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Calcium and voltage dependence of single Ca^{2+}-activated K^+ channels from cultured hippocampal neurons of rat

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Calcium and voltage dependence of the Ca^{2+}-activated K^+ channel, K(Ca), was studied at the single-channel level in cultured hippocampal neurons from rat. The K(Ca) channel has approx. 220 pS conductance in symmetrical 150 mM K^+, and is gated both by voltage and by Ca^{2+} ions. For a fixed Ca^{2+} concentration at the inner membrane surface, $[Ca]_i$, channel open probability, P_o, increases e-fold for 14 mV positive change in membrane potential. At a fixed membrane potential (0 mV), channel activity is first observed at $[Ca]_i = 10^{-6}$ M, and increases with Ca^{2+} concentration approximating an adsorption isotherm with power 1.4. The $[Ca]_i$ required to half activate ($P_o = 0.5$) the channel is $4 \cdot 10^{-6}$ M. When compared to other preparations, the K(Ca) channel from hippocampal neurons reported here shows the lowest Ca^{2+} sensitivity and the highest voltage sensitivity. These findings are interpreted in evolutionary terms.

Introduction

Ca^{2+}-activated K currents have been described in a large number of preparations (Meech (Ref. 26, review), Adams et al. [1], Brown and Griffith [7], Romey and Lazdunski [29], Thomas [32], Deitmer and Eckert [8], Pennefather et al. [28], Zbicz and Weight [36]). In excitable cells in vivo, these K currents are thought to be activated by Ca^{2+} ions entering the cell during the rising phase of the action potential, and help to repolarize the cell membrane after the action potential (Thompson [33], Adams et al. [1]). Evidence supporting this hypothesis is the finding that intracellular injection of Ca^{2+} ions indeed activates K conductance (Brown and Brown [6], Gorman and Thomas [12], Adams et al. [1]), and that Ca^{2+}-channel blockers such as Cd^{2+}, and Ca chelating agents (BAPTA) prolong the duration of the action potential (Lancaster and Nicoll [16]).

More recently, the ionic channels that underly this conductance have been observed at the single-channel level, and their sensitivity to voltage and $[Ca]_i$ have been studied (Marty [21], Barrett et al. [2], Latorre et al. [17], Wong et al. [35], Maruyama et al. [23,24], Gallin [11], Ewald et al. [9], Benham et al. [3], McCann and Welsh [25], Smart [31]). Although these channels share several common features such as a high conductance, K^+ selectivity, voltage sensitivity, and dependence on $[Ca]_i$, a considerable variation exists from preparation to preparation.

This paper extends the investigation of the K(Ca) channel to mammalian hippocampal neurons, and reports on a study at the single-channel level on the voltage and $[Ca]_i$ dependence of the K(Ca) channel activity in this preparation.

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Methods

Experiments were carried out on hippocampal neurons dissected from 18-d-old rat embryos, and plated on collagen/polylysine-coated culture dishes containing basic tissue culture medium (N5) supplemented with a fraction of horse serum (Kaufman and Barrett [15]). Neurons were kept in culture for 2–4 weeks before the experiment.

The patch clamp method (Hamill et al. [13]) was used to record single-channel currents in the inside-out configuration. A homemade patch clamp amplifier was used for voltage clamping and current amplification. Single channel currents were filtered at 0.5 kHz ($\approx$ 3 dB), digitized at 400 μs sampling rate, and analyzed with a laboratory computer system (Nova 4, Data General). Single-channel records were taken in steady-state conditions attained by stepping the membrane voltage to the desired level, and maintaining that value for tens of seconds to minutes. Recording began several seconds after the step potential, to allow relaxation processes subsequent to the voltage step to reach completion.

Current amplitude histograms were constructed from 20–120 s stretches of current records, and used to measure single-channel current and channel open probability, P_o. Single-channel current was measured as interpeak distance, and channel open probability calculated from the integral areas of the amplitude histogram peaks. If a membrane patch had only one active channel, amplitude histograms produced a maximum of two peaks, corresponding to closed- and open-channel levels. In this case, the open probability is given by the ratio between the area of the open-channel peak, A_o, and the sum of the areas of the two (closed- and open-channel) peaks, $(A_c + A_o)$. When the patch contained two active channels, amplitude histograms had a maximum of three peaks (see Figs. 2B and 3B), corresponding to: (1) both channels closed (peak centered at 0; relative area A_c); (2) either channel open (next on the right; relative area $A_{o,1}$); (3) both channels open (rightmost; relative area $A_{o,2}$). Assuming the two channels to be independent in their gating, the following relations hold,

$$A_c = (1-P_o)^2 \quad A_{o,1} = 2P_o(1-P_o) \quad A_{o,2} = P_o^2$$

where P_o is the probability of an individual channel being in its open state. This probability can be directly measured from the relative areas of the open-channel peaks, according to the equation

$$P_o = (A_{o,1} + 2A_{o,2})/2$$

If a membrane patch contained more than two channels (not an infrequent occurrence), this type of analysis was not carried out, and the experiment discarded.

Intra- and extracellular solutions contained 150 mM of either NaCl or KCl, 2 mM Mops, 1 mM EGTA, and a varied amount of $CaCl_2$ to obtain the desired level of free Ca^{2+}. With 1 mM EGTA the desired free Ca^{2+} concentration and the corresponding amount of $CaCl_2$ in the solution were calculated using the stability constants for the binding of Ca^{2+} and H^+ to EGTA as described by Barrett et al. [2]. The solutions were adjusted to pH 7.3. Effective change of test solutions at the cytoplasmic surface of the patch in the inside-out configuration was obtained with a perfusion system similar to that described in Franciolini and Nonner [10]. Experiments were carried out at 22–25°C.

Results

Ca^{2+}-activated K^+ channels were identified primarily by their characteristic large conductance (approx. 220 pS in symmetrical 150 mM KCl; Fig. 1), and by their sensitivity to voltage and $[Ca]_i$ (Figs. 2–5). Other criteria include complex kinetics with channel openings typically occurring in bursts that lasted milliseconds, and containing many short closures typical of the activity of K(Ca) channels seen in other preparations (see for instance Magleby and Pallotta [19,20]). Membrane patches, obtained with 3–5 MΩ-resistance pipettes (thus an estimated free membrane patch area of about 5 μm^2 (Sakmann and Neher [30])) usually contained 2–4 active K(Ca) channels.

Fig. 1 plots single-channel current amplitude against membrane potential from two typical experiments. Single-channel conductance is 220 pS in symmetrical 150 mM KCl, and the channel does not rectify within the voltage range studied (from +60 to −60 mV). Typical single-channel records in these conditions are shown in the inset.

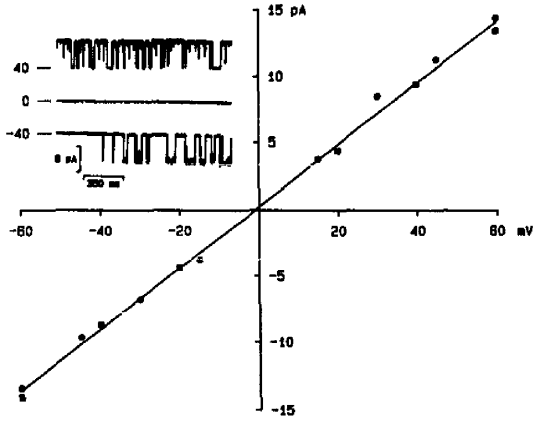

Fig. 1. Single-channel current-voltage relation of the K(Ca) channel. The membrane patch in the inside out configuration was bathed with symmetrical 150 mM KCl. $[Ca]_i$ was $4 \cdot 10^{-6}$ M. Results from two different experiments are shown, and the line fitted to the data points gives a slope conductance of approx. 220 pS. Inset: Examples of current records at different voltages, as used to construct the current-voltage plots.

Fig. 2 shows the voltage dependence of the K(Ca) channel activity in an excised inside-out patch. The membrane patch was bathed with 150 mM KCl at the cytoplasmic side, and 150 mM NaCl extracellularly (150-KCl//150-NaCl). Single-channel currents, recorded at different voltages and fixed $[Ca]_i$ (10^{-5} M), are shown in Fig. 2A. A maximum of three equidistant current levels in the recordings indicate the presence in the membrane patch of two active K(Ca) channels. The decrease in single-channel current with hyperpolarization is a consequence of a reduction in the driving force for outward K^+ ions, and eventually makes the current unmeasurable at -60 mV. In these bi-ionic conditions (150-KCl//150-NaCl), currents are outward at all the voltages shown. Na^+-supported inward currents were never observed even at very negative voltages (-80 mV, not shown). A reversal potential for these large conductance K channels could not be determined, indicating that Na^+ ions are not measurably permeant through this K(Ca) channel.

As the patch is depolarized, the channels open more frequently, and double openings can be seen (Fig. 2A) reflecting an increase in open probability. This increase in channel open probability upon depolarization is better illustrated in Fig. 2B, which shows current amplitude histograms at different voltages. These histograms were constructed from long stretches of single-channel recordings including the segments shown in Fig. 2A. The direct relation between P_o and voltage is indicated by the increase of the cumulative area of open-state peaks (both single- and double-openings) with depolarization. At -60 mV, only the closed-state peak (centered at zero; both cannels closed) is present in the amplitude histogram. The absence of peaks corresponding to open states (either single or double), however, is not proof that the two channels present in the patch never open at -60 mV. Since at this voltage K^+ ions are close to the K equilibrium potential, even if the two channels opened no measurable current would flow. By extrapolating single-channel current (interpeak distance in Fig. 2B) to -60 mV, a value lower than 0.3 pA would be obtained, which is not resolvable with our recording conditions. Upon depolarizing the membrane patch to -45 mV, a second peak appears that corresponds to one channel open. Double openings do not occur measurably at this potential, as indicated by the absence of a third peak in the amplitude histogram. Further depolarization to -30, -15, and 0 mV produces a third peak (just visible at -30 mV) which indicates the occurrence of double openings, as a result of the higher channel open probability. The widening of interpeak distance (as result of larger single-channel current) is a direct results of the increase in K driving force.

Fig. 3 illustrates the dependence of K(Ca) channel activity on $[Ca]_i$. A and B show, respectively, single-channel currents and the corresponding amplitude histograms recorded at different $[Ca^{2+}]_i$ (indicated). The membrane potential was 0 mV. At $4 \cdot 10^{-7}$ M $[Ca]_i$, virtually no openings are observed in the current trace (Fig. 3A), and only the peak corresponding to the closed state (centered at zero) can be seen in the corresponding amplitude histogram accumulated over 46 s. Openings appear sporadically at 10^{-6} M $[Ca]_i$, and more frequently at the elevated $[Ca]_i$. Double openings are observed at high $[Ca]_i$, where the open probability is high. The top trace, taken at physiologically extreme $[Ca]_i$ ($2 \cdot 10^{-5}$ M), was selected to show some of the long closed intervals that occasionally appeared at this Ca^{2+} concentra-

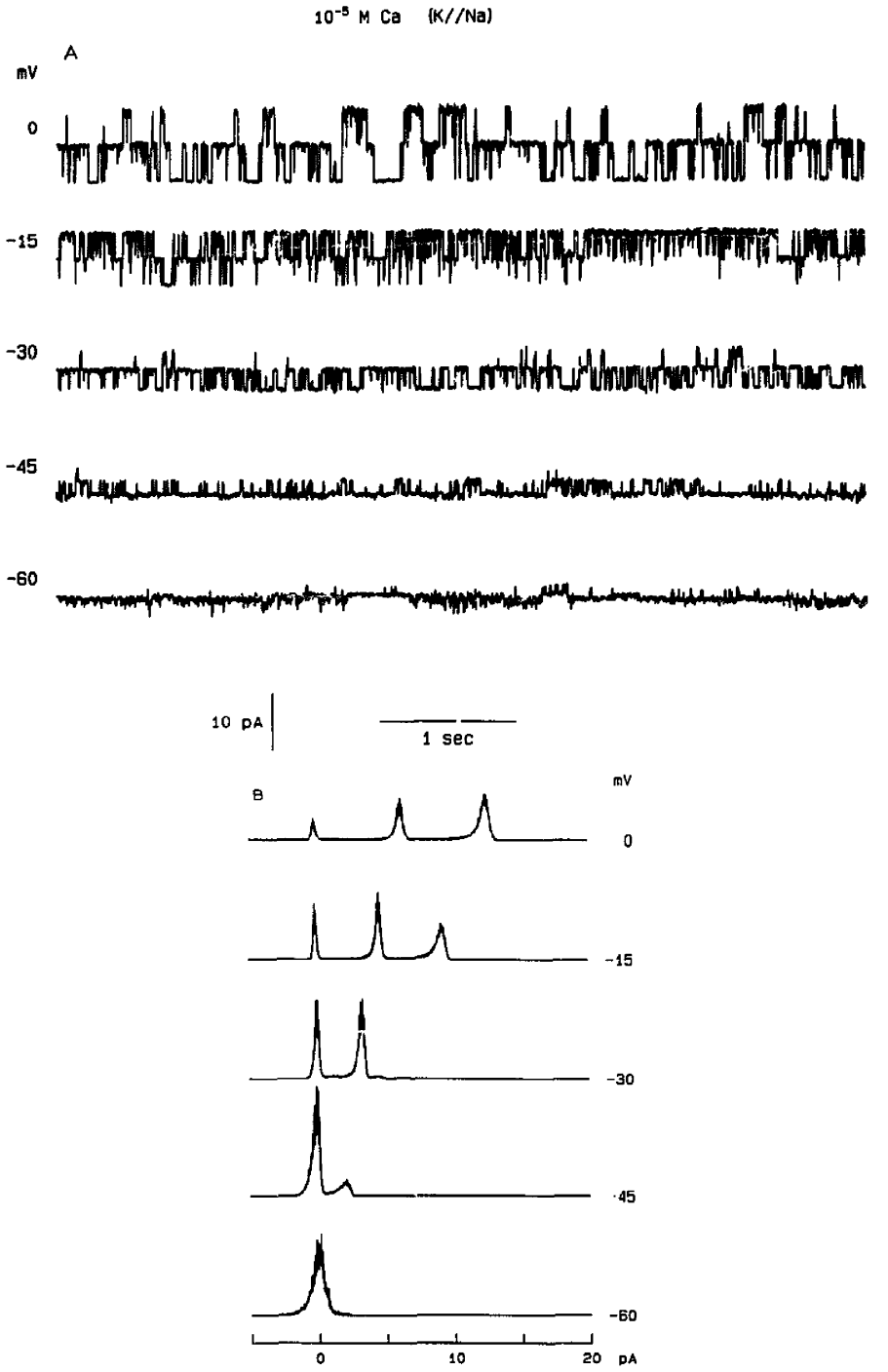

Fig. 2. Voltage dependence of the K(Ca) channel activity in an excised, inside-out patch of somatic membrane from hippocampal neurons of rat. (A) Segments of single-channel records showing K^+ currents through K(Ca) channels at varying (indicated) membrane potentials. Perfusing conditions were 150-KCl//150-NaCl. $[Ca]_i$ was 10^{-5} M. A maximum of two levels of openings in the segments shown indicate the presence in the patch membrane of two active channels. (B) Amplitude histograms derived from 20–120 s of single-channel activity. The corresponding segments in (A) contributed in part to the histograms. The measured currents, sampled every 400 μs, were binned at 0.04 pA resolution, and the number of observations in each bin plotted against the current range for each bin.

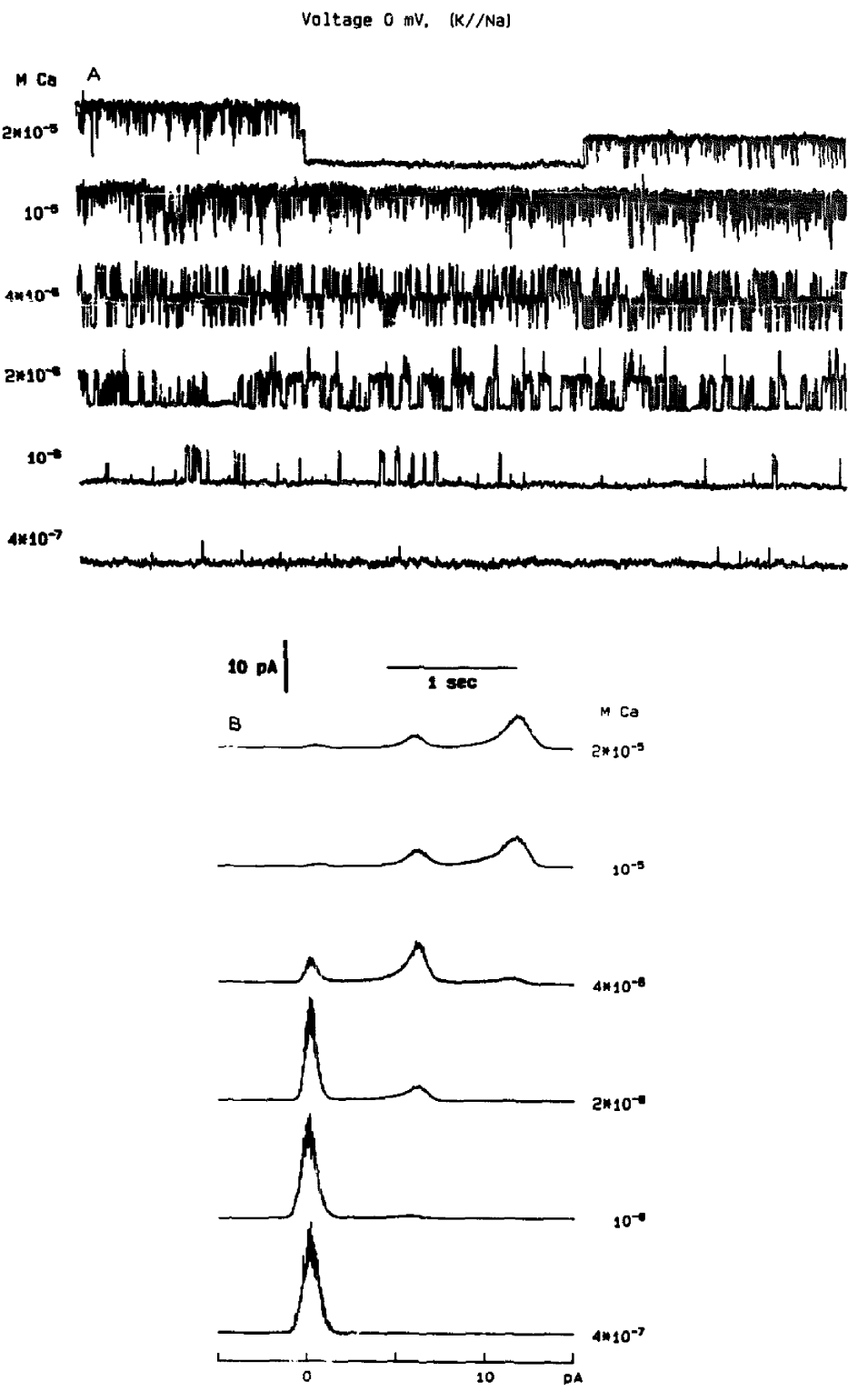

Fig. 3. Dependence of the K(Ca) channel activity on $[Ca]_i$. (A) Segments of single-channel records showing K^+ currents through K(Ca) channels at varying $[Ca]_i$. Perfusing condition for this membrane patch in the inside-out configuration was 150-KCl//150-NaCl. Membrane potential was 0 mV. Again, two levels of openings indicate the presence in the patch of two active channels. (B) Amplitude histograms obtained as described in the legend to Fig. 2B.

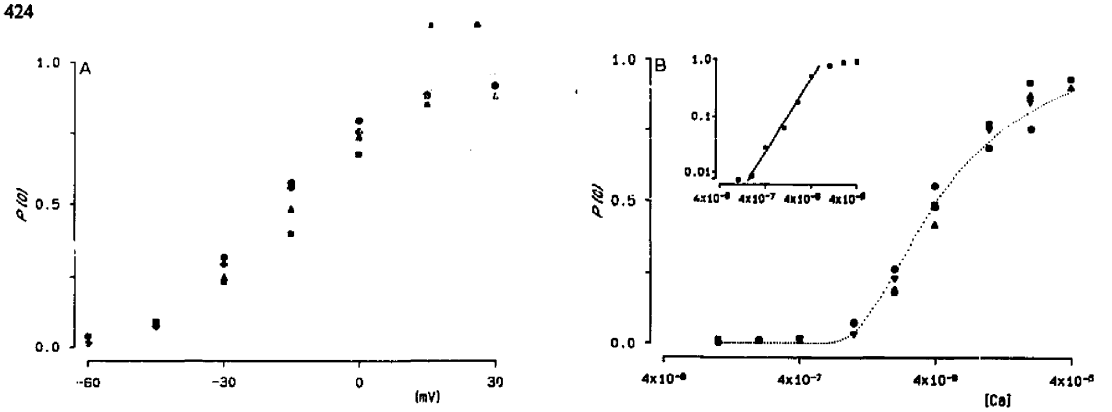

Fig. 4. Quantitative analysis of voltage and $[Ca]_i$ dependence of the K(Ca) channel activity. (A) Plot of the channel open probability, P_o, as function of membrane potential. $[Ca]_i = 10^{-5}$ M. Different symbols are from separate experiments. The dotted curve is a fit for the experimental points according to the equation $P_o = (1 + \exp[nF(V - V_o)/RT])^{-1}$, with $n = 1.75$, and V_o, the voltage at which $P_o = 0.5$, equal to -15 mV. (B) Plot of channel open probability, P_o, as function of $[Ca]_i$. Membrane potential, 0 mV. Different symbols are from separate experiments. The dotted curve is the fit to experimental data with the equation $P_o = (1/(1 + K_d/[Ca]))^n$, with $n = 1.4$, and $K_d = 4 \cdot 10^{-6}$ M. Inset: plot of the same data (mean values) on logarithmic co-ordinates. Solid line indicates a slope of 1.28.

tion. Similar long closures have been reported in the literature, and interpreted as reflecting an inactivated state of the K(Ca) channel (Barrett et al. [2]), or as a blockade of the channel by high internal Ca^{2+} (Marty [21]; see also Vergara and Latorre [34] and Benham et al. [3]).

A quantitative analysis of the voltage and $[Ca]_i$ dependence of K(Ca) channel activity is illustrated in Fig. 4, which plots the channel open probability as a function of membrane potential at a fixed $[Ca]_i$ (10^{-5} M). A fit of the data with a Boltzmann distribution (see figure legend) yielded the parameter $n = 1.75$. This value, interpreted as the equivalent charge that has to move across the entire membrane field to open a K(Ca) channel, shows that this channel has a considerably smaller voltage dependence than the Na^+ channel (n = ca. 6), or the delayed rectifyer K^+ channel (n = approx. 4.5) (Hodgkin and Huxley [14]). The open probability of the K(Ca) channel increases e-fold for each 14 mV positive change in membrane potential.

Fig. 4B plots the open probability as a function of $[Ca]_i$, at a fixed membrane voltage (0 mV). Open probabilities at low $[Ca]_i$ are consistent with a Hill coefficient of approx. 1.3 (see inset), and data over the entire $[Ca]_i$ range are also approximated by the 1.4 power of an adsorption isotherm (dotted curve; see legend to Fig. 4 for details). $[Ca]_i$ required to half-activate ($P_o = 0.5$) the channel is $4 \cdot 10^{-6}$ M (0 mV).

Discussion

This paper reports on a study of the voltage and $[Ca]_i$ sensitivity of the K(Ca) channel in excised membrane patches of cultured hippocampal neurons from rat. In symmetrical 150 mM KCl, single-channel conductance is 220 pS, which is similar to other large conductance K(Ca) channels (180 pS, Marty [21]; 200 pS, Benham et al. [3]; and Smart [31]; 210 pS, Wong et al. [35]; 220 pS, Barrett et al. [2]) but much larger than the conductance of the K(Ca) channels reported on *Helix* neurons (19 pS, Lux et al. [18]; 40–60 pS, Edwald et al. [9]) and on cultured skeletal muscle (10–14 pS, Blatz and Magleby [5]). Based on the large conductance as well as on the $[Ca]_i$ and voltage sensitivity, this channel can be classified as a BK channel (Marty [22]).

The activity of the channel is a function of both membrane potential and $[Ca]_i$. At any given $[Ca]_i$, channel activity can be modulated by varying the membrane potential. The channel is preferentially active at depolarized voltages. The percent of time the channel spends in the open state increases with a voltage dependence of 14 mV per e-fold change of P_o (Fig. 4A). This voltage sensitivity is

nearly twice as strong as that reported for the macroscopic Ca^{2+}-activated K^+ currents in *Aplysia* neurons (25 mV per e-fold change in K^+ conductance: Gorman and Thomas [12], or for single K(Ca) channels of clonal anterior pituitary cells and smooth muscle cells (28 and 30 mV, respectively: Wong et al. [35] and Benham et al. [3]). The voltage sensitivity, however, is closer to that reported for K(Ca) channels from *Helix* neurons (20–23 mV: Edwald et al. [9]), and rat muscle fibers (15 mV: Barrett et al. [2]).

The voltage sensitivities reported above were measured at different $[Ca]_i$. Whereas previous observations (Barrett et al. [2], Edwald et al. [9], Benham et al. [3]) have shown that an increase in $[Ca]_i$ can reduce the voltage dependence of K(Ca) channels, such effects are only observed at relatively high $[Ca]_i$, 10^{-6} M or higher. Most of the values reported above refer to the range between 10^{-8} and 10^{-6} M, and if allowance could be made for the different $[Ca]_i$ used, differences in voltage dependencies among the various preparations would tend to be increased. In fact, the highest voltage sensitivities reported (Barrett et al. [2]; this study) were obtained at the highest $[Ca]_i$. Thus, most of the variability is likely to derive from the preparation and may be indicative of different functional roles of the K(Ca) channels in cells as diverse as secretory cells, muscle fibers, and neurons.

The activity of the K(Ca) channel is also modulated by $[Ca]_i$. Activity is first observed at $[Ca]_i = 10^{-6}$ M, and increases with $[Ca]_i$ following an approximation of an adsorption isotherm distribution. The $[Ca]_i$ at which the channel is half activated ($P_o = 0.5$) is $4 \cdot 10^{-6}$ M. When compared with other data, the K(Ca) channel described in this paper falls at the lower end of the calcium sensitivity range. (To compare Ca^{2+}-sensitivities, the $[Ca]_i$ required to activate the K(Ca) channel to $P_o = 0.5$, at 0 mV, was taken). The channel shows a Ca^{2+}-sensitivity similar to the large-conductance K(Ca) channels from rat muscle fibers ($5 \cdot 10^{-6}$ M: Barrett et al. [2]), and rat sympathetic neurons ($5 \cdot 10^{-6}$ M: Smart [31]), but has a Ca^{2+}-sensitivity more than one order of magnitude smaller than that found in a number of other preparations (pituitary cells, $5 \cdot 10^{-7}$ M: Wong and Lecar [35]; pancreas acinar cells and salivary glands, $5 \cdot 10^{-7}$ and 10^{-8} M, respectively: Maruyama et al. [23 and 24]; snail neurons, $5 \cdot 10^{-8}$ M: Edwald et al. [9]; smooth muscle, $4 \cdot 10^{-7}$ M:Benham et al. [3]). The K(Ca) channels with lower Ca^{2+} sensitivity are from embryonic tissues, whereas those showing higher sensitivity are from adult tissues, suggesting that sensitivity to Ca^{2+} ions increases with development. This concept is supported by the finding that K(Ca) channels from young neurons of *Xenopus* acquire Ca^{2+} sensitivity upon maturing in culture (Blair and Dionne [4]). However, hypotheses about the cellular mechanism by which the sensitivity to Ca^{2+} can be acquired with maturation have not yet been proposed.

From the double-logarithmic plot of open probability vs. $[Ca]_i$ (Fig. 4, inset) a slope value of 1.28 is obtained, which indicates that the binding of more than one Ca^{2+} ion is required to open the channel. This result is similar to that reported by Benham et al. [3] on smooth muscle, and by Gorman and Thomas [12] on *Aplysia* neurons (macroscopic measurement). An approximation of a third power relationship has, however, been observed in snail neurons (Meech and Thomas [27], macroscopic measurements), in embryonic muscle fibers (Barrett et al. [2]), and in pituitary cells (Wong et al. [35]).

In summary, when compared to other preparations, the K(Ca) channel from rat hippocampal neurons reported here shows the strongest voltage sensitivity, and the lowest Ca (threshold) sensitivity. The slope value of approx. 1.3 from P_o vs. $[Ca]_i$ (logarithmic) relationship (Fig. 4, inset) instead, falls well within the range seen for this channel in other preparations.

Two pharmacologically distinct Ca^{2+}-activated K^+ currents, which give rise to the fast and slow after-hyperpolarizations (Ahp), have been described in several preparations (Romey and Lazdunski [29], Deitmer and Eckert [8], Pennefather et al. [28]). Studies at the single-channel level in rat muscle fibers have characterized their molecular counterparts, showing the presence of BK and SK channels that, respectively, underlie the fast and slow Ahp (Blatz and Magleby [5]). The large conductance K(Ca) channel described here thus presumably supports the Ca^{2+}-activated K^+ current that produces the fast Ahp.

In hippocampal neurons the suppression of this current (by TEA, intracellular injection of Ca^{2+} chelating agents, or blockers of Ca^{2+} channels) in addition to eliminating the fast Ahp, also increases the duration of the action potential (Lancaster and Nicoll [16]). The suppression of the fast Ahp, however, does not affect the frequency of firing in response to a sustained depolarizing pulse (Lancaster and Nicoll [16]), which led the authors to suggest that the major role of the BK channels is to shorten the time to repolarize the membrane after an action potential, and to prevent prolonged depolarizations that would be accompanied by an increase in $[Ca]_i$ (see also Romey and Lazdunski [29].

The large conductance, Ca^{2+} and voltage sensitivity of the K(Ca) channel described here are in accordance with this interpretation. Depolarization and Ca^{2+} influx that accompany an action potential would both result in an activation of these channels resulting in an outward K^+ current which would, in conjunction with the delayed rectifier K^+ channels and the A current, repolarize the membrane. (However, from the results presented here, it is not possible to say whether in vivo voltage activates the K(Ca) channels more quickly than Ca^{2+} ions, or viceversa. This paper reports in fact only data from steady-state experiments; the relevant kinetic experiments have not been done. This point ought to be kept in mind in reading the following paragraph, where an evolutionary interpretation of the results is presented.)

The finding that the K(Ca) channel from hippocampal neurons shows, when compared to the other K(Ca) channels from different preparations, the strongest voltage dependance and the weakest Ca dependence, is here tentatively interpreted in evolutionary terms. In order to obtain faster transmission of signals, structural changes were adopted which would emphasize the importance of fast stimuli such as voltage, as compared to the slower, diffusion-limited, Ca^{2+}-sustained stimuli, wherever speed of signalling was important. In this scheme, excitable tissues of evolutionary more primitive organisms (*Helix, Aplysia*) or tissues not primarily concerned with speed of signaling (smooth muscle fibers, secretory cells) being less strongly (at least in this respect) subjected to evolutionary pressure have not aquired mechanisms relevant to transmission of fast stimuli. The result may be that they depend more on Ca^{2+}-sustained signals and less on voltage than excitable tissues of vertebrates (hippocampal and sympathetic neurons, and skeletal muscle fibers) which have been adapted for fast transmission and consequently show the strongest voltage dependence and the weakest Ca^{2+} dependence.

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