 mM), then the activity decreased to reach low values of open probability, with a typical oscillatory pattern (Fig. 3c) as already described [22].

In few experiments, performed in both cell-attached and inside-out configurations, the effects of calmidazolium were studied on erythrocytes bathed in physiological solution instead of high K^+, obtaining substantially similar results (data not shown).

To test the effects of the CaM antagonists at various free Ca^{2+} concentrations and in different states of channel regulation, inside-out membrane patches were perfused with solutions of different composition.

As reported by the authors [22], Gardos channels

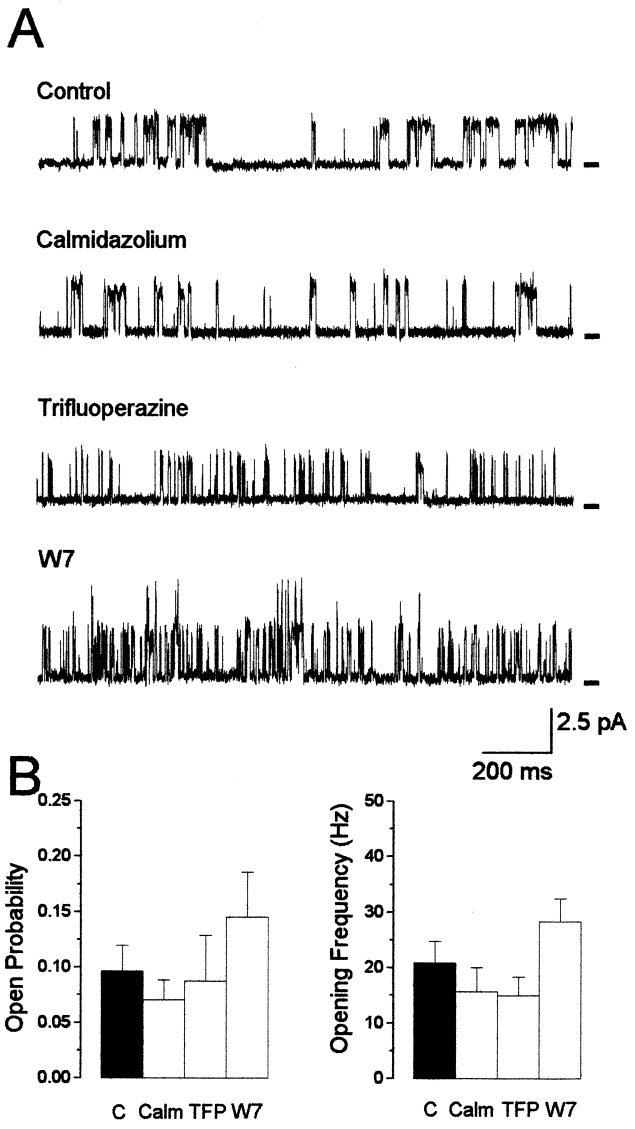

Fig. 1. CaM antagonists do not inhibit IK_{Ca} channels activity recorded in the cell-attached (c.a.) configuration. (A) The current traces from top to bottom are samples of channel activity in control cells, in cells treated for 15–60 min with calmidazolium (500 nM), trifluoperazine (10 μM) and W7 (25 μM). The mark at the right of each record corresponds to the current with all channels closed. The membrane potential was held at −80 mV. Data are filtered at 1 kHz. Inward currents are displayed as upward deflections. (B) The mean values ± S.E.M. of the open probability (left graph) and opening frequency (right graph) were measured from 64 membrane patches: 26 control (C), 21 calmidazolium (Calm), eight trifluoperazine (TFP) and nine W7, respectively. Silent patches (7.1% in control and 7.3% in treated cells) were not included in the statistical analysis.

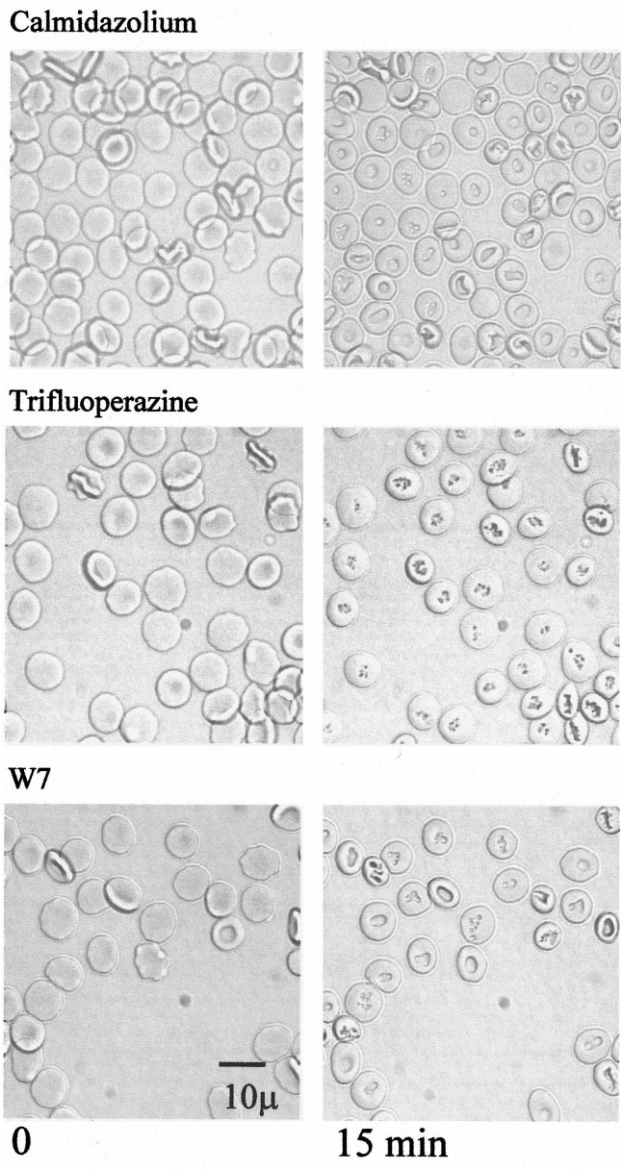

Fig. 2. DIC images of erythrocytes acquired just after drug application (left) and after incubation for 15 min (right) with calmidazolium (500 nM), trifluoperazine (10 μM) and W7 (25 μM).

are silent when perfused with solutions containing 0.1 μM free Ca^{2+} and maximally activated at 10 μM Ca^{2+}. In Fig. 4 the different levels of activity induced after excision, by three free Ca^{2+} concentrations in the presence of W7, are displayed. It is evident that the calcium sensitivity of these IK_{Ca} channels is maintained in presence of the CaM antagonist.

It has been shown [22] that the transient applica-

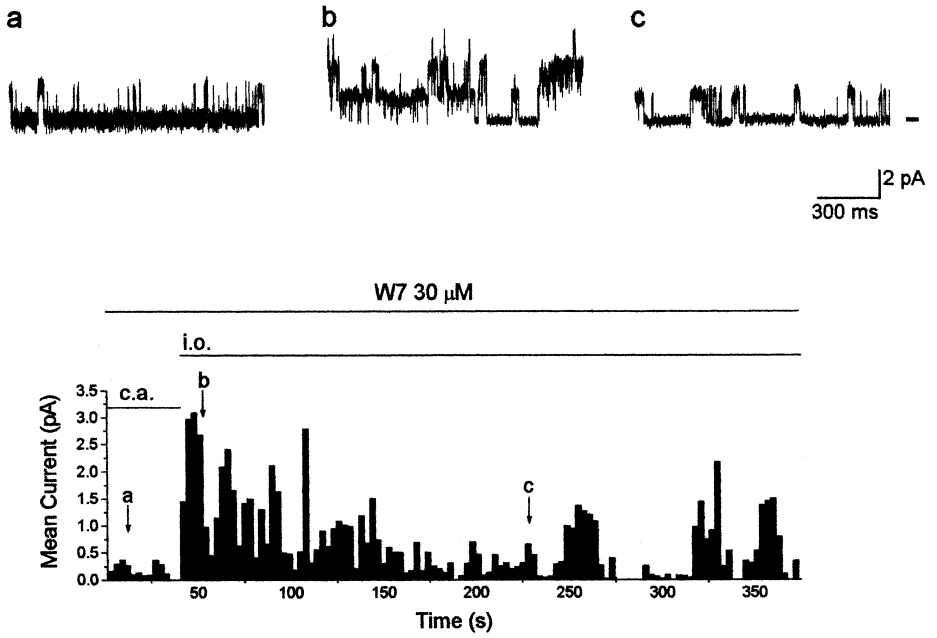

Fig. 3. Cell-attached (c.a.) and inside-out (i.o.) activity of the same IK_{Ca} channels in the presence of 30 μM W7. The time course of the mean current recorded from a patch containing at least three channels, is illustrated in the plot. Patch activity in the c.a. configuration is shown in the first upper record (a). The second and third traces are samples of activity at times marked b and c as indicated by the arrows. The membrane potential was held at −80 mV. Filtering at 1 kHz. The mark on the right of the records corresponds to the current with all channels closed. Inward currents are displayed as upward deflections. Each column of the plot represents the mean patch current calculated from a consecutive 3-s long data segment.

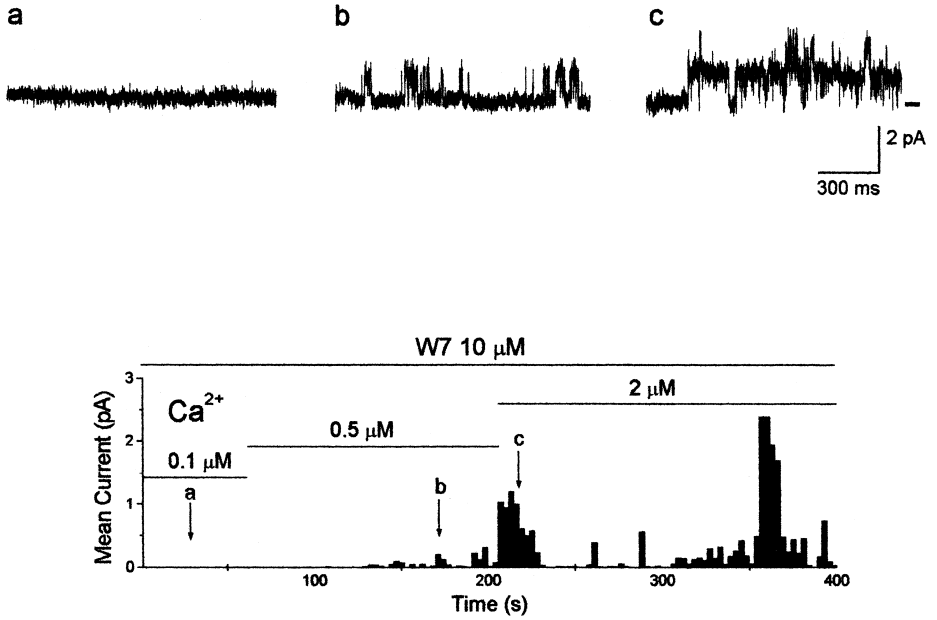

Fig. 4. Calcium sensitivity of IK_{Ca} channels in the presence of W7. An inside-out patch containing at least two IK_{Ca} channels was perfused with solutions containing free Ca^{2+} concentrations of 0.1 (a), 0.5 (b) and 2 μM (c), in the presence of 10 μM W7. The traces are samples of activity at the times a, b and c as indicated by the arrows. The membrane potential was held at −80 mV. The baseline current with all channels closed is indicated by the tick on the right of the records. Inward currents are upward deflections. Filtering at 1 kHz. Each column in the plot represents the mean patch current calculated from a consecutive 3-s long data segment.

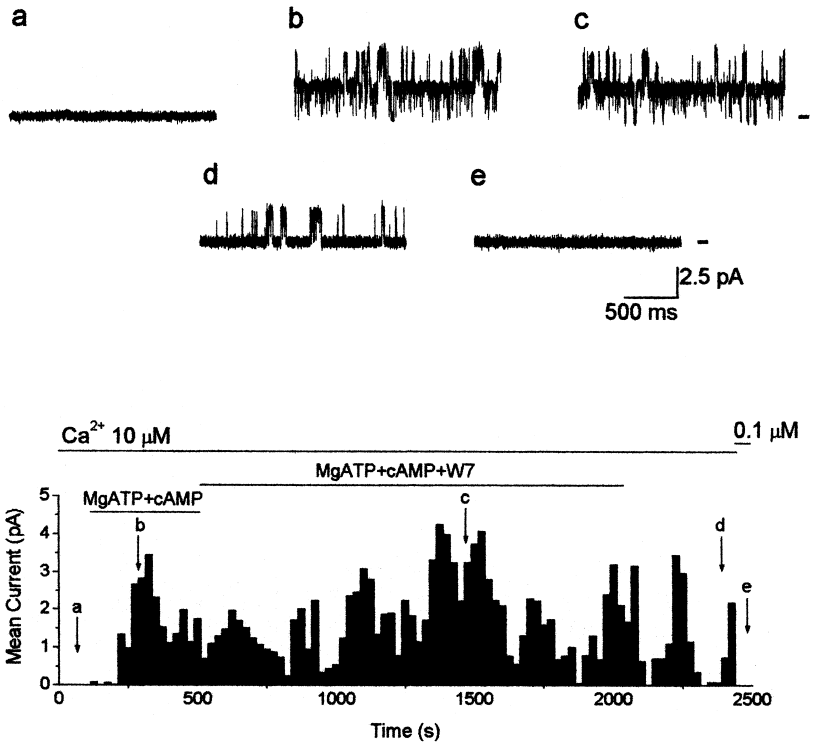

Fig. 5. Lack of inhibitory effect of W7 on the activity of a membrane patch containing two IK_{Ca} channels, which have been previously up-modulated. The plot illustrates the mean current values recorded 6 min after patch excision, and complete activity run-down (a). An increase in the mean current value was induced by perfusion with a phosphorylation promoting mixture, containing MgATP (1 mM) and cAMP (1 mM) (b). The addition of 10 μM W7 (c) does not inhibit the channels activity. The removal of PKA-stimulating solution reduces the mean current (d) and the activity is extinguished by a free Ca^{2+} concentration of 0.1 μM (e). The traces are samples of activity at the times a, b, c, d and e as indicated by the arrows. The membrane potential was held at −80 mV. The channel closed current is indicated by the mark on the right of the records. Upward deflections are inward currents. Filtering at 1 kHz. Each column in the plot represents the mean patch current calculated from a consecutive 25-s long data segment.

tion of a phosphorylation mixture to the cytoplasmic side of excised patches activated an endogenous PKA inducing a reversible channel up-regulation. Fig. 5 shows the absence of inhibition of the up-regulation of two Gardos channels by W7. After a complete run-down in 10 μM free calcium, the channels were silent (Fig. 5a), then their activity was up-regulated by the phosphorylation promoting cocktail activating endogenous PKA (Fig. 5b). The addition of W7 did not reduce the mean patch current (Fig. 5c). The removal of the phosphorylation cocktail led to a decrease in channel activity (Fig. 5d) and, at this stage, the reduction of free Ca^{2+} to 0.1 μM interrupted channel activity (Fig. 5e).

The CaM antagonists were unable to inhibit Gardos channels at high concentrations [28,29]. Fig. 6 illustrates the absence of inhibition in the presence of W7 100 μM, a concentration that starts producing erythrocyte hemolysis [30]. Similar results (data not shown) were obtained both in cell-attached and inside-out configurations with W7 100 μM or with calmidazolium 2 μM, a dose that inhibits almost completely the CaM-dependent fraction of Ca^{2+} ATPase activity [28,29].

4. Discussion

In previous experiments [22,23] we found that in the cell-attached configuration, at millimolar $[Ca^{2+}]_o$, the opening frequency of IK_{Ca} channels from human erythrocytes was extremely low. After excision, in the inside-out configuration, channel activity first increased and then ran-down. Furthermore, channel activity was restored by perfusing the inner side of the patch with cAMP and MgATP. These findings

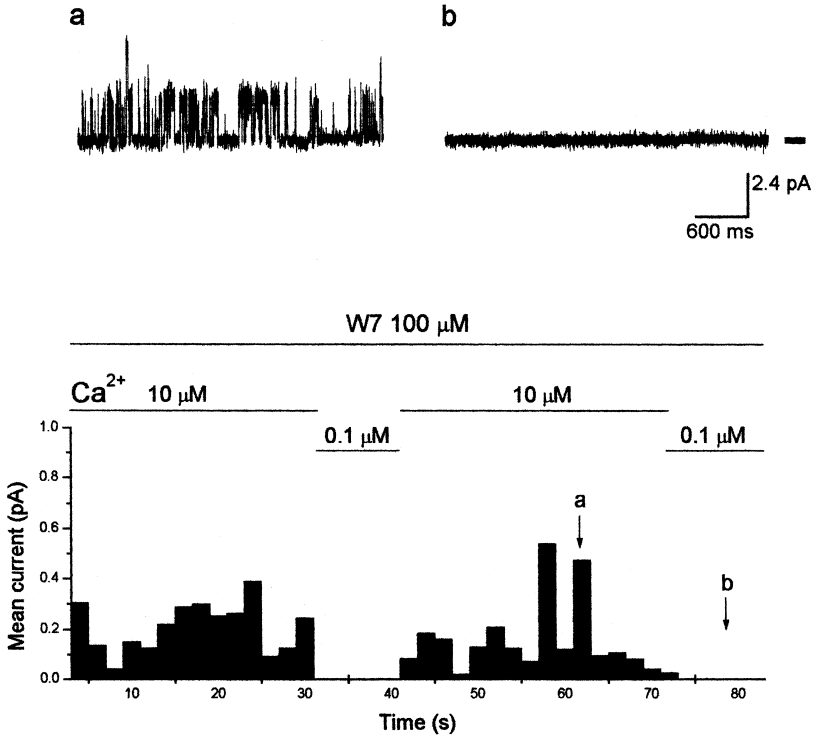

Fig. 6. Activation–deactivation cycles of two IK_{Ca} channels induced by changes of free calcium on the cytoplasmic side of an inside-out patch, in the presence of W7 100 μM. The traces are samples of activity at the times a and b as indicated by the arrows. The membrane potential was held at −80 mV. The channel closed current is indicated by the mark on the right of the records. Upward deflections are inward currents. Filtering at 1 kHz. Each column in the plot represents the mean patch current calculated from a consecutive 2.5-s long data segment.

provided a direct demonstration that Gardos channels are up-regulated by endogenous PKA, whose presence has already been revealed [31–33]. The occurrence of channel run-down after excision, as well as after interruption of perfusion with the PKA-stimulating cocktail, suggested that dephosphorylation reactions participate in channel down-regulation [34].

The main change induced by PKA activation consisted in an increase of the channel Ca^{2+} sensitivity [22], as reported for other types of Ca^{2+}-activated K^+ channels [35].

There is evidence that CaM acts as Ca^{2+} sensor in IK_{Ca} channels. It coprecipitates with the channel in specifically transfected cell lines, and its mutant form, which is defective in calcium sensing but retains binding to the channel, reduces the IK_{Ca} current [12]. In addition, IK_{Ca} channels lacking the CaM-binding region do not function properly [13]. The experiments described here were designed to test whether CaM antagonists inhibit the native IK_{Ca} of human erythrocytes. In our experiments all three CaM antagonists induced clear-cut changes in cell shape, presumably by CaM-dependent cytoskeletal modifications [27,36], but were ineffective in inhibiting IK_{Ca} channel activity. This was observed both in the cell-attached configuration at the steady $[Ca^{2+}]_i$ (reported to be about 1×10^{-7} M [37]), as well as in the inside-out configuration, at $[Ca^{2+}]_i$ ranging from 10^{-7} to 10^{-5} M. Moreover, the IK_{Ca} channel was reversibly up-modulated by activating endogenous PKA, in the presence of CaM antagonists. Assuming that CaM acts as the Ca^{2+} sensor of IK_{Ca} channels of erythrocytes, the absence of inhibitory effects may be taken as an indicator of the unavailability of hydrophobic sites to which CaM antagonists can bind in order to prevent the interactions between CaM and the channel. Thus, the evidence presented here is consistent with a constitutive association of CaM with the native Gardos channels. However, the present evidence cannot rule out the possibility that the Ca^{2+} sensitivity of IK_{Ca} channel of erythrocytes is independent of CaM.

Our results are in keeping with some measurements of Ca^{2+}-dependent K^+ efflux obtained in both intact cells and subcellular preparations [15,18]. However, the published information on the participation of CaM in the control of Ca^{2+}-dependent K^+ channels of human erythrocytes is contradictory. Lackington and Orrego [14] reported that many CaM antagonists (including trifluoperazine and W7) inhibited the net loss of K^+ induced by divalent cation ionophore in intact red cells. Similarly, Yingst and Offman [17] found that trifluoperazine inhibited Ca^{2+}-dependent K^+ transport in cell ghosts. On the contrary, Plishker [15] found that phenothiazines stimulate the activation of Ca^{2+}-dependent K^+ transport in red cells and attributed such an effect to the inhibition of the Ca^{2+} pump. Pape and Kristensen [16] have reported an activation of Ca^{2+}-dependent K^+ transport in inside-out vesicles by purified CaM, but this contrasts with the failure to evidence such an effect by Alvarez et al. [18].

It is not easy to account for these discrepancies. On one hand, it is well known that the experimental approach can affect measurements of the erythrocyte K^+ efflux. On the other hand, it has been pointed out that most CaM antagonists are strongly lipid-soluble and could have non-specific effects due to alterations of the membrane physical properties [17,18].

In the present work the patch-clamp technique was exploited to unambiguously identify the Gardos channel; the absence of inhibition of native IK_{Ca} channels by CaM antagonists was found both in cell-attached and in cell-free patches, whose channels retained both Ca^{2+} sensitivity and modulation.

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