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BIOPHYSICAL AND PHARMACOLOGICAL PROPERTIES OF LARGE CONDUCTANCE CA^{2+}-ACTIVATED K^+ CHANNELS IN N1E-115 CELLS

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The large conductance Ca^{2+}-activated K^+ channels in differentiated mouse neuroblastoma N1E-115 cells have been studied using patch-clamp single-channel current recording in excised membrane patches. These channels displayed a unitary conductance of 200 pS under symmetrical K^+ concentrations. Effects of blockade by TEA^+, Cs^+ and Ba^{2+} were different and argued for distinct action mechanisms. The open probability of these channels increased with increasing internal calcium and membrane potential. Maximum sensitivity of these channels ranged over physiological variations of internal calcium at membrane potentials close to zero, suggesting a physiological role for these channels in regulating the membrane potential and Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

The Ca^{2+}-activated K^+ channels -BK(Ca)- couple cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane potential to K^+ flux and membrane excitability in excitable cells including neurons (1-3). In all cells with voltage-gated Ca^{2+} channels, this link allows regulation of $[Ca^{2+}]_i$ (3). Presence of Ca^{2+} voltage-gated channels (4) and BK(Ca) channels (5, 6) was previously demonstrated on differentiated N1E-115 murine neuroblastoma cells.

The present study is the first report on the pharmacological properties of BK(Ca) channels in N1E-115 cells using single-channel recording techniques. We show that these channels are blocked by Ba^{++}, Cs^+, and TEA^+ probably through distinct action mechanisms. We describe their calcium and voltage sensitivities and suggest possible physiological roles for these channels since they are effectively controlled at membrane potentials close to zero within the $[Ca^{2+}]_i$ range of 5.10^{-7} to 10^{-6} M which includes the determinated levels of $[Ca^{2+}]_i$ in stimulated cells (7).

Materials and methods

Cell culture: N1E-115 cells were grown in 25 cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 50 $\mu g.ml^{-1}$

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gentallin. Cultures were maintained at 37° in CO_2 incubator. Cells were subcultured with no enzymatic dissociation. Cell differentiation was initiated in 35 mm Petri dishes containing DMEM supplemented with 0.5% FCS and 1.5% dimethylsulphoxide (DMSO) (8). Cells were used for experiments 8-30 days after induction of differentiation.

Patch-clamp recording: We have used the patch-clamp technique in the excised inside-out and outside-out configurations (9). Pipette resistance was typically 6 MΩ. The Ag/AgCl electrodes were connected to a patch-clamp amplifier (RK300 Bio-Logic, Claix, France) with a 100 GΩ feedback resistor. After filtering through a 5-pole Tchebitcheff low-pass filter (-3 dB at 3 kHz), data were stored on a digital tape recorder Bio-Logic DTR1200 and analyzed off-line. Chart records were displayed with a Gould Windograf chart recorder after filtering at 300 Hz and digitizing at 800 Hz. Sequences of potential pulses or ramps were generated by a programmable stimulator Bio-Logic SMP300. All experiments were performed at room temperature (20-23 °C).

Data analysis: Single channel current records were later digitized at 9600 Hz and analyzed with Biopatch (Bio-Logic software). Multigaussian adjustments of the amplitude distributions were used to determine unitary currents, stationary probabilities. The functional independence between channels was verified by comparing the observed stationary probabilities with the values calculated according to the binomial law. The number of active channels in a patch, N, was taken as the maximum number of channels simultaneously open under conditions of maximum open probability. The open probabilities, Po, were evaluated using an iterative process to minimize the chi square (χ^2) calculated with a sufficiently large number of independent observations.

Solutions and chemicals: Rapid changes in perfusion solutions could be performed by moving the patch pipette into a different perfusion stream using a multiple perfusion system. All solutions were buffered with 10 mM HEPES and adjusted to pH 7.35 with either NaOH or KOH. Solution bathing the cells contained (mM): NaCl, 150; KCl, 3; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10. Patch electrodes usually contained (mM): KCl, 145; $MgCl_2$, 2. Perfusion solutions contained (mM): KCl 145, $MgCl_2$ 2. In case of experiments with asymmetrical K^+, the excised membrane patch was exposed to a perfusion solution containing (mM): NaCl, 150; KCl, 3; $MgCl_2$, 1. When needed, and for concentration of free calcium in the submicromolar range, we used a standard EGTA/$CaCl_2$ buffer: the internal solution (perfusion solution in inside-out, pipette in outside-out) was supplemented with 1 mM EGTA and the concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was adjusted by adding an appropriate amount of $CaCl_2$ (10). DMEM and FCS were obtained from Gilco. Apamin was from Sigma, and charybdotoxin from Latoxan (Rosans, France).

Results

In cell-attached-patch configuration in differentiated N1E-115 cells, a large conductance channel was often found that was only active at strong depolarizing potentials. In inside-out configuration, this channel is sensitive to cytoplasmic application of calcium (Fig. 1A). Under symmetrical [K^+] (145 mM), the current-voltage relationship is linear between -60 and 60 mV and gives a conductance of 180-200 pS in excised patches or cell-attached configurations. The conductance is reduced to 125 pS and a slight rectification is observed under physiological [K^+] asymmetry (Fig. 1B). In all cases, the reversal potential is close to the equilibrium potential of K^+, showing the high selectivity of these channels for K^+ over Na^+.

Effects of TEA^+, Cs^+ and Ba^{++}. Current records shown in Figure 2A are from the same patch when the external membrane surface is sequentially exposed to no blocking agent, 1 mM TEACl and 1 mM CsCl. When exposed to 1 mM TEA^+, these K^+ channels display a highly reduced unitary current size (20% of its normal value). The current size is further reduced by 1 mM Cs^+. However the effects of TEA^+ and Cs^+ differ in one aspect: the open probability of the channel is clearly enhanced in the presence of Cs^+, whereas no obvious change in the gating kinetics of the channel is observed in the presence of TEA^+. These K^+ channels are more

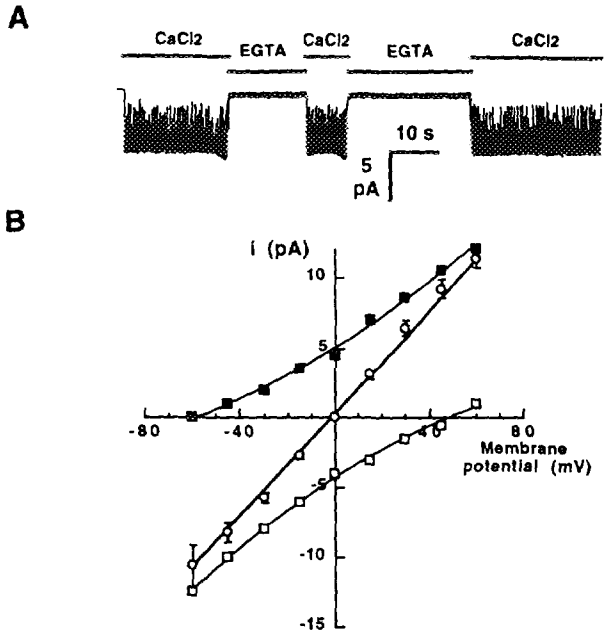

Figure 1. Ca^{2+} sensitivity and current voltage relationship of BK(Ca) channel in N1E-115 cells. **A.** The open probability of the channel follows without delay the concentration of internal calcium. Inside-out patch with 145 mM K^+ in the pipette and bathed in a solution containing 150 mM NaCl, 3 mM KCl and either 2 mM CaCl2 or 1 mM EGTA. Membrane potential: 0 mV. **B.** Current voltage relationship with asymmetrical K^+ concentrations in outside-out patch (■) $[K^+]_{in}$ / $[K^+]_{out}$ = 145 mM / 3 mM, $[Na^+]_{out}$ = 150 mM and inside-out patch (❑) $[K^+]_{in}$ / $[K^+]_{out}$ = 3 mM / 145 mM, $[Na^+]_{in}$ = 150 mM. The I/V relationships in symmetrical 145 mM K^+ (○) have been established at various $[Ca^{2+}]_i$ from the same inside-out patch shown in Figure 3A and the unitary current sizes were given as the mean values ± SD.

sensitive to application of TEA^+ on the external face than on the interior face: the current size is reduced to 50% of its normal value with 30 mM internal TEA^+ whereas a more pronounced effect is obtained with only 1 mM external TEA^+ (Fig. 2B). A slight effect of the membrane potential is also observed: the inhibitory effect of TEA^+ increased as the TEA^+-free membrane face is made more negative. Given its high conductance, its activation by intracellular calcium, its high selectivity to K^+ and its blockade by TEA^+ and Cs^+, this channel appears to belong to the BK(Ca) channel family (1). This assignment is confirmed by two other sets of experiments. In the inside-out configuration, when 2 mM Ba^{++} are applied to the cytoplasmic face of the channel, the open probability diminished as a result of long-lived closed events (Fig. 2C). The effect of Ba^{++} is more pronounced at positive voltages (outward K^+ currents) than at negative voltages. In the outside-out configuration, the present BK(Ca) channel is also blocked by externally applied 100 nM charybdotoxin, but is insensitive to 100 nM apamin (data not shown).

Regulation by Ca^{2+} and voltage. Currents through two identical BK(Ca) channels in an inside-out patch at various concentrations of free Ca^{2+} at the inner membrane surface ($[Ca^{2+}]_i$) and various membrane potentials (Vm) are shown in Figure 3A. All records were realized under the same patch. The effect of $[Ca^{2+}]_i$ and applied voltage on the channel gating is obvious. With

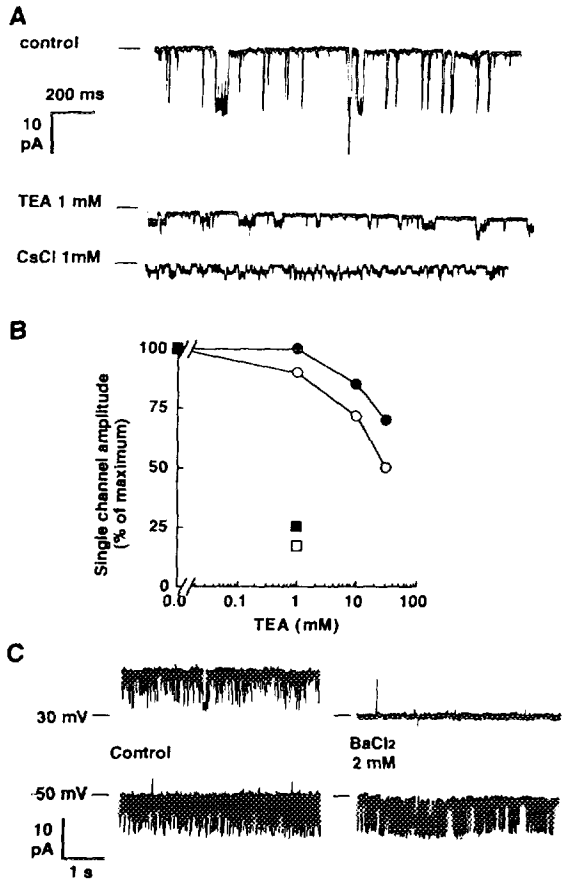

Figure 2. BK(Ca) channel blockade by different ions. **A.** Effects of external TEA^+ and Cs^+ on the unitary current amplitude. The channel in the outside-out configuration and in symmetrical 145 mM KCl was sequentially exposed to 1mM TEACl and 1 mM CsCl. Membrane potential: - 60 mV, $[Ca^{2+}]_i = 10^{-6}$ M. **B.** Dose-response curves for the inhibitory effect of TEA^+. The membrane patch, either in the inside-out (circles) or in the outside-out configuration (squares), was exposed to various concentrations of TEA^+. Single channel current size was measured in symmetrical K^+ (145 mM) and at - 60 mV (❑), -40 mV (●) or 60 mV (○, ■). Each symbol type represents data obtained from a different membrane patch. **C.** Current records from an inside-out patch in symmetrical K^+ (145 mM) and clamped at 30 mV or -50 mV were shown before and after exposure of the inner face of the channel to 2 mM $BaCl_2$. $[Ca^{2+}]_i = 5$ μM.

500 nM $[Ca^{2+}]_i$ and at negative voltage only sporadic openings occur. When $[Ca^{2+}]_i$ is increased twofold (1 μM) or when the membrane is depolarized (Vm ≥ 15 mV), the open probability enhances dramatically and double openings occur with high frequency. $[Ca^{2+}]_i$ has no effect on the unitary conductance of the channel (Fig. 1B). Long-lived nonconducting states appeared when $[Ca^{2+}]_i$ is elevated or when Vm is made more positive (Fig. 3A). This is particularly obvious at 60 mV and at pCa 5.3 where stays in the silent state are as frequent as stays in the active state. A statistical study of the duration of silence and burst periods is not valid since our records are too short to include enough events. In these records, the estimation of the open probabilities meets two difficulties: a putative cooperative behavior between these two channels and the existence of

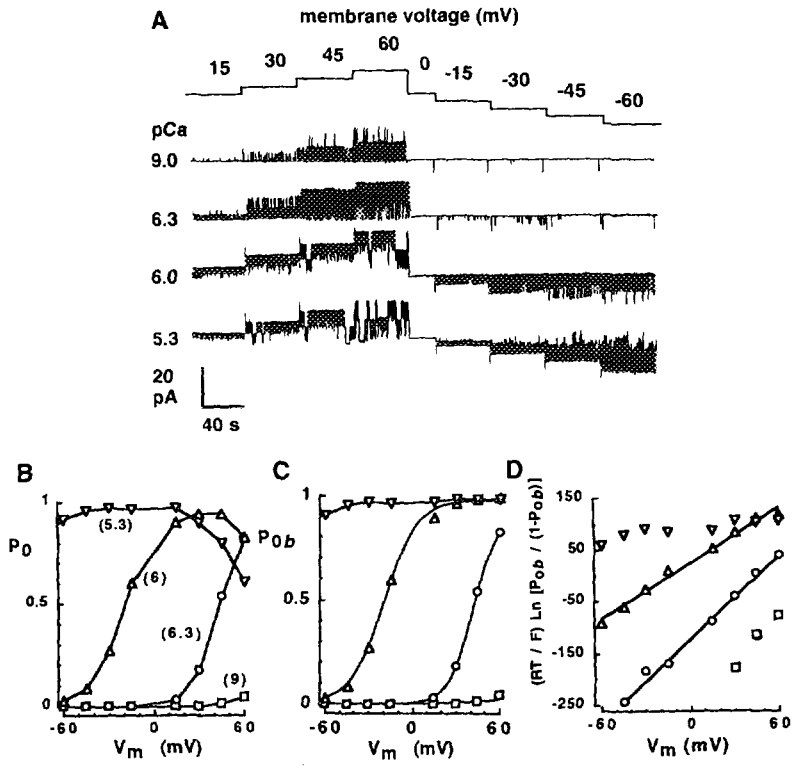

Figure 3. Ca^{2+} and voltage dependence of BK(Ca) channel activity. **A.** Current records from an inside-out patch containing two active BK(Ca) channels in symmetrical K^+ (145 mM). The cytoplasmic face of the membrane was exposed to various concentrations of free calcium and the current was monitored for one minute at the indicated voltage. **B.** Open probability of the two channels shown in A as a function of voltage and at various $[Ca^{2+}]_i$: pCa 9 (□), 6.3 (○), 6 (Δ), 5.3 (∇). **C.** Same as in B except that closed events lasting more than 500 ms were not considered before calculation of Po. The voltage dependence of Po inside bursts of activity (P_{ob}) is then adequatly described by the Boltzmann equation: $P_{ob} = \{ 1 + \exp[\zeta F(Vo-Vm)/RT] \}^{-1}$. **D.** A linear relationship between Ln (P_{ob} / (1-P_{ob})) and Vm is observed.

these long-lived nonconducting states. However, three observations suggest that these two channels open and close independently (data not shown): (i) during periods where the two channels are active, the probabilities of 0, 1, and 2 channels being open are described by a binomial distribution; (ii) when one channel switches to the long-lived silent state the open probability of the remaining active channel is equal to the open probability calculated from periods where the two channels are active; (iii) the mean dwell-times, Mkc, in the states "k = 0, 1, 2 closed channels" measured from non-blockade periods are akin to the mean closed and open times (Mc and Mo) measured from periods where only one channel is active:

$Mkc = [k.Mc^{-1} + (2-k).Mo^{-1}]^{-1}$ This suggests that when one channel switches from the normal to the silent state, it has no effect on the gating kinetics of the remaining active channel. In Figure 3B the open probability of the BK(Ca) channel, calculated from the whole current traces shown in Figure 3A, has been plotted as a function of membrane potential. At low $[Ca^{2+}]_i$ (pCa 6.3) where no long-lived silent states occur, the effect of voltage is exclusively positive. By

contrast, at pCa 5.3 the open probability decreases with voltage as a result of silent events. An intermediate case is observed at pCa 6 since a biphasic curve is obtained. The open probability inside periods of bursts (Po_b) has been calculated after exclusion of closing events lasting longer than 500 ms (Fig. 3C). Po_b increases with Vm and $[Ca^{2+}]_i$. The Po_b - voltage relationship is well described by the Boltzmann relation (11):

$$P_{ob} = \{1 + \exp [\ \zeta.F.(V_0 - V_m) / RT\ ]\}^{-1}$$

where V_0 is the voltage at which $P_{ob} = 0.5$. ζ can be interpreted as the equivalent charge that has to move across the entire membrane field to open the BK(Ca) channel. The experimental values of ζ and V_0 could be evaluated as respectively the slope and the root of the linear relationship between $(RT / F).Ln [P_{ob} / (1-P_{ob})]$ and Vm (Fig. 3D). ζ diminishes from 2.7 at pCa 6.3 to 1.8 at pCa 6 indicating that the voltage dependence of the gating slightly diminishes as $[Ca^{2+}]_i$ increases (Po_b increased e-fold per 13 mV at pCa 6 and per 9 mV at pCa 6.3). At high $[Ca^{2+}]_i$ (pCa 5.3), no voltage dependence of the open probability is observed since Po_b is close to unity even at -60 mV. The amount of bound Ca^{2+} that is required for the Ca^{2+}-induced channel opening is commonly deduced from a Hill plot where Po/(1-Po) is plotted against $[Ca^{2+}]_i$ on double logarithmic co-ordinates. Given the low number of calcium concentrations tested here, we could not give a Hill coefficient with confidence.

Discussion

In the present study, the properties of the BK(Ca) channel have been examined in excised membrane patches of differentiated N1E-115 cells by using the patch-clamp technique. The present channel resembles in many aspects those found in other membrane preparations (12). The open probability increases sharply on membrane depolarization. The relationship between Po and voltage is shifted to lower voltage as $[Ca^{2+}]_i$ increases. The positive effect of calcium and depolarization on the channel opening is counteracted by long-lived periods of closure that occur at high voltage (> 30 mV) and elevated $[Ca^{2+}]_i$ (> 1 µM). These long-lived periods of silence have been previously described for BK(Ca) channels in other membrane preparations and have been proposed to correspond to a long-lived voltage-dependent binding of Ca^{2+} to the conduction system which blocks the K^+ flux (13). Blockades by Ba^{++}, Cs^+ and TEA^+ of the present BK(Ca) channel also appear to be comparable to that observed in most previous studies (13-15). When added internally, Ba^{++} induces long periods of blockade without affecting the unitary conductance of the channel. The effect of Ba^{++} is reminiscent of the effect of Ca^{2+} which can block, at high concentration, the K^+ flux for several seconds. Therefore, both blockades have been interpreted as the binding of Ba^{++} or Ca^{2+} on the same site in the channel (13). By contrast, externally applied TEA^+ has no effect on the gating kinetics of the channel but decreases the apparent conductance. The effect of externally applied Cs^+ is more complex since the reduction in unitary current size is counteracted by an enhancement in the open probability. This effect is explained by the "foot in the door" model which states that, when residing in the channel pore, the blocker hinders channel closing (16). Ca^{2+} sensitivity of the BK(Ca) channel in N1E-115 cells have been studied to a lesser extent by Leinders and Vijverberg (6). We observed a large increase

in the open probability of the BK(Ca) channel at membrane potentials close to zero when $[Ca^{2+}]_i$ was changed over the range from 0.5 to 1 μM, that is, under physiological variations of $[Ca^{2+}]_i$ in stimulated cells (7). Similar changes in the open probability were obtained with tenfold higher $[Ca^{2+}]_i$ by Leinders and Vijverberg. Moreover, we did not observe a negative cooperative behavior in multiple channel patches. Some of these discrepancies may result from differences in used culture conditions and differentiation process and in recording signal analysis (we have studied single channel transitions with a higher time resolution). However, it must be reminded that there is a large variability among the BK(Ca) channels (12, 17) and that Ca^{2+} and voltage sensitivities for a BK(Ca) could be modified by others existing metabolic regulations (18).

The role of these BK(Ca) in N1E-115 remains to define. Our analysis of calcium and voltage sensitivities in inside-out patch configuration indicate that the BK(Ca) channels found in N1E-115 cells would open only during strong depolarizations under physiological $[Ca^{2+}]_i$, as confirmed by observations in cell attached configuration (data not shown). At normal resting potential, these channels would open only for micromolar concentrations of calcium. However, under conditions where $[Ca^{2+}]_i$ increase is accompanied by depolarization, these channels would become very active. These conditions could occur when Ca^{2+} channels open. We speculate that BK(Ca) channel opening would play a important physiological role in the return to the resting potential and so lead to a negative feedback on the opening of voltage-dependent Ca^{2+} channels.

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