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Calcium- and voltage-activated potassium channels in adrenocortical cell membranes

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Current flowing through single Ca- and voltage-activated K channels has been recorded from cell-attached and inside-out excised membrane patches of cultured Y-1 adrenocortical cells. In intact cells, single-channel current amplitude and the time a channel stays in the open state increase with membrane depolarization. In excised patches bathed in symmetrical 130 mM K solutions, single-channel conductance is 170 pS. This value is constant in the membrane potential range of ±50 mV but decreases at larger hyper- and depolarizations. Channel open probability is heavily influenced by the concentration of ionic Ca at the inner surface of the membrane in the range between 0.01 and 10 μM. When internal Ca concentration is close to 0.01 μM, channels are usually closed even at large depolarizing voltages. With larger Ca concentrations, channel open probability increases and its voltage dependence is greater. These channels are uniformly distributed in the plasma membrane, since one to four channels were seen in more than 99% of the patches isolated in this study. There are previous reports suggesting a role for calcium ions in the secretory response of adrenocortical cells to ACTH. Therefore, it is possible that, as in other endocrine cells, these K channels modulate Ca influx across the plasma membrane and thus contribute to regulate steroid biosynthesis and release.

Introduction

Ca- and voltage-activated K channels with large unitary conductance have been recently identified in a variety of cell membranes [1–4]. These channels are of importance to regulate secretion in those cells that store the secretory material as cytoplasmic granules and where Ca entry through voltage-dependent channels of the plasma membrane triggers exocytosis [5]. Activation of the K channels by depolarization and by the rise of intracellular Ca causes membrane repolarization which in turn results in a decrease of further Ca influx. In this paper, we report the properties of voltage- and Ca-activated K channels found in membrane patches of Y-1 adrenocortical cells. These results give further information about Ca-dependent K permeability of cell membranes and directly demonstrate the presence of voltage- and ion-gated ionic channels in steroid-secreting cells. External Ca is needed for an adequate response of adrenocortical cells to ACTH [6], and although in these cells there appears to be no significant amount of stored steroids, it has been reported that calcium ions participate in steroidogenesis and release of newly synthesized hormone [7–9]. Secretagogues increase Ca uptake in adrenal glands [8] and induce membrane-potential changes in adrenocortical cells which are possibly mediated

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

by calcium ions [10]. As in other endocrine cells, K channels found in the present study may contribute to regulate steroid hormone secretion by modulating Ca influx across the plasma membrane.

Materials and Methods

Single-channel currents were recorded from cell-attached and inside-out excised membrane patches of cultured Y-1 cells, which are a transformed line of murine adrenocortical cells, using the patch-clamp techniques described by Hamill et al. [11]. Y-1 cells were purchased from the American Type Culture Collection (U.S.A.) and cultured in Ham's F-10 medium with 5% fetal bovine serum and antibiotics added. Cells were used for recording 2–24 h after plating in small culture dishes. Patch pipettes were fabricated by a 2 stage pull from hematocrit capillaries (Hirschmann, F.R.G.) with a vertical puller (David Kopf, model 700D, U.S.A.) and the tip fire polished under microscope control. Pipette resistance ranged between 2 and 6 MΩ. The composition of bath and pipette solutions is given in the figure legends. During the experimental protocol, culture dishes were placed on the stage of an inverted microscope (Nikon, model Diaphot, Japan) with Nomarski optics. Micropipettes were moved under visual control by means of a three-dimensional hydraulic micromanipulator (Narishige, model MO-103, Japan) and electrically connected to the headstage of a patch-clamp amplifier built in our laboratory. The indifferent electrode was a chlorided silver wire connected to ground. The patch-clamp amplifier is based on the design by Sigworth [12] and has a FET operational amplifier (Burr Brown 3523, U.S.A.) wired as a current-to-voltage converter with a 8.5 GΩ feedback resistor. Membrane potential of the isolated patch could be modified by applying a d.c. potential at the non-inverting input of the headstage amplifier. The preparation was electrically isolated by a metallic shield and placed on a heavy granite table sitting on inner tubes for shock absorption. Single-channel current and pipette potential were displayed on an oscilloscope and stored on an analog manetic tape for off-line analysis. The cut-off frequency of the recording system was about 200 Hz which results in a rise-time of approx. 2 ms. When stored recordings of single-channel currents were replayed they were low-pass filtered at 1 kHz. In all figures, outward and inward currents are respectively shown above and below zero-current level. Experiments were performed at room temperature (20–23°C).

Results and Discussion

Fig. 1 illustrates the basic properties of the large K channels as seen in situ (cell-attached conformation). After a micropipette is sealed against the membrane ($> 10^9$ Ω seal resistance), channel openings and closings appear as square steps of outward current with a fixed amplitude and variable duration. Channel openings and closings lasting less than about 2–3 ms are filtered due to the limited frequency response of the recording amplifier and appear with an amplitude smaller than the normal unitary current. However, we cannot exclude that some of these brief events actually represent partial openings and closings (see below). On membrane patch depolarization, the outward current steps increase in amplitude and duration. In the example shown in the figure, there appears to be only a channel in the patch that at a depolarization of 20 mV from the cell-resting potential stays in the closed state most of the time. At this voltage, openings are brief and last less than 100–200 ms. When the patch is depolarized by 60 mV, the duration of the openings as well as single-channel current amplitude increase. During long-lasting openings, the channel flickers between the open and closed states which results in filtered events of very short duration. Another characteristic of these channels is the presence of intermediate conductance sub-states which are indicated in the figure by arrows. When the channel is in these conformations, the current steps have smaller amplitude than when it is fully open. From a sub-state, the channel can go to the fully open or closed states which indicates that the steps of reduced amplitude are due to sub-states of the same channel and not to the opening of a different channel type. Ca-dependent K channels recorded in excised patches from embryonic rat muscle cells [2] have an intermediate conductance sub-state with a current amplitude 40% of the normal value which is usually preceded by an

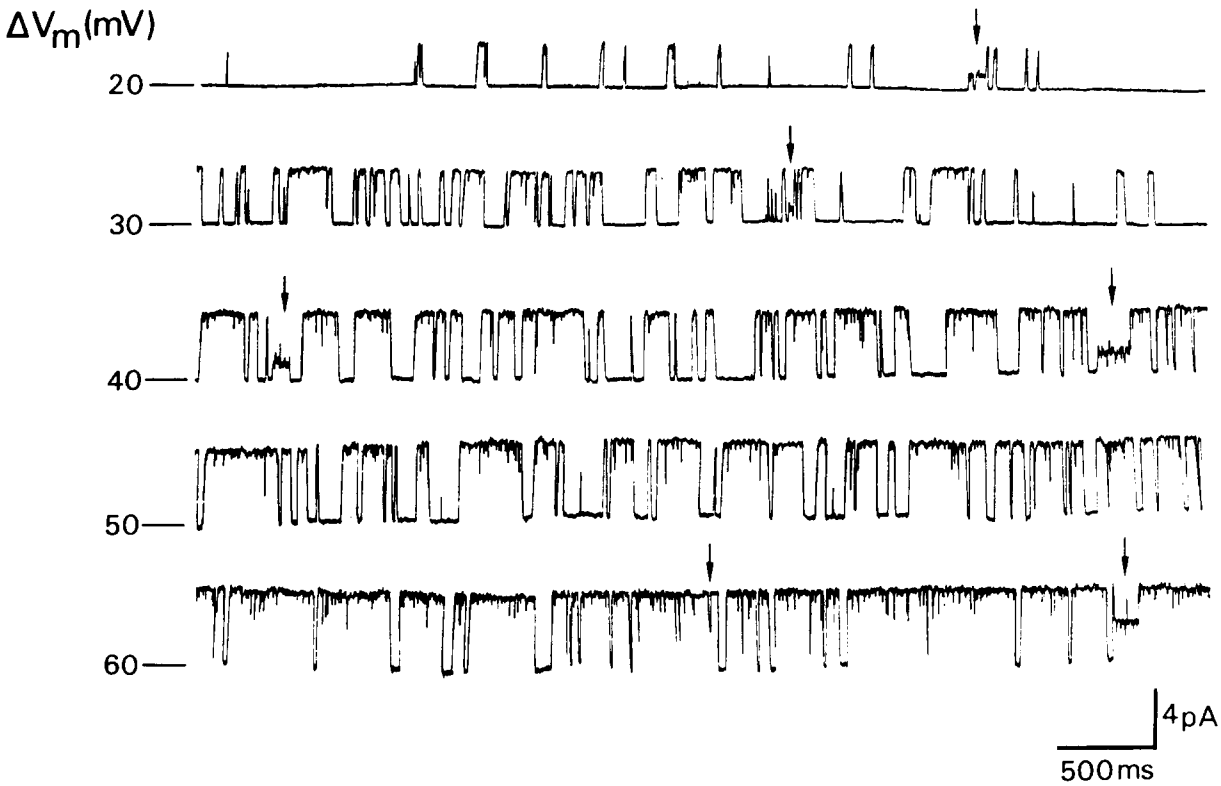

Fig. 1. Single K channel currents recorded from an adrenocortical cell membrane patch in situ. Membrane potential of the patch was changed by polarizing the pipette interior with respect to bath ground. Numbers on the left of each trace indicate, in mV, the amplitude of the depolarization from the cell-resting potential. Bars drawn close to the records indicate zero current level. Single-channel openings appear as square steps of outward current. At the resting potential, single-channel current amplitude was about 2 pA (see also Fig. 3). Depolarization activates the channel and increases single-channel current amplitude (6.8 pA at +60 mV). Arrows have been drawn over intermediate conductance sub-states of the channel. Pipette and bath solution composition was in mM: NaCl, 140; KCl, 2.8; $CaCl_2$, 1; $MgSO_4$, 2; Hepes, 10 (pH = 7.3).

open-channel state of normal conductance. The large K channels seen in Y-1 cells seem to have at least two different reduced conductance sub-states. A sub-state appears to be entered from the closed state and results in a single-channel current amplitude of about 35% of the value seen in the normal conducting state (arrows in records at 20, 30 and 40 mV in Fig. 1). Reduced currents due to a different sub-state that appears to be entered directly from the normal open state have an amplitude of about 55% of the normal unitary current (arrows in record at 60 mV in Fig. 1). We have observed these two different reduced conductance sub-states in several cell-attached and excised membrane patches; however, the number of observations is still limited and so we cannot rule out other possible intermediate conductance conformations of the channel. In addition, these channel may have sub-states of short lifetime which cannot be detected because of the limited frequency response of our recording system.

Since the single-channel currents shown in Fig. 1 can also be consistent with the opening and closing of a voltage-dependent Cl channel, this possibility has been discarded by recordings from excised patches in which the composition of external and internal solutions is known. Changing K concentration on both sides of the membrane results in single-channel currents the direction and equilibrium potentials of which are the expected for a K current. In addition, replacement of 99% chloride ions by sulfate keeps unaltered single-channel current amplitude.

To determine if K channels seen in adrenocorti-

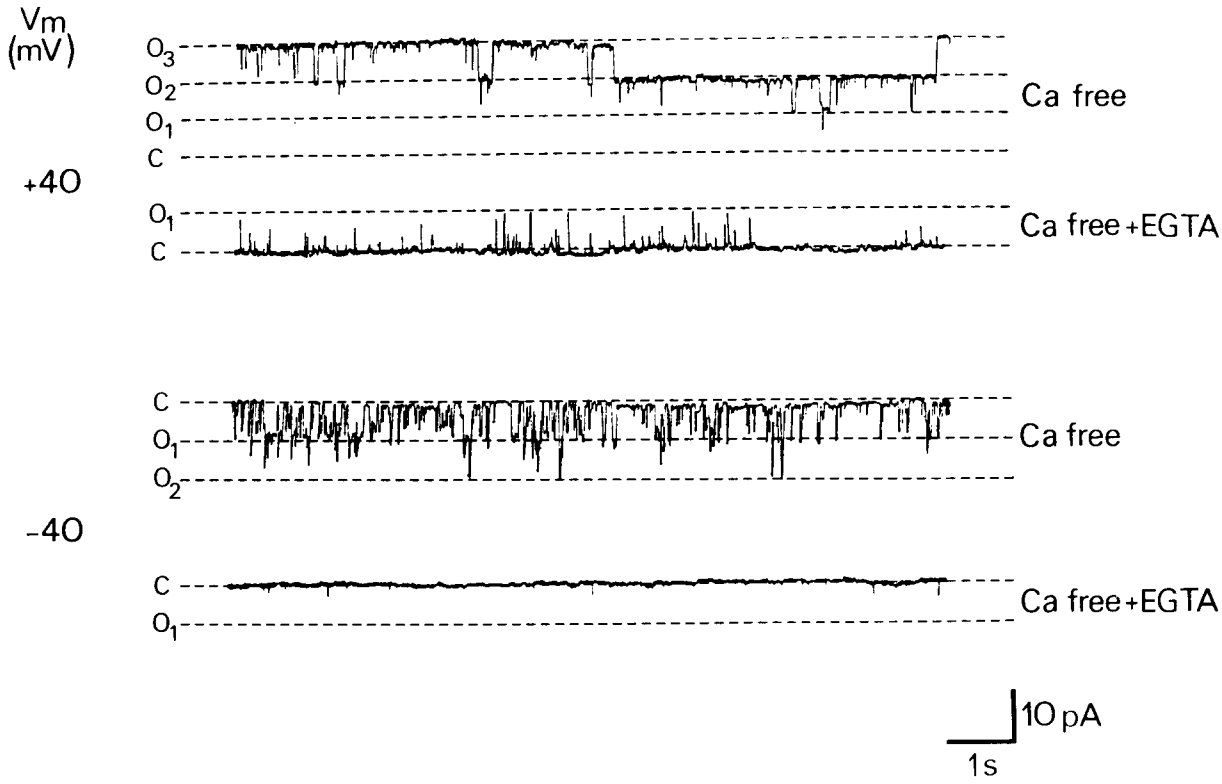

Fig. 2. Single K channel currents recorded from an inside-out excised patch in symmetrical high-K solutions. Excised patches were obtained by pulling the pipette away from the cell and brief exposure of the tip to air. Solutions were the same on both sides of the membrane and had in mM: KCl, 130; NaCl, 10; $MgSO_4$, 2; Hepes, 10 and no Ca added. pH = 7.3. In this Ca-free solution, true ionic Ca concentration was about 2–3 μM as estimated with a Ca electrode. The same patch was also exposed to an internal solution with the same composition as before but with EGTA at a final concentration of 30 μM. This last solution had an ionic Ca concentration of about 0.01 μM. At a membrane potential of +40 mV (pipette voltage negative with respect to bath ground) and with the Ca-free internal solution, the opening and closing of the three channels included in the patch appear as superimposed outward current steps of 7 pA. Removal of internal Ca by EGTA reduces the number of open channels. Successive current levels due to the simultaneous opening of 0, 1, 2, 3 channels are indicated in the figure by the lines C, O_1, O_2, O_3. When the patch membrane potential is held at −40 mV (pipette voltage positive with respect to bath ground), inward current steps of 7 pA are also seen with the Ca-free internal solution; channel open probability at this voltage is smaller than at a membrane potential of +40 mV. Reducing Ca concentration results in a nearly permanent closure of the channels.

cal cells are activated by intracellular calcium ions, we have performed experiments on inside-out excised membrane patches with variable concentrations of ionic Ca at the inner surface of the membrane. In several patches Ca, up to 1 mM, was added to the pipette solution and was without effect on the voltage dependence of channel activation and single-channel conductance. Apparently, any Ca flowing through the excised membrane patch does not seem to effectively increase Ca concentration at the inner surface of the membrane, because this ion rapidly diffuses away without activating the K channels [1,2]. By contrast, intracellular ionic Ca has a profound effect on channel activation. Fig. 2 illustrates that a decrease in internal ionic Ca concentration in the range 0.01–1 μM markedly reduces the open-state probability of the channels. All recordings of the figure are from the same excised patch and were obtained in symmetrical 130 mM K solutions. At a membrane potential of +40 mV, and with a Ca-free internal solution (no Ca added, 2–3 μM ionic Ca), the three channels of the patch are usually open and the amplitude of the outward current fluctuates between two and three discrete levels. Occasionally, as illustrated by the trace chosen for

the figure, a channel stays closed and current amplitude fluctuates between two and one discrete levels. When EGTA, at a final concentration of 30 μM, is added to the internal solution (which results in an ionic Ca concentration close to 0.01 μM), the three channels of the patch are closed most of the time and there appear only brief openings of a channel at a time. Removal of internal Ca has the same effect at a membrane potential of -40 mV. In the Ca-free solution (2–3 μM ionic Ca), inward current amplitude fluctuates between zero and one discrete level; the opening of a second channel only appears sporadically. At this membrane potential, the probability for channel opening is smaller than at $+40$ mV, which demonstrates that in excised patches, the voltage dependence of channel activation remains unaltered. When the membrane patch is held at -40 mV, the replacement of the Ca-free solution by the one with EGTA has a drastic effect on channel open probability; the three channels of the patch stay closed and openings, if any, are undetectable.

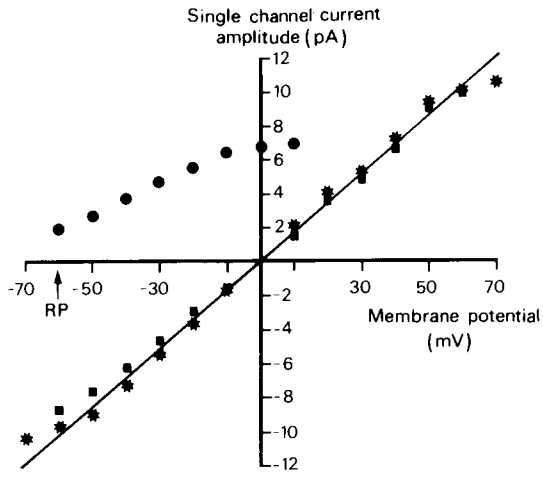

Fig. 3. Single-channel current amplitude as a function of membrane potential. In cell-attached patches (circles), single-channel current increases with depolarization although the *I-V* curve shows non-linearities. In excised patches (other symbols), with symmetrical high-K solutions, the *I-V* curve is linear in the membrane potential range of ± 50 mV. The slope of the eye-fitted straight line gives a value of 170 pS for the single-channel conductance. Current amplitude increase saturates at large positive and negative voltages. The equilibrium potential is close to 0 mV. Current amplitude and single-channel conductance are independent of Ca concentration at the inner surface of the membrane (squares, 2–3 μM; stars, 50 μM). Solutions were the same as in Figs. 1 and 2.

Fig. 3 is a plot of single-channel current amplitude as a function of membrane potential. The *I-V* curve obtained from a patch in situ (circles) has been drawn assuming a cell-resting potential of -60 mV, which is close to the resting-potential values obtained by us in Y-1 cells with intracellular microelectrodes (Tabares, L. and López-Barneo, J., unpublished observations). In the membrane potential range between -30 and -10 mV, single-channel conductance, as calculated by the slope of the curve, is 85 pS; however, this value decreases at membrane potentials close to 0 mV and to the cell-resting potential. In excised patches, there are no appreciable differences between the *I-V* curves when the internal solution has an ionic Ca concentration of 50 μM (stars) and 2–3 μM (squares). In the voltage range between ± 50 mV, the points can be well fitted by a straight line with a slope of 170 pS. Single-channel conductance decreases by hyper- and depolarizations above 50 mV.

Fig. 4 summarizes the effect of membrane potential and intracellular ionic Ca on channel open-state probability. Circles are values calculated from single-channel currents recorded in situ. The points have been drawn assuming a cell-resting potential of -60 mV (see above) and clearly show the voltage dependence of channel activation. The percent of time a channel stays in the open state increases e-fold by a positive change of 10 mV in the membrane potential. The other symbols are data points obtained from excised patches. In all cases, depolarization increases channel open-state probability, although this effect is heavily influenced by internal ionic Ca concentration. In the presence of high Ca (50 μM, stars), channels are usually open over a broad range of membrane potentials; open-state probability decreases at potentials below -40 mV. By contrast, in a low internal Ca solution (approx. 0.01 μM, triangles), channel open probability is close to zero even at large depolarizing voltages. In several patches, we have observed that with an internal Ca concentration above 0.5 mM, the voltage dependence of channel activation is nearly lost. The curve obtained for channel open-state probability as a function of membrane potential in the cell-attached conformation is intermediate between the

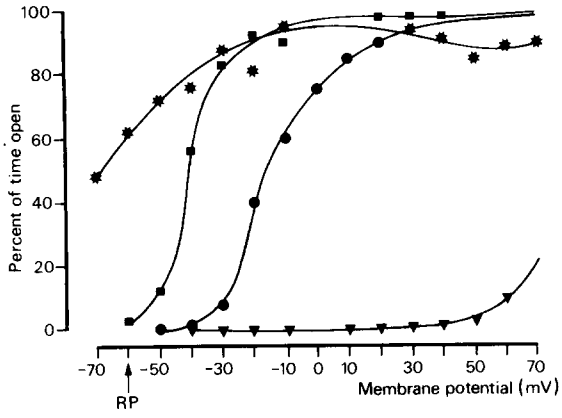

Fig. 4. Percent of time a channel is open as a function of membrane potential and intracellular ionic Ca concentration. Data points have been obtained from cell-attached (circles) and inside-out excised (other symbols) patches. Lines drawn over the points have been fitted by eye. All measurements were done by hand from continuous recordings lasting 40–60 s displayed on the screen of a storage oscilloscope; therefore, brief openings and closures may have been underestimated. Data points belonging to cell-attached recordings have been plotted assuming a cell-resting potential of −60 mV. At this membrane voltage channel open probability is near zero and increases very steeply between −30 and 0 mV. In excised patches, the time a channel stays in the open state is also a function of membrane potential but the voltage dependence of channel activation is affected by ionic Ca concentration at the inner surface of the membrane. With 50 μM Ca (stars), channel open probability is close to 1 in a wide range of membrane potentials. By contrast, with a Ca concentration of approx. 0.01 μM (triangles), channels are usually closed at the same membrane voltages. When internal Ca concentration is about 2–3 μM (squares), the curve is intermediate between the ones obtained with 50 μM Ca and in the cell-attached conformation. Solution composition was the same as in Figs. 1 and 2.

ones obtained in excised patches with internal Ca concentrations of 2–3 and 0.01 μM. Therefore, it could be inferred that the intracellular ionic Ca concentration in Y-1 cells is close to 0.1 μM, which is a value not far from estimates in other vertebrate cells [2,5]. However, it must be kept in mind that in cell-attached patches, depolarization may also indirectly activate the K channels by increasing Ca influx across the plasma membrane and/or inducing Ca release from intracellular stores, since any of these events would result in an elevation of cytosolic Ca. With internal Ca concentrations above 50 μM, we have repeatedly observed a decrease of channel open-state probability at large depolarizations. This effect may be due to channel inactivation induced by the simultaneous action of membrane depolarization and calcium ions.

Ca-activated K channels are uniformly distributed over the plasma membrane of Y-1 cells since one to four channels were present in more than 99% of the patches isolated for this study. We have seen other ionic channel types in Y-1 cell membranes the properties of which are being studied [13]. These other channels are difficult to identify unless the large K channels are blocked or their activation inhibited by internal Ca concentrations below 0.01 μM.

The present work demonstrates the presence in adrenocortical cell membranes of K channels with large unitary conductance activated by membrane depolarization and intracellular ionic Ca. Most properties of these channels are similar to those reported for K channels identified in chromaffin [1] and GH_3 anterior pituitary [3] cells as well as in other tissues [5], which indicates the wide distribution among vertebrate cells of this membrane conductance. In the endocrine cells referred to above, Ca- and voltage-activated K channels contribute to regulate Ca entry required for 'stimulus-release coupling' [5]; therefore, it is conceivable that in Y-1 adrenocortical cells, which secrete glucocorticoids in response to ACTH [14], the K channels described here also participate in the regulation of steroid secretion. It is well established that external Ca is needed for optimal steroid production and output in response to ACTH [6,7,15] and that secretagogues increase Ca uptake and induce Ca-mediated membrane potential changes in adrenocortical cells [8,10]. Although in these cells stimulation primarily activates biosynthesis and there is no significant amount of stored hormones, Ca influx across the plasma membrane may be involved in both release of newly synthesized hormone and activation of steroid biosynthesis. While some steroids are released by simple diffusion across the lipid phase of the membrane, those molecules having several hydroxyl groups follow more complex releasing kinetics [9]. It has been suggested that these compounds are associated to a steroid-containing intracellular organelle and are secreted by exocytosis [9,16,17]. In cultured adrenocortical cells, ACTH induces a rearrangement of microtubules and mi-

crofilaments which seems to be important for organelle association as well as cholesterol mobilization and transport to mitochondria required for steroidogenesis [14,18,19]. Ca influx can possibly participate in these events since local ionic Ca concentration is an important signal determining cytoskeleton organization [20].

In summary, changes in intracellular ionic Ca concentration are probably involved in the secretory response of adrenocortical cells to ACTH. These cells have voltage- and Ca-activated K channels uniformly distributed in the plasma membrane. As in other endocrine cells, these channels may serve as a link between internal ionic Ca and membrane voltage-dependent ionic conductances and therefore contribute to the regulation of steroid biosynthesis and release.

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