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Characterization of a Calcium-Activated Potassium Channel from Rabbit Intestinal Smooth Muscle Incorporated into Planar Bilayers

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Summary. Interaction of vesicles from a microsomal fraction of rabbit intestinal smooth muscle with planar bilayers promotes the incorporation of a large conductance potassium-selective channel. The channel conductance fluctuates between two states: closed and open and the fraction of time the channel dwells in the open state is a function of the electric potential difference and the calcium concentrations. This channel seems to correspond to a Ca-activated K channel described by other authors in smooth muscle cells with the patch-clamp technique. Single-channel conductance is a saturating function of the potassium concentration. The relationship between conductance and concentration cannot be described by a hyperbolic function, suggesting multiple occupancy of the channel. The single-channel conductance is 230 pS in symmetrical 0.1 M KCl. Current is a linear function of the applied voltage in the range between −100 and +100 mV, at concentrations of 0.1 M KCl or higher. At lower concentrations, current-to-voltage curves bend symmetrically to the voltage axis. Sodium, lithium and cesium ions do not pass through the channel and the permeability for Rb is 66% that of potassium. All these alkali cations and Ca^{2+} block the channel in a voltage-dependent manner. A two-site three-barrier model on Eyring absolute reaction rate theory can account for the conduction and blocking characteristics.

Key Words smooth muscle · Ca-activated K channel · conduction · selectivity · bilayers

Introduction

Gastrointestinal smooth muscle cells do not elicit action potentials when they are in their normal ionic environment. If calcium ion concentration is above normal, action potentials can be elicited by current stimulation. Walsh and Singer [21] have carefully studied the characteristics of the action potentials in dispersed cells. They found that inward current is carried by calcium ions since the rate of rise and the maximum membrane potential displacement of the action potential are dependent on calcium ion concentrations. Sodium ions seem to carry an insignificant current. Outward current is carried by potassium ions since the rate of depolarization is decreased by TEA and barium ions, which are potassium channel blockers.

Voltage-clamp experiments on isolated smooth muscle cells have confirmed that the inward current is carried by calcium ions and revealed that the outward current is carried by potassium ions through a Ca-activated conduction system. Outward current is inhibited in the absence of inward Ca currents. In this condition some delayed potassium current remains which is evidence of a second type of potassium conductance [22].

Single-channel currents corresponding to the above-mentioned conductance systems have been demonstrated using patch-clamp recording. A Ca channel has been identified in smooth muscle cells of toad stomach [18]. Two types of K channels have been detected in rabbit jejunum smooth muscle cells: one with low conductance which is voltage dependent and insensitive to calcium [3], the other has a conductance of 200 pS and is sensitive to Ca concentration and slightly sensitive to electric potential [4].

In this paper, we report the incorporation of fragments of smooth muscle membranes into planar bilayers, as an attempt to have a more accessible system which may allow us to readily control the ionic composition of the channel environment in order to further characterize it. With this method we have detected the Ca-activated K channel and we describe its conduction and alkali ion selectivity properties. Potassium conduction was studied over a wide range of concentrations and membrane potentials. The data collected was sufficient to define a model for potassium conduction based on Eyring absolute reaction rate theory, following the approach of Hille and Schwarz [9].

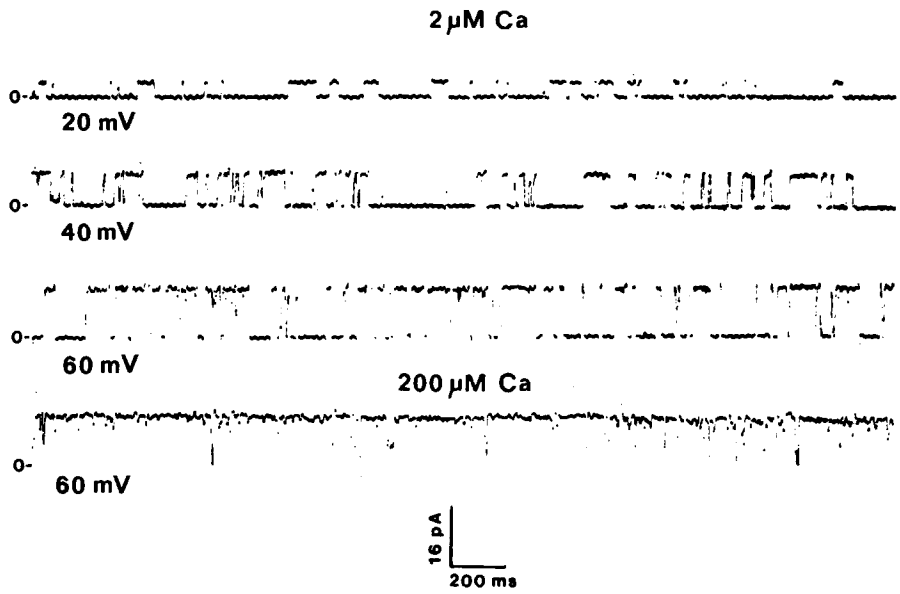


Fig. 1. Single-channel fluctuations. Experiments were made in PE bilayers with symmetrical 0.1 M KCl, 5 mM MOPS-K (pH 7.0) solutions. Bandwidth: 1 kHz. Zero-current level is indicated at the left of each trace. Upward deflections indicate channel openings

Materials and Methods

Membrane Preparation

Three buffer solutions were used in the membrane preparation procedure. Buffer 1 is a solution containing 0.15 M NaCl, 10 mM Tris-Cl, pH 7.4. Buffer 2 is a solution containing 0.75 M KCl, 10 mM Tris-Cl, pH 7.4. Buffer 3 is a solution containing 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4.

Microsomes of rabbit intestinal smooth muscle were prepared according to the following procedure: the animal was killed by a blow on the head, the abdomen was opened and the small intestine removed, rinsed and internally flushed with cold buffer 1 (4°C). The intestine was then cut in 15-cm pieces which were everted, rinsed again with buffer 1 and blotted for removal of mucus. The intestine was then cut into 2-cm pieces and 60 grams of tissue were suspended into 180 ml of buffer 1. Mucosal cells and soft components of the tissue were removed by strong mechanical vibration using a vibromixer for 2 min. Gross pieces of muscle and connective tissue were separated from the mucosal cells by passing the suspension through a Buchner funnel. The pieces were then suspended in 250 ml of buffer 2, and homogenized in a Waring Blender® at low speed for 20 sec. The homogenate was spun down at 9,000 × *g* for 30 min and the supernatant was discarded. The pellet containing dispersed cells was resuspended in 250 ml of buffer 3, and homogenized for 30 sec at 3/4 maximum speed in a tissue homogenizer (Tekmar Co., Model SLR-1810, Cincinatti, Ohio). The homogenate was then centrifuged at 15,000 × *g* for 30 min. The pellet containing intact cells, mitochondria and nuclei was discarded and solid KCl was added to the supernatant to a final concentration of 0.75 M to solubilize contractile proteins. After standing in ice for 30 min, the suspension was centrifuged at 100,000 × *g* for 1 hr. The pellet containing the microsomes was resuspended in 10 ml of a solution containing 0.25 M sucrose, 10 mM MOPS-K, pH 7.4, separated in 0.1 ml aliquots and left overnight at 2°C, to allow penetration of potassium. Next day, the aliquots were frozen in a mixture of acetone and dry ice and stored at −80°C for later use.

The microsomal fraction prepared as described contained about 10 mg/ml of proteins. 5′-nucleotidase was used as marker enzyme for plasma membrane and was concentrated about 3 times between the first homogenate and the final microsomal suspension.

Electrical Measurements

The current passing through the membrane was measured with a two-electrode voltage clamp, described in detail by Alvarez and Latorre [1]. One side of the chamber was connected to a current-to-voltage converter and the other to a waveform generator. Both connections were made using Ag/AgCl electrodes. To minimize the effect of junction potentials, the electrodes were connected through agar bridges made in 1 M KCl. The current was filtered with a low-pass active filter, amplified and stored in digital form on videotape using an encoding interface of our own design. The system allowed us to measure currents as small as 1 pA at 1 kHz bandwidth. The formation of the membrane was monitored measuring the capacitance by applying a square pulse of 10-msec duration and a frequency of 100 Hz. Membrane capacitance was about 250 to 400 pF. The sign convention for the voltage is defined once the channel is incorporated. The voltage is defined as applied on the intracellular side of the channel, which is the calcium-sensing side. The probability of either orientation was roughly 0.5.

Bilayer Formation and Channel Incorporation

Bilayers were formed according to the method of Mueller et al. [17] in a 300-μm hole made on a Teflon® partition separating two compartments of a Teflon chamber. Membrane material was a lipid solution containing 10 mg/ml brain phosphatidylethanolamine (PE) (Avanti Polar, Birmingham, Alabama) in *n*-decane (Sigma, St. Louis, Mo.). Both sides of the chamber were filled with identical KCl solutions containing 5 mM MOPS-K, pH 7.0.

Aliquots of the microsomal fraction containing 10 to 100 μg

of proteins were added to the side of the chamber connected to the waveform generator. To favor fusion, before adding the vesicles, usually the KCl concentration of this side was made 5 times more concentrated than the other side by adding an adequate volume of a 3 M KCl solution. Fusion was also favored by making the vesicle side 1 mM in $CaCl_2$.

Channel incorporation was observed as square-current fluctuations at 0 mV, current driven by the concentration difference between both sides of the bilayer. The vesicles were then washed away and both solutions made of the same composition by perfusion of the vesicle side compartment. Single-channel current was measured by applying constant potentials ranging between −100 and +100 mV, at 10-mV intervals, during 1 to 2 min.

Channel conductance was calculated from the amplitude of the current jumps associated with the opening or closing of a single channel and the applied potential. The experiments were done at different KCl concentrations to study the conductance-concentration relationship of the channel.

Gating Characteristics

The fraction of the time the channel dwells in the open state was calculated by analyzing the current fluctuations in a fast recorder using thermic paper (General Scanning, Watertown, Massachussetts). The calculation was made measuring the time the channel is in its open state and dividing by the total recording time. The study was made at different electrical potentials and calcium ion concentrations.

Selectivity Measurements

The selectivity of the channel was studied measuring the zero-current voltage when the membrane separated symmetrical KCl concentration plus some other alkali chloride at one side of the membrane. A current-voltage curve was first made in symmetrical conditions, then a known concentration of the other salt was added to one side of the chamber and the current-voltage curve was repeated. Zero-current voltages for both curves were interpolated by tracing a line connecting the data points and the difference between the two values was used to calculate a permeability ratio using the Goldman-Hodgkin-Katz equation.

Results

Channel Incorporation

When the vesicles are added to the membrane chamber, in the presence of a KCl concentration gradient across the membrane, the incorporation of channels is visualized as the sudden appearance of discrete fluctuations in the current at zero membrane potential. Most of the time several channels are incorporated at the same time. Further incorporation is reduced when the vesicles are perfused away.

After the gradient is removed by perfusion of the vesicle-containing compartment, current fluctuations can be visualized by applying an electrical potential. Figure 1 shows typical records of single-channel fluctuations. It can be observed that the time the channel dwells in the open state is both a function of the Ca^{2+} concentration and the applied potential. The effect of calcium is to increase the time the channel dwells in the open state and is exerted only from one side. The channel incorporates with its Ca-sensitive side, which is the intracellular side, facing either side of the chamber. The potential is defined as intracellular, and the extracellular side is defined as zero. The effect of the electric potential is such that the fraction of the time the channel spends in the open state increases as the potential is made more positive.

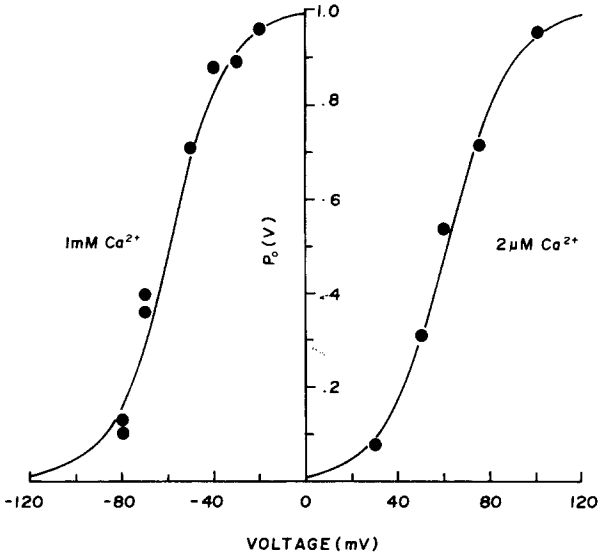


Fig. 2. Fraction of time that the channel dwells in the open state as a function of the applied voltage at two different Ca^{2+} concentrations: 2 μM and 1 mM. The experimental points were fitted to the following equation: $P_o(V) = (1 + \exp(-nF(V - V_o)/RT))^{-1}$. Both curves were obtained in the same membrane. Saline was 0.1 M KCl, 5 mM MOPS-K

The effects of Ca^{2+} and potential on the fraction of the time the channel stays in the open state ($P_o(V)$) is illustrated in Fig. 2. It is observed that the curve is shifted towards the positive potentials as the calcium concentration is reduced. The curve is displaced but does not change in shape. This behavior is similar to that described by Latorre et al. [12] for a Ca-dependent K channel from T-tubule membrane from skeletal muscle. We have not, however, studied the kinetics of the smooth muscle channel in detail because it is very difficult to obtain membranes with only one channel and when we have more than one channel, they may not show the same orientation. On the other hand the behavior of

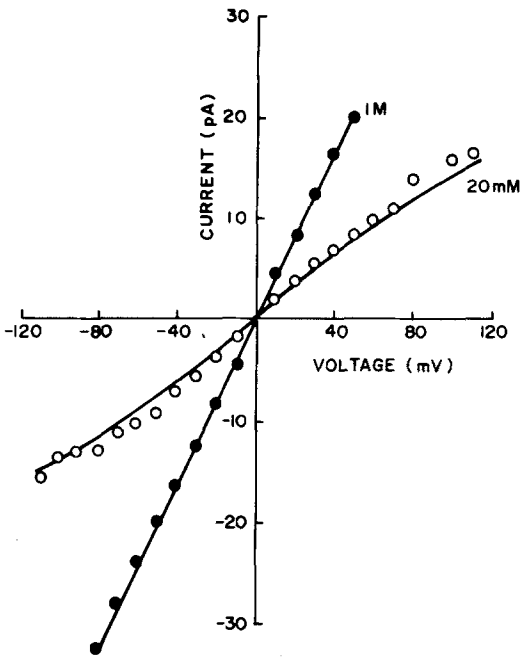


Fig. 3. Current-voltage relationship. The current through the open channel was determined by measuring the height of the current jumps at different potentials. Data were obtained in PE bilayers under symmetric KCl solutions buffered at pH 7 with MOPS-K. Solid lines represent the relation predicted by the three-barrier model. Symbols: ○, 20 mM KCl; ●, 1 M KCl

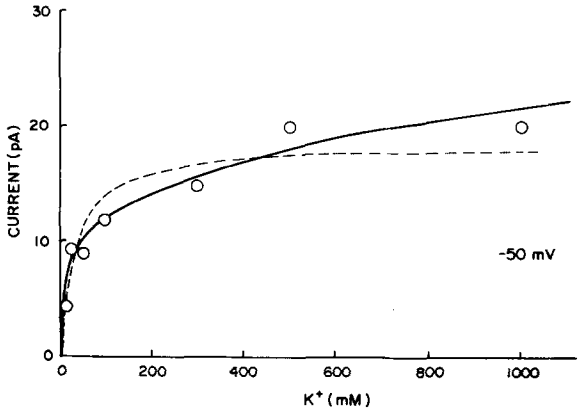


Fig. 4. Channel current at −50 mV as function of K^+ concentration. Open symbols represent experimental values in PE bilayers in the presence of symmetric KCl solutions of different concentrations buffered at pH 7 with MOPS-K. The solid line represents the relation predicted by the model. The dotted line represents a Lineweaver-Burk fit of the experimental values

the channel is not entirely stationary; we often observed sudden shifts in the probability of opening.

Channel Conductance

Channel conductance in conditions of symmetrical 100-mM KCl solutions is 230 pS. The single-channel conductance is independent of electrical potential in the −100 to +100 mV interval when the KCl concentration is 100 mM or higher. At lower KCl concentrations, the current-to-voltage curves bend symmetrically to the voltage axis. These results are shown in Fig. 3.

Channel conductance is a saturating function of salt activity, as shown in Fig. 4. The saturation of the current with ion activity suggests the presence of saturable sites inside the channel. However, it deviates markedly from a hyperbolic function, suggesting multiple ion occupancy of the channel [9].

Channel Selectivity

The current-voltage curves made in the presence of a KCl concentration difference across the membrane give reversal or zero-current potentials which correspond to a perfectly cation-selective channel. For example, the reversal potential observed when the membrane separated 500 mM KCl in the intracellular side from 100 mM KCl in the extracellular side (activities: 325 and 77 mM, respectively) was 35.2 ± 1.3 mV compared to 36.3 mV predicted by the Nernst equation.

When the membrane separated solutions containing 50 mM KCl on both sides and 50 mM RbCl on the intracellular side, a reversal potential of 13 mV was obtained. This value gives a permeability ratio P_{Rb}/P_K of 0.66. Li^+, Na^+ and Cs^+ did not produce any shift in the current-to-voltage curves when added to the intracellular side of the chamber. Because of this, it is not possible to define a selectivity sequence for the alkali ions. It is not possible either to measure single-channel currents in any of the alkali cations other than potassium.

Channel Blockade

The addition of Li, Na, Cs or Ca chlorides to the intracellular side of a channel separating symmetrical KCl solutions produced an apparent reduction in single-channel conductance which is voltage dependent: outward currents are lower than inward currents. This finding suggests that these ions have access to a region inside the channel from where they cannot go further, blocking the passage of potassium. Figure 5 illustrates the blocking effect of Na and Cs ions.

Discussion

Comparison with the Smooth Muscle Ca-Activated K Channels

Ca-activated K channels have been found in gastrointestinal smooth muscle cells by the patch-clamp technique. In cell-free patches of dispersed cells from amphibian stomach, Berger et al. [5] and Singer and Walsh [18] found a 200-pS K channel which needs the presence of intracellular calcium to open and is voltage sensitive. These channels behave very much like the one described in this paper, which was incorporated in bilayers. The blocking effect by intracellular sodium we describe in this paper was also found by Singer and Walsh [18] in toad stomach cell patches.

However, other potassium channels have been described using the patch-clamp technique. Some of them are insensitive to voltage and others are not activated by calcium [3, 5]. We have not observed these channels with the bilayer technique. It is possible that these channels are labile and are lost during the membrane preparation. It is also possible that they cannot function in solvent-containing bilayers.

Comparison with Other Ca-Activated K Channels

Many other Ca-activated K channels have been described in different tissues and all of the high-conductance channels are very similar [10].

Channel Insertion

The Ca-activated K channel from smooth muscle, described in this paper, is almost identical to the T-tubule channel described by Latorre et al. [12]. The most notorius difference refers to the orientation of the channel incorporation into the planar bilayer. While in the case of the T-tubule, channel incorporation occurs almost in 100% of the cases with the Ca receptor side facing the side of the chamber where the vesicles are added, the smooth muscle channel can be inserted with the probability in either orientation. This difference suggests that, contrary to what occurs with the T-tubule vesicles, the plasma membrane vesicles obtained from smooth muscle cells can be inside-out or inside-in. The difference can be explained on the basis of the structural difference between plasma membrane and T-tubules: the first is a sheet of membrane which can easily be thought to vesiculate in any configuration. The T-tubule, on the other hand, because of its tubular structure, is easier to vesiculate with its natural extracellular side looking to the inside.

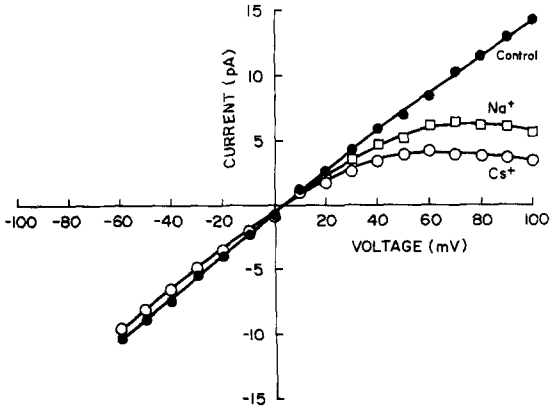


Fig. 5. Blockade of the channel by Na^+ and Cs^+ from the intracellular side. Current-voltage relationships were obtained in symmetric 50 mM KCl with 10 mM NaCl or CsCl added to the intracellular solution. Ca^{2+} concentration was 200 μM in the intracellular side

Channel Gating

The fact that the smooth muscle channel incorporated with any orientation together with the difficulty in getting single-channel membranes, makes it extremely difficult to study the gating kinetics quantitatively. However, we can say at least some things about this process:

1. The channel fluctuates only between two conductance states: open and closed. However, we usually observe long periods where the channel remains closed, interrupted by bursts of activity. This behavior indicates that there are several molecular configurations with conductance corresponding to the closed conductance state.
2. The mean open times are of the order of 10 to 100 msec.
3. Calcium and electrical potential increase the open time and decrease the closed time within a burst of activity.
4. Open-time probability changes spontaneously with time. It is also observed that open-time probability changes from membrane to membrane at the same Ca^{2+} concentration. These changes produce shifts of the $P_o(V)$ *vs.* C curves of about 50 mV at the same Ca^{2+} concentration.

All these gating characteristics are very similar to those of the Ca-activated K channels from T-tubule membranes [12] and skeletal muscle membranes [13, 14]. This suggests that we are in the

Table. Permeability ratios P_X/P_K determined from reversal potential shifts in different Ca-K channels

Ion (X)	Smooth muscle	T-tubule[1]	Chromaffin cell[2]	Cultured muscle cell[3]
K^+	1.0	1.0	1.0	1.0
Rb^+	0.66	0.7[4]	0.83	0.67
Cs^+	*	*	*	<0.05
Li^+	*	*	—	<0.02
Na^+	*	*	<0.03	<0.01

* = unmeasurable.
[1] Vergara [19].
[2] Yellen [24].
[3] Blatz and Magleby [6].
[4] Naranjo and Latorre (*unpublished results*).

presence of the same channel. This idea seems reasonable if we think that smooth and skeletal muscle cells probably have the same origin.

Channel Selectivity

The Ca-activated K channel from smooth muscle shows a striking similarity in its alkali cation selectivity with Ca-activated K channels from other preparations. The Table shows a comparison of the permeability ratios of alkali cations to K^+ in different Ca-activated K channels. It can be observed that in all of them Rb^+ is appreciably permeant; on the other hand Li^+, Na^+ and Cs^+ permeabilities are extremely low.

Conduction Mechanism

Any model proposed to describe the conduction mechanism through this channel must explain the following observations:

1. The current-to-voltage relationship is linear at higher-potassium concentrations and bends towards the voltage axis at low-potassium concentrations.

2. The channel current saturates with cation activity, suggesting saturable binding sites inside the conducting pathway. The conductance-activity function is not a hyperbola, but rather the sum of two hyperbolas. This suggests multiple ion occupancy of the channel. Blatz and Magleby [6] have found a hyperbolic behavior for the Ca-activated channel from cultured muscle cells. A hyperbolic curve was also obtained for the T-tubule channel [11] when the saline was buffered with Tris, a cation which blocks the channel [19]. This blockade originated an artifactual "Michaelis-Menten" behavior. When the experiments were repeated in solutions

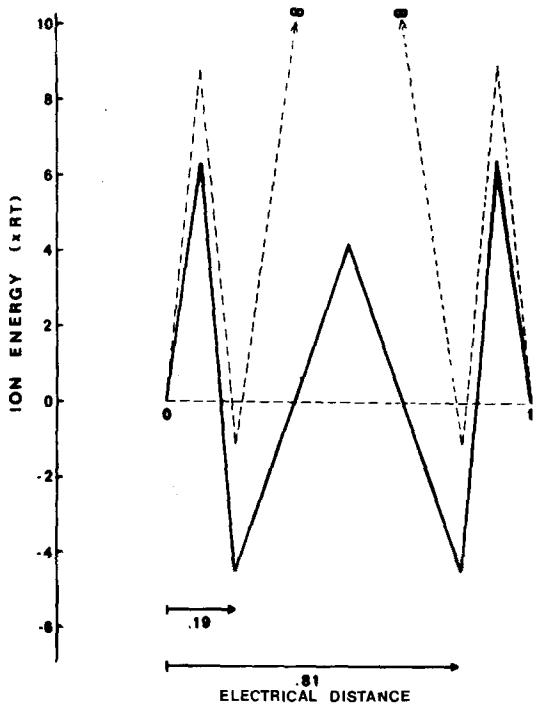


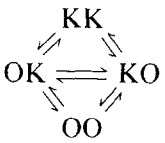
Fig. 6. Potassium energy profile inside the Ca-activated K channel. Solid lines represent the energy profile for single occupancy and dotted lines represent the energy profile for double occupancy of the channel. These values were obtained from the fitting procedure applied to experimental current-to-voltage curves, according to the three-barrier model

buffered with MOPS, the same nonhyperbolic function as in the smooth muscle channel was obtained [16, 20]. Blatz and Magleby [6] used N-methyl-D-glucosamine as buffer and it is possible that this cation also blocks the channel.

3. Other alkali cations which cannot cross the channel, interfere with the potassium transport in a way that suggests competition by saturable sites inside the channel.

In many channels, the conduction mechanism can be described with models in which the channel presents saturable binding sites where the ions bind specifically. The sites are separated by energy barriers the ion "jumps" in its pass through the channel. This kind of model has been successful in explaining the conduction and blockade of many channels, as the gramicidin channel [2], the hemocyanin channel [7], the Na channel [8] and the Torpedo electroplax Cl channel [23].

We present here a three-barrier model for the smooth muscle Ca-activated K channel. A diagram of the model with possible states of occupancy of the channel in the presence of K^+ can be represented as follows:



and was taken from Hille and Schwarz [9]. The channel can be empty (OO), with one ion (OK, KO) or with two ions (KK). We considered the following experimental findings which place constraints on the model:

1. The symmetry of the current-to-voltage curves leads to a symmetrical model. This means that the energy peaks and wells must be symmetrically located and have the same heights when viewed from either side of the membrane.

2. The shape of the conductance-activity relationship suggests more than one ion at a time inside the channel. For simplicity, we start with a two-ion model, which implies two binding sites or wells.

3. The voltage dependence of the blocking effect by other alkali ions locates the binding sites under the influence of the electric field created by the applied voltage. This means that the two binding sites must be located in the region where the voltage drops.

Once the model is stated, the expected current can be solved as a function of voltage, potassium concentration and a set of model-dependent parameters. The set of parameters that better describe the experimental currents was found using a nonlinear least-squares curve-fitting computer program. With these parameters, the shape of the current-to-voltage curves was reproduced and also the progression of the conductance with ion concentration. This can be observed as continuous lines in Figs. 3 and 4.

The values of the parameters are summarized in Fig. 6. These values show that the entry of a second K is 12 times more difficult than the first one, and that the exit of a K is 2.5 times easier when there are two potassium ions in the channel than when there is only one. These last observations explain the slope of the current-concentration curve at high potassium, but the double occupancy is so improbable that a decrease in conductance at concentrations as high as 1 M cannot be observed, as has been demonstrated for other two-ion channels [9].

Channel Blockade

Channel blocking by the nonpermeant alkali ions can be explained in terms of the model. To do this we assume that a blocking ion can enter the channel up to the first well but cannot cross the central barrier. Now, two new parameters are added: the peak height and well depth for the blocker. These two parameters can be found by adjusting the experimental current-to-voltage curves in the presence of a blocker. For cesium added to the intracellular side of the channel, we found that these two adjustable parameters are not sufficient to describe the blockade but if the position of the well for the blocker is allowed to be different from the potassium, a good fit can be found. Figure 7 shows current-to-voltage curves in the presence of three different intracellular cesium concentrations. Points are experimental results and solid lines are predicted by the model, with the position for cesium at 0.43 instead of 0.20 found for potassium when there is no blocker present.

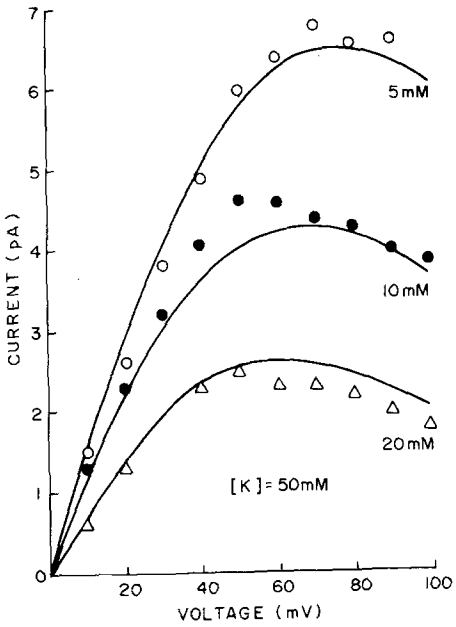


Fig. 7. Inhibition by Cs^+ from the intracellular side. Current-voltage relationships were obtained in symmetric 50 mM KCl and adding Cs^+ to the intracellular side to final concentrations of 5, 10 and 20 mM. Symbols represent experimental data; solid lines are the relationships predicted by the model

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