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Modulation of Ca^{2+}-activated K^+ channels of human erythrocytes by endogenous protein kinase C

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Abstract

Single IK_{Ca} channels of human erythrocytes were studied with the patch-clamp technique to define their modulation by endogenous protein kinase C (PKC). The perfusion of the cytoplasmic side of freshly excised patches with the PKC activator, phorbol 12-myristate 13-acetate (PMA), inhibited channel activity. This effect was blocked by PKC_{19-31}, a peptide inhibitor specific for PKC. Similar results were obtained by perfusing the membrane patches with the structurally unrelated PKC activator 1-oleoyl-2-acetylglycerol (OAG). Blocking of this effect was induced by perfusion with PKC_{19-31} or chelerythrine. Channel activity was not inhibited by the PMA analog 4α-phorbol 12,13-didecanoate (4αPDD), which has no effect on PKC. Activation of endogenous cAMP-dependent protein kinase (PKA), which is known to up-modulate IK_{Ca} channels, restored channel activity previously inhibited by OAG. The application of OAG induced a reversible reduction of channel activity previously up-modulated by the activation of PKA, indicating that the effects of the two kinases are commutative, and antagonistic. Kinetic analysis showed that down-regulation by PKC mainly changes the opening frequency without significantly affecting mean channel open time and conductance. These results provide evidence that an endogenous PKC down-modulates the activity of native IK_{Ca} channels of human erythrocytes. Our results show that PKA and PKC signal transduction pathways integrate their effects, determining the open probability of the IK_{Ca} channels.

Keywords: Erythrocyte; IK_{Ca} channel; Calcium-activated potassium channel; Gardos channel; Protein kinase C; Phosphorylation

1. Introduction

Calcium-activated K^+ channels (K_{Ca}) transduce fluctuations in intracellular calcium concentration into changes of potassium permeability and of membrane potential in both excitable and nonexcitable cells. K_{Ca} channels are divided into three subfamilies: BK_{Ca}, IK_{Ca} and SK_{Ca} according to their big-, intermediate- and small-conductances, respectively [1]. These sub-types also differ in their amino acid sequences, pharmacological profiles and calcium sensitivities. 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, they mediate the calcium-induced K^+ efflux [6], which probably contributes to the elimination of senescent red blood cells [7]. The function of calcium-activated K^+ permeability in human erythrocytes still remains poorly understood. Since Gardos channels have been used as a model to study the pathophysiology of myotonic dystrophy [8], a human systemic disease involving defective expression of myotonin protein kinase [9–11], it is important to elucidate the role of protein kinases in the modulation of these channels. The data available confirm that PKA is an important regulator of IK_{Ca} in general [12] and of Gardos channels in particular [13]. However, a detailed description of the role of PKC on single Gardos channels has not been reported [14–18]. The lack of information prompted us to study the effects of endogenous PKC activation or inhibition on the activity of single IK_{Ca} channels of human erythrocytes. Here we report a clear-cut PKC-dependent

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down-modulation of Gardos channel unitary currents in excised patches. We also provide evidence that PKC and PKA have antagonistic effects in determining the channel activity level.

2. Materials and methods

2.1. Preparation

Experiments were performed on blood samples from 10 healthy volunteers (age 25–55 years) after their informed consent. 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, Hepes 10, pH adjusted to 7.4 with NaOH, bovine serum albumin (BSA) (1 mg/ml). Erythrocytes were kept at 5 °C (hematocrit 2%) in the washing solution supplemented with $CaCl_2$ 1.8 mM and glucose 10 mM (storage solution), and used within 2 days. Storage of red blood cells at 5 °C in this solution might alter their internal ionic composition [19]. However, all measurements of Gardos channel activity were performed in inside–out configuration and the general cell conditions (biconcave shape without membrane indentations and intracellular calcium level below 50 nM, by fura-2 imaging) did not seem detectably altered by these storage conditions. Aliquots of this suspension were diluted at low density in tissue culture Petri dishes (Falcon 3001, Becton Dickinson, UK) in the storage solution without BSA. Inside–out experiments were performed in a high K^+ solution, containing (mM): KCl 120, $MgCl_2$ 1, Hepes 10, pH adjusted to 7.2 with KOH and free calcium concentrations in the range 0.1–10 μM. These solutions were obtained by buffering with 1 mM EGTA (ethyleneglycol-bis[β-aminoethylether]*N*,*N*-tetraacetic acid) or 2 mM BAPTA (1,2-bis[*o*-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), according to the MaxC software by C. Patton (Stanford University). Recordings were performed at room temperature (20–24 °C) after at least 5 min of incubation in the storage solution.

2.2. Patch-clamp recordings

Single channel currents were recorded in inside–out configuration [20] with an Axopatch 1 D amplifier (Axon Instruments, CA, USA) and were low-pass filtered at 1 kHz, using a four pole Bessel filter. Pipettes were pulled in two stages from disposable capillaries (Blaubrand 7087) and were coated with Sigmacote (Sigma). Pipettes were systematically checked for both resistance (10 MΩ) and "bubble number" (3.5 cc) [21]. The pipette solution contained (mM): KCl 120, $CaCl_2$ 1, Hepes 10, pH adjusted to 7.2 with KOH, and was filtered with a 0.2 μm Millipore filter. Cell-free patches were tested with solutions of different composition applied to the internal side by a rapid solution changer (Biologic RSC 200, France), the motor speed was set to allow the change of the tube facing the patch pipette to occur within 100 ms.

2.3. Chemicals

Protein kinase C inhibitor peptide 19–31 (PKC_{19-31}), 1-oleoyl-2-acetylglycerol (OAG) and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem (Inalco, Milan, Italy) while all other reagents were obtained from Sigma (Milan, Italy).

2.4. Data analysis

Data were analyzed on-line with software developed in our laboratory for the purpose and simultaneously stored on a modified videotape recorder [22]. 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, percentage of open time, as well as mean open and closed times (for patches with single channels) 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

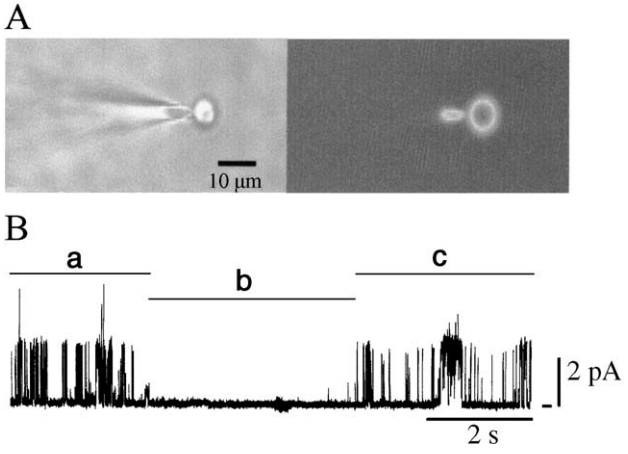

Fig. 1. Visualization of the portion of membrane included in a patch. The two images were acquired in Nomarski differential interference (A left) and in fluorescence (excitation 480 nm, emission 520 nm, A right). The erythrocyte was loaded with fluo-4 AM and permeabilized to calcium with ionomycin (2 μM) just after pipette sealing. After excision in the inside–out configuration, the cytoplasmic side of the membrane patch, containing two Gardos channels, was perfused with high K^+ solution containing 1 μM (a and c) and 0.1 μM (b) free calcium (B). Channel activity was quickly interrupted by the reduction of $[Ca^{2+}]_i$. The membrane potential was held at −80 mV. Filtering 1 kHz. The channel closed current is indicated by the mark on the right of the record. Upward deflections are inward currents.

transported by the duration of analyzed data. The mean frequency of events was obtained by calculating the ratio between the number of opening transitions and the total record 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. Only records with a single channel were used to study channel kinetics. The open and closed dwell time distributions were fitted with dual exponential functions, whose time constants were estimated by minimizing χ^2 using a Levemberg–Marquardt algorithm. Mean channel open and closed times were determined by the arithmetic means of dwell time data. The effect of drugs on channel activity was determined by measuring the mean patch current during the last 2 min of drug application.

Sigma Plot 3 and Sigma Stat 2 (Jandel Scientific) were used for mathematical transformations and statistical analysis. Results are expressed as means ± standard error of the mean. Differences between groups were examined with the

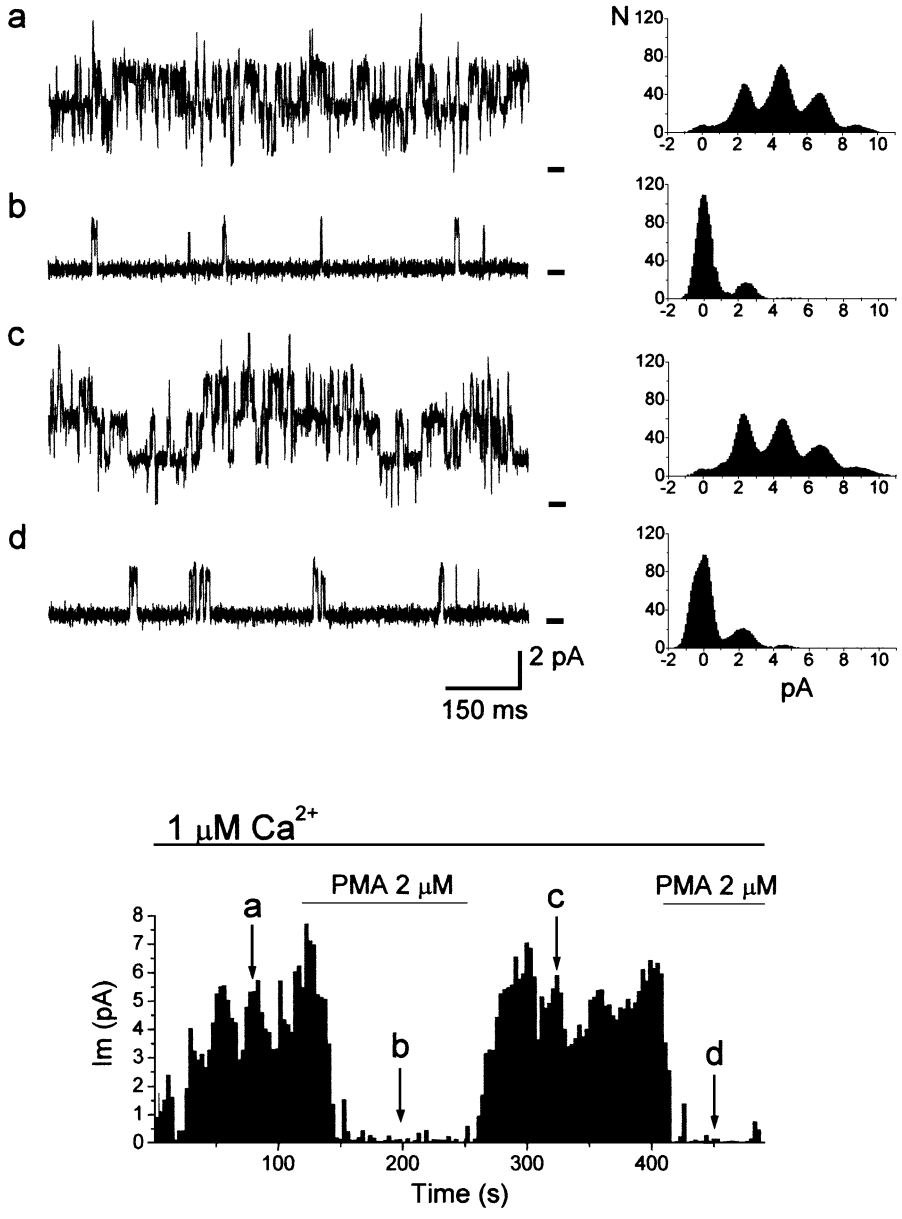

Fig. 2. Reversible effect of PKC activation on channel activity. The cytoplasmic face of a membrane patch containing four Gardos channels was perfused by high K^+ solution containing 1 μM calcium with or without PMA. The membrane potential was held at − 80 mV. Filtering 1 kHz. The channel closed current is indicated by the mark on the right of each record. Inward currents are displayed as upward deflections. Columns in the plot represent the mean patch current calculated from 3-s-long consecutive data segments. The current traces are samples of activity at the times marked a, b, c and d by the arrows. The corresponding all-points histograms are displayed on the right of each trace.

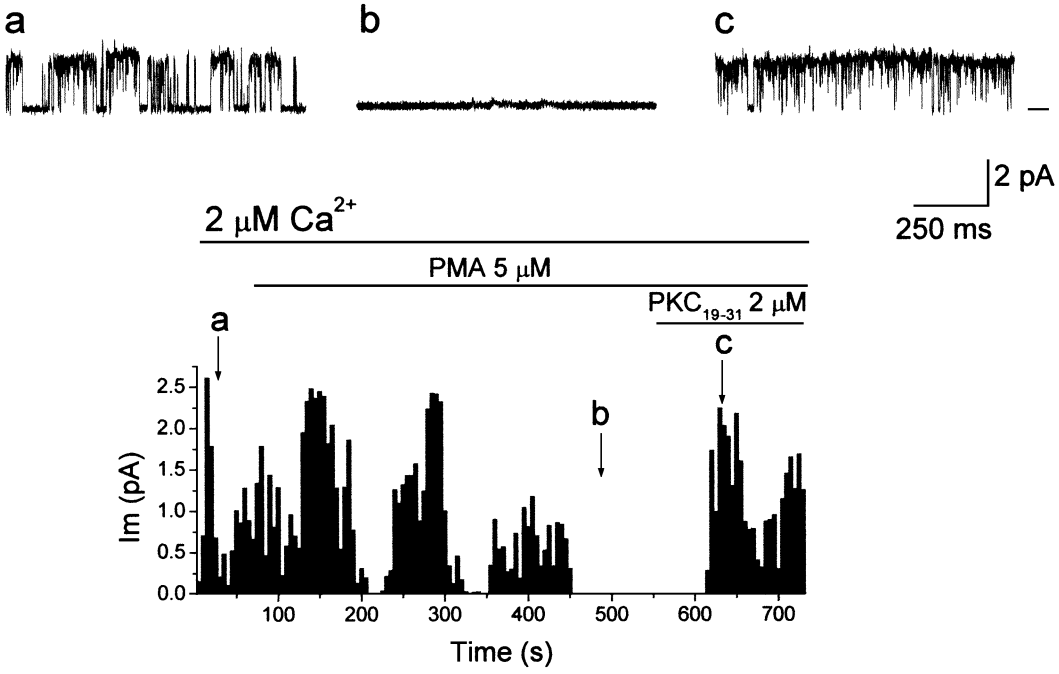

Fig. 3. Slow inhibition of a single Gardos channel by PMA in 2 μM Ca^{2+} and restoration of activity by addition of PKC_{19-31}. The membrane potential was held at − 80 mV. Filtering 1 kHz. Each column of the plot represents the mean patch current calculated from a consecutive 5-s-long data segment. The current traces are samples of activity at the times marked a, b, and c by the corresponding arrows.

Student's *t*-test and a *P* value of less than 0.05 was taken as significant.

3. Results

Unitary currents of Gardos channels were identified by their conductance value, inward rectification, voltage independence of open probability, kinetics and Ca^{2+}-sensitivity, all features which have been described elsewhere [8,13,20,23–26]. Because of the pipette shape and the sealing procedure used, membrane patches consisted of a large membrane area which was imaged using a fluorescent calcium indicator (Fig. 1A). Cell-free inside–out patches presumably preserved complex cytoskeletal structure and enzyme machinery, as indicated by the consistent presence

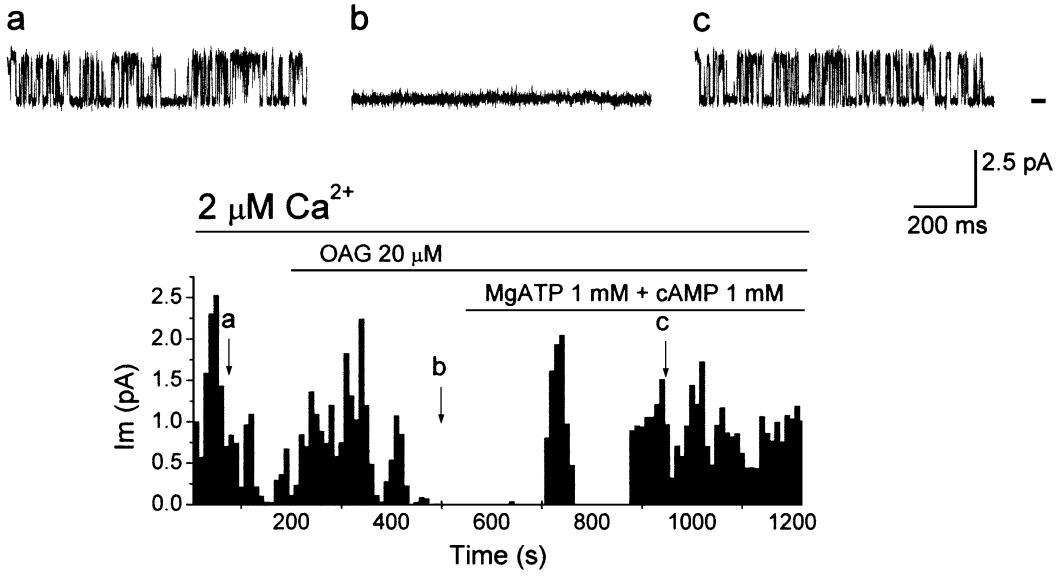

Fig. 4. Slow inhibition of a single Gardos channel by OAG in 2 μM Ca^{2+} and restoration of activity by PKA activation. The membrane potential was held at − 80 mV. Filtering 1 kHz. Each column of the plot represents the mean patch current calculated from a consecutive 10-s-long data segment. The current traces are samples of activity at the times marked a, b, and c by the corresponding arrows.

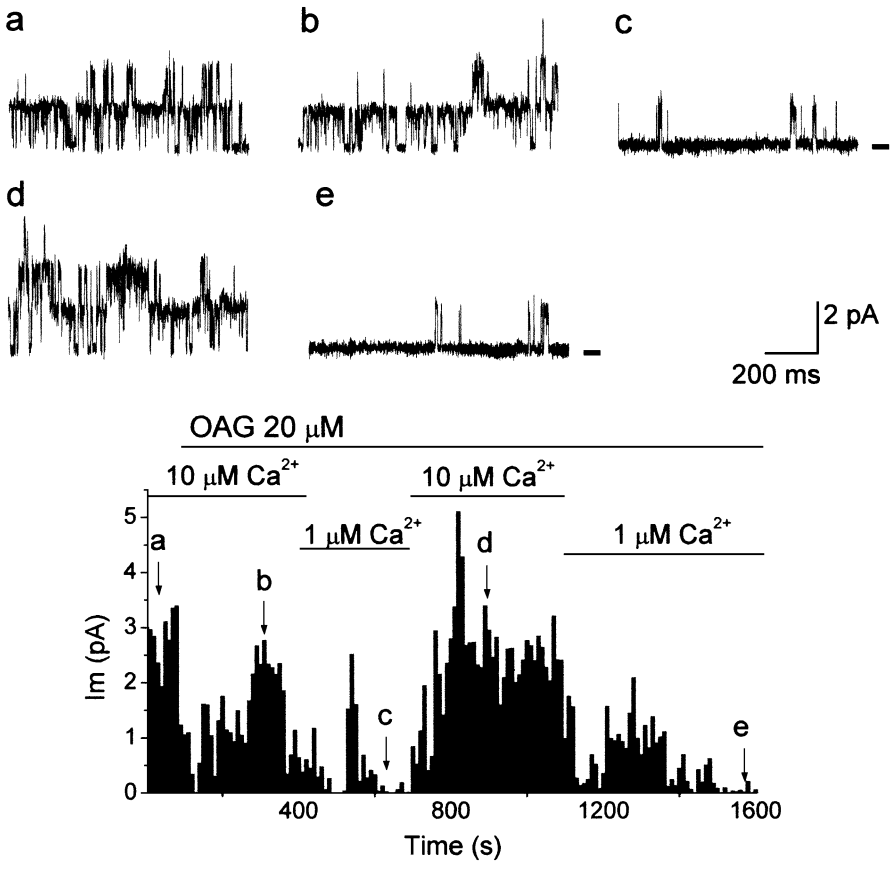

Fig. 5. Effect of Ca^{2+} on the channel activity during OAG application. A membrane patch containing three Gardos channels was perfused with OAG while the calcium concentration was changed twice from 10 to 1 μM. Lack of inhibition at higher $[Ca^{2+}]_i$ can be noted. The membrane potential was held at −80 mV. Filtering 1 kHz. The bin in the plot is 10 s. The current traces are samples of activity at the times marked a, b, c, d and e by the corresponding arrows.

of modulatory activity by kinases and phosphatases. Furthermore, unrestricted access to the internal face of the ion channels was allowed, as indicated by the quick interruption of activity when perfused by a low-calcium solution (Fig. 1B). The typical activity pattern of Gardos channels has been reported in a previous paper [13]. Briefly, in the cell-attached configuration, the channel activity was consistently low, because of the low calcium permeability and of the large-capacity Ca^{2+} pump of the human erythrocyte membrane. The activity rapidly increased after excision, when the free calcium concentration in the solution perfusing the inner side of the patch was higher than 0.2 μM. A spontaneous reduction of activity (run-down) occurred within minutes after excision. Channel activity was reversibly restored under perfusion of the membrane patch with a PKA-activating cocktail, containing 1 mM cAMP, 1 mM MgATP and 1 mM theophylline. Current up-modulation was mainly due to an increase of the opening frequency and was proportional to calcium concentration. These results demonstrated that the ion channels are up-regulated by an endogenous PKA, perhaps intimately associated with the channel itself. On the other hand, the occurrence of channel run-down both after excision and after interruption of perfusion with the stimulating cocktail was evidence for

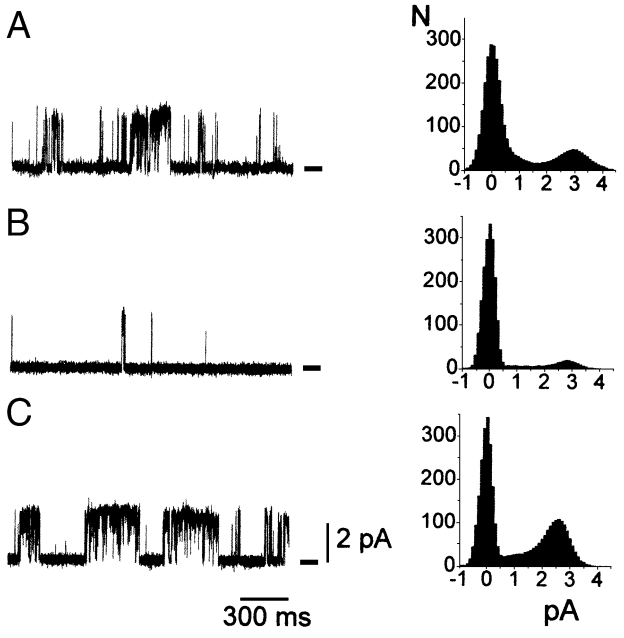

Fig. 6. Samples of activity of a single channel before (A) and after addition of PMA (B) and PMA + PKC_{19-31} (C). The corresponding all-points histograms are displayed on the right of each trace. Holding membrane potential −80 mV, filtering 1 kHz.

Table 1
PMA down-modulates the activity of a single Gardos channel, this effect is abolished by addition of PKC_{19-31}

	Opening frequency (Hz)	Mean channel open time (ms)	Mean channel closed time (ms)	Single channel current (pA)
Control	10	4.2	94.8	2.9
PMA	1.7	4.9	586.4	2.9
PMA + PKC_{19-31}	33.5	4.2	24.9	2.7

ongoing dephosphorylation reactions, which down-regulate channel activity [13]. Accordingly, in this study the effects of PKC activation were analyzed either before activity run-down or after PKA activation.

The perfusion of freshly excised inside–out patches with the PKC activator PMA strongly reduced the open probability, in a reversible mode. Fig. 2 illustrates such an effect. At the beginning of the record, corresponding to the excision, openings increased in frequency because the calcium content of the perfusing solution was 1 μM. The time course of the mean patch current (I_m) during two consecutive stimulations with PMA (2 μM) is displayed. The mean value of I_m was reduced from 3.65 to 0.26 pA with a lag time of about 20 s. Wash-out was followed by an increase of activity, within about 15 s, while readmission of PMA induced a new inhibition. Repetitive stimulation never showed changes in patch responsiveness. In seven experiments, addition of PMA decreased I_m to 17.3 ± 7.7% of the control value, while it was restored to 106.5 ± 14.1% after wash-out.

The delay in getting a clear-cut inhibitory response to the PKC activator was widely variable from patch to patch. Two main factors were found to affect this delay. First, long lag times (minutes) were found to be associated with multi-channel patches in which the distribution of the times spent at different conductance levels clearly deviated from the binomial distribution, suggesting that modulation differentially affected neighbouring ion channels. This aspect clearly needs further investigation, although it is beyond the scope of the present study. As far as the second factor is concerned, calcium concentration in the perfusing solution were found to affect both lag times and potency of channel inhibition. Channel activity was almost completely abolished by PMA in 0.5 μM Ca^{2+}, it was reduced to less than 20% of the control activity in the Ca^{2+} range 1–2 μM, with delays increasing to minutes in 2 μM Ca^{2+}, whereas the inhibition was undetectable in 10 μM Ca^{2+}.

Fig. 3a and b illustrates a slow inhibition by PMA in 2 μM Ca^{2+}. Moreover, channel inhibition was reversed by addition of PKC_{19-31}, a peptide inhibitor specific for PKC, as shown in Fig. 3c. In three experiments, I_m was reduced to 14.5 ± 0.6% of the control value by PMA to increase to 207.6 ± 73.6% by addition of PKC_{19-31}. Similar effects were observed using calphostin C (1 μM) as the PKC inhibitor.

The PMA analog 4α-phorbol 12,13-didecanoate (4αPDD), which is uneffective on PKC, did not inhibit channel activity and did not maintain the channel inhibition previously induced by PMA. In three experiments, I_m was reduced to 11.1 ± 3.2% of the control value by PMA and restored to 83.2 ± 13.4% by substitution of PMA with 4αPDD.

Channel inhibition was also obtained by substituting PMA with the structurally unrelated PKC activator OAG

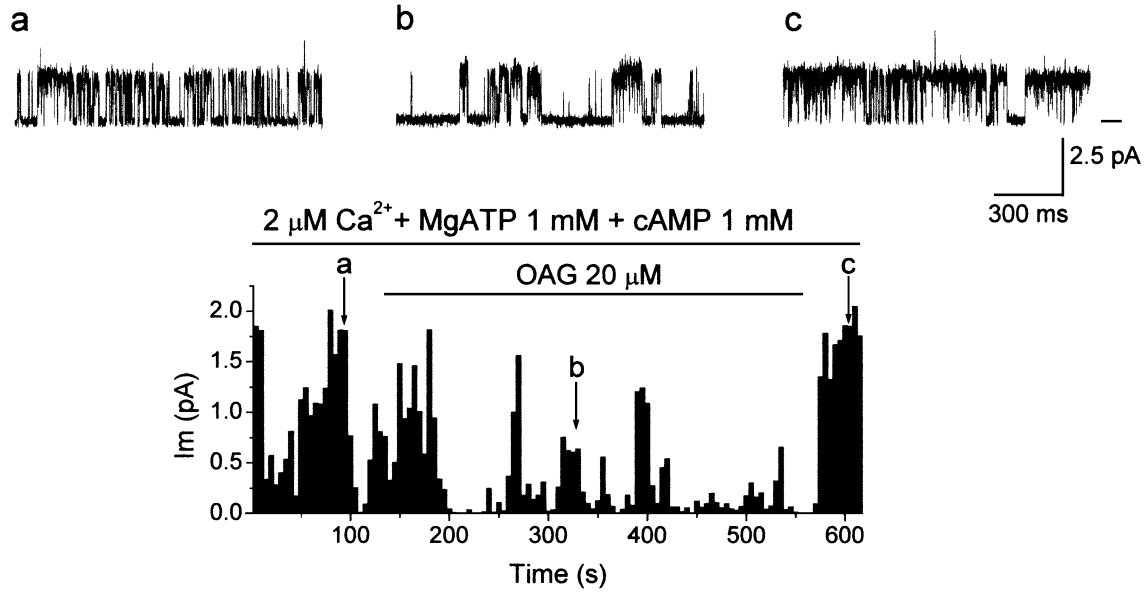

Fig. 7. Application of OAG, during up-modulation by PKA, reversibly reduces the activity of two Gardos channels. Holding membrane potential −80 mV, filtering 1 kHz. Each column of the plot represents the mean patch current calculated from a consecutive 5-s-long data segment. The current traces are samples of activity at the times marked a, b, and c by the corresponding arrows.

(see Fig. 4a and b), and the activity was resumed upon the addition of PKC_{19-31} (data not shown).

Fig. 5 shows how different calcium concentrations allow or counteract channel inhibition by a PKC activator.

The PMA-induced down-modulation of the mean current is mainly due to a decrease of the opening frequency. Samples of activity recorded from a patch containing a single channel are displayed in Fig. 6. Table 1 summarizes the values of opening frequency, mean channel open and closed times and single channel current, measured in the experiment illustrated in Fig. 6. The bursting activity of these channels indicates the existence of at least two closed states, PMA mainly affected the long closures.

Finally, the effects of concurrent activation of PKA and PKC on channel activity was studied. Channels which had been previously down-modulated by OAG were also perfused with a PKA-activating solution without theophylline, as shown in Fig. 4. It can be seen that the inhibition induced by OAG was then removed by the simultaneous activation of PKA. Reversing the order of presentation of protein kinase activations did not seem to alter the specific effects of the two kinases on channel activity. Fig. 7 illustrates how channel activity, up-modulated by PKA, can be reversibly depressed by the simultaneous application of OAG.

Moreover, the PKC inhibitor chelerythrine removed channel inhibition induced by OAG during PKA activation, as shown in Fig. 8. The relative potency of the two modulations was not further investigated because some findings suggested the occurrence of a variable basal channel inhibition in the absence of PKC activators. In fact, when applied alone, PKC_{19-31} attenuated the activity run-down following excision (data not shown). Furthermore, as indicated, addition of PKC_{19-31} after PMA, besides removing channel inhibition, often raised channel activity beyond the control level.

4. Discussion

The structurally unrelated PKC activators PMA and OAG produced a similar inhibition of IK_{Ca} channels of human erythrocytes when applied in the inside–out configuration. The reduction in the patch current was associated with a clear-cut decrease in the opening frequency with minor changes in the mean channel open time and no change in unitary current. The PMA analog 4αPDD, which is known to have no effect on PKC, did not mimic the effect produced by PMA. Addition of PKC_{19-31},

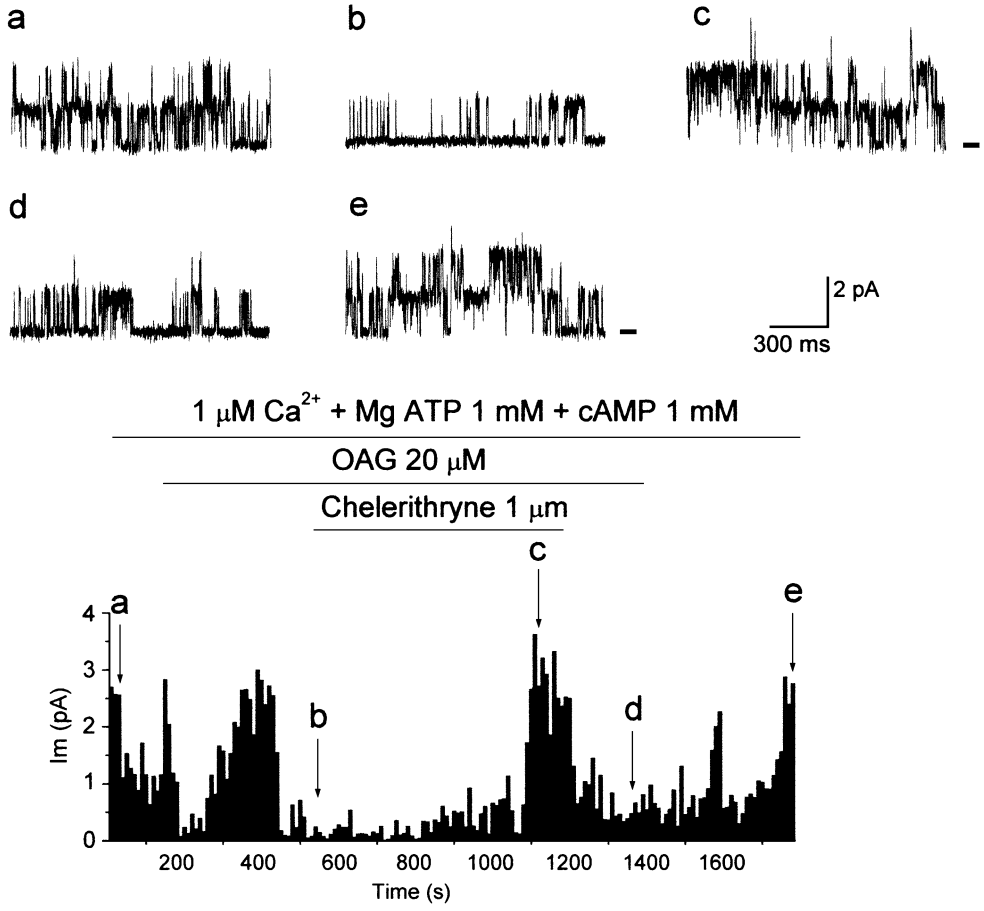

Fig. 8. Chelerythrine restores activity previously inhibited by OAG during up-modulation of two Gardos channels. Columns in the plot represent the mean patch current calculated from 10-s-long consecutive data segments.

which acts as a pseudosubstrate maintaining the enzyme PKC in its inactive form [27], removed either PMA or OAG inhibition, often resulting in higher activity than in control conditions. Similar effects were observed using calphostin C, a potent and irreversible PKC inhibitor that affects the phorbol-ester binding site [28], or chelerythrine, which interacts with the catalytic domain of PKC by competitive inhibition of adenosine thriphosphate binding [29]. These findings rule out the hypothesis that the observed effects are due to PKC activators per se [18] and indicate that activation of an endogenous PKC was responsible for Gardos channel down-modulation. Four isoforms of PKC have been detected in human red blood cells α, ζ [16], μ and ι [30]. The α-isoform is probably involved in the effects on Gardos channels reported here, because channel inhibition was induced by both OAG and PMA. The short latency of channel inhibition measured in some patches at low calcium concentration (see the example illustrated in Fig. 2), as well as the consistency of the mechanism found in most of the cell-free membrane patches, make it unlikely that a complicated biochemical cascade is responsible for the PKC-induced channel inhibition. Although our results cannot allow us to identify the molecular target of phosphorylation, they are consistent with a direct modulation of the channel itself or of an associated regulatory protein.

When PKC_{19-31} was applied alone, it attenuated the activity run-down following excision. Furthermore, the activity induced by addition of PKC_{19-31} during PMA treatment in many cases was higher than in controls. Accordingly, in our experimental conditions, the mechanism which underlays channel inhibition seems to work at a background level, in the absence of PKC activators, and this mechanism is augmented by PKC activation. Here, the picture emerging for IK_{Ca} channels of human erythrocytes is similar to the regulation of most of the structurally unrelated BK_{Ca} channels in smooth muscle, which are up-modulated by PKA and down-modulated by PKC [31]. Modulation of IK_{Ca} channels by PKC has been studied: (a) by measurements of Ca^{2+} pump transport activity and of K^+ efflux in sickle red blood cells [16]; (b) by whole-cell current recording in CHO and HEK293 cells expressing the canine isoform cIK1 [14]; (c) by single channel recording in T84 cells expressing endogenous hIK1 channels [18] and (d) by measurements of ^{86}Rb influx in normal and sickle human erythrocytes [15]. Fathallah et al. [16] found that incubation with PMA inhibited both Ca^{2+} pump and charybdotoxin-sensitive Ca^{2+}-stimulated K^+ efflux in sickle red blood cells. As far as cIK1 modulation is concerned, it has been reported that cIK1 currents are regulated in two ways by PMA, with an indirect acute activation and with a long-term inhibitory effect on the PKC site at T329, disclosed after overnight incubation with the drug. The authors suggest that this second effect might be due to altered recycling of cIK1 channels in the plasma membrane [14]. Devor and Frizzell [18] found that the inhibition of hIK1 channels of T84 cells by the PKC activator DIC_8, previously reported [17], was not reproduced by treatment with the PKC catalytic subunit, concluding that PKC does not acutely regulate hIK1 channels in T84 cells. Rivera et al. [15] found that charybdotoxin-sensitive Rb influx in human erythrocytes was inhibited by PKC inhibitors and suggested that activation of Gardos channels by endothelin-1 might be mediated by PKC.

Our study provides evidence for an acute and reversible inhibition of single Gardos channels by PKC activation in human red blood cells.

The difference in the acute effects (inhibition vs. activation) of PKC on native hIKs and on cIK1s is remarkable. It should not be due to the technical approaches used by Wulf and Schwab [14] and by us, since both whole-cell and inside–out configurations of the patch-clamp technique involve cytoplasm dialysis. Also, the nature of these channels, native hIK in erythrocytes and heterologous cIK1 in CHO and HEK293 cells, does not seem to explain the differences of the acute effects of PKC activation. In fact, native cIK1 channels in canine MDCK cells are similarly activated by PKC [32]. Moreover, unlike hIK, cIK1 channels lack PKA sensitivity. Thus, it is likely that human and canine isoforms of IK_{Ca} channels are inserted in specific molecular assemblies, resulting in a PKC action that is opposite or synergistic with Ca^{2+}, respectively, in controlling channel gating. This picture is in keeping with tissue-specific actions of other protein kinases on BK_{Ca} channels [31]. It is not possible with the available data to explain the results reported by Rivera et al. [15] for native human Gardos channels. They found a reduction of charybdotoxin-sensitive Rb influx in erythrocytes permeabilized to divalent ions with A23187 and preincubated with inhibitors of PKC. Since Rivera et al. did not activate PKC pharmacologically, in principle, their results might be induced by the several cellular mechanisms which are controlled by the increase of $[Ca^{2+}]_i$. In Rivera et al.'s [15] experiments PKCα should be translocated by high calcium but its activity might depend on indirect calcium-promoted phospholipase C activity. Our findings deal with a more delimited system and involve persistent PKC activation and its inhibition by exogenous drugs. On the other hand, our experimental conditions allow: (i) direct identification of IK_{Ca} channels; (ii) reversibility of the effects; (iii) rigorous control of the medium composition on both sides of the membrane; (iv) parsimonious use of pharmacological tools; (v) the preservation of integrated modulation by endogenous kinases and phosphatase in excised patches. In these conditions, the acute and reversible contribution of PKC to the channel modulation is a reduction of the opening frequency. In addition, we showed that increasing $[Ca^{2+}]_i$ over 1 μM progressively masks the inhibitory effect of PKC modulation. Our findings are in agreement with the reduced Ca^{2+}-stimulated K^+ efflux in sickle cells reported by Fathallah et al. [16]. These authors used high $[Ca^{2+}]_i$ and PMA to translocate and activate

PKC and used 4αPDD as negative control, as in our experiments. Moreover, our data are in keeping with the observation that PMA treatment prevented deoxygenation-induced SS cell dehydration [33], making it unlikely that an excitatory net effect might be found when measurements are made on whole cells.

The lack of inhibition of K^+ efflux in normal red cells reported by Fathallah et al. [16] was attributed by the authors to a lower level (about half) of membrane PKCα activity. In our experiments, single channel recording probably overcame this difficulty because of its molecular detection level.

Our results clearly indicate that PKC and PKA promote regulatory mechanisms of Gardos channels. Their actions may integrate signals mediated through these parallel pathways, determining the channel opening frequency. The results of concurring activations of PKC and PKA reveal that neither of the two have any precedence in order to determine the effect induced by the other, as in the reported convergent regulation of sodium channels [34]. However, while the level of activation of the Gardos channel by PKA is enhanced by the increase of $[Ca^{2+}]_i$ [13], the inhibition by PKC is progressively reduced by the same factor. Thus, if concurrently stimulated, the two signal transduction pathways are expected to balance their effects at a basal or low $[Ca^{2+}]_i$, whereas at high $[Ca^{2+}]_i$ the activation by PKA should prevail. This unbalance might be critical in determining elimination of senescent red blood cells, in which an age-dependent decrease of PKC activity has been reported [35].

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