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Properties and the Cytoskeletal Control of Ca^{++}-independent Large Conductance K^+ Channels in Neonatal Rat Hippocampal Neurons

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Abstract. A member of the family of Ca^{++}-independent large conductance K^+ channels (termed BK channels) was identified in patch clamp experiments with cultured neonatal rat hippocampal neurons. Permeation was characterized (at 5 mmol/l external, 140 mmol/l internal K^+; 135 mmol/l external Na^+) by a conductance of 107 pS, a ratio $P_{Na}/P_K \sim 0.01$, and outward rectification near the reversal potential. Channel activity was not voltage-dependent, could not be reduced by internal TEA or by a shift of internal pH from 7.4 to 6.8, i.e., discriminating features within the Ca^{++}-independent BK channel family. Cytosolic proteolysis abolished the functional state of hippocampal Ca^{++}-independent BK channels, in contrast to the pronase resistance of hippocampal Ca^{++}-activated BK channels which suggests structural dissimilarities between these related channels.

Cytoskeletal alterations had an activating influence on Ca^{++}-independent BK channels and caused a 3–4-fold rise in P_o, but patch excision and channel isolation from the natural environment provoked the strongest increase in P_o, from 0.07 ± 0.03 to 0.73 ± 0.04. This activation process operated slowly, on a minute time scale and can be most easily explained with the loss of a membrane-associated inhibitory particle. Once activated, Ca^{++}-independent BK channels reacted sensitively to a Mg-ATP supplemented brain tissue extract with a P_o decline, from 0.60 ± 0.06 to 0.10 ± 0.05. Heated extracts failed to induce significant channel inhibition, providing evidence for a heat-unstable molecule with reassociates with the internal channel surface to reestablish channel inhibition. A dualistic channel control, by this membrane-associated molecule and by the cytoskeleton seems possible.

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Key words: Maxi K^+ channels — Colchicine — cytochalasin B — Cytoskeleton

Introduction

Large conductance K^+ channels comprise a highly K^+ selective channel family which is characterized by a Ca^{++}-dependent gating process (for review *see* Latorre, 1994; Breitwieser, 1996). These BK_{Ca} channels can be found in many excitable and nonexcitable tissues and play an important role, for example, in the regulation of neuronal excitability and refractoriness, or in balancing the amount of neurotransmitter release from presynaptic terminals. Permeation and gating have their structural equivalent in the evolutionary conserved pore-forming α-subunit which has a membrane topology similar to the putative architecture of voltage-gated K^+ channels. However, each of the four domains of the α-subunit consists of ten instead of six transmembrane segments (Adelman et al., 1992; Butler et al., 1993). Functional BK_{Ca} channels can be formed by the α-subunit alone and, indeed, an association with a smaller β-subunit (Knaus et al., 1994) is lacking in several tissues (Tseng-Crank et al., 1996) although coexpression of both subunits which bind covalently to each other can tremendously enhance the Ca^{++} sensitivity of the channel (Tseng-Crank et al., 1996). The tissue-specific expression and distribution of the β-subunit (Tseng-Crank et al., 1996) may be, therefore, a molecular equivalent of the well-established heterogeneous Ca^{++} sensitivity of BK_{Ca} channels (for review *see* Latorre, 1994). The Ca^{++} binding site is not necessarily provided by the β-subunit but is thought to be accommodated in the C-terminal region of the α-subunit.

Several biophysical studies during the last few years have challenged the generally accepted view that large

conductance K^+ channels are essentially ligand-gated. Ca^{++}-insensitive K^+ channels with unitary conductances between 200 pS and 268 pS (at symmetrical K^+ concentration of 140 mmol/l) which, thus, meet the criterion of a maxi K^+ channel have been reported in adult (Green et al., 1991; Khan et al., 1993; Dopico et al., 1994) or dedifferentiated (Ehrhardt, Frankish & Isenberg, 1996) smooth muscle cells and in various embryonic neuronal tissues (Bulan et al., 1994; Mienville et al., 1996). These channels attain a considerably high open probability even in EGTA-buffered, Ca^{++} chelating conditions. Intriguing questions relate to their classification either as a variant of BK_{Ca} channels devoid of a Ca^{++} binding site or as a member of a distinct channel family. Their existence in embryonic and dedifferentiated cells casts some doubts that the Ca^{++}-independent large conductance K^+ channels (termed BK channel) play a major role in adult cells if their expression are associated with an immature cellular state. Without an effective control of channel activity by signal molecules, cytosolic factors or voltage, Ca^{++}-independent BK channels would disadvantageously generate a background K^+ conductance of fixed and perhaps considerable magnitude.

The present patch clamp experiments with cultured hippocampal neurons identified a Ca^{++}-independent BK channel with specific properties. This supports the hypothesis that Ca^{++}-independent BK channels represent a heterogeneous channel family. Evidence will be presented in favour of an effective control of channel activity by a membrane-associated inhibitory molecule and by the cytoskeleton. Ca^{++}-independent BK channels can also be distinguished from BK_{Ca} channels by their vulnerability towards proteolytic enzymes, a first argument for structural dissimilarities between both channel types.

Materials and Methods

Elementary K^+ currents through two types of K^+ channels were recorded in the cell-attached and in the inside-out configuration of membrane patches from the soma of cultured neonatal rat hippocampal neurons with an L-M/EPC5 amplifier by using the standard patch clamp technique (Hamill et al., 1981). Bipolar neurons with a spherical soma and lacking morphological signs of cell damage dominated in the cell culture and were selected for the patch clamp experiments. The neurons were kept in depolarizing conditions during the experiments by bathing them in isotonic K^+ solutions. With borosilicate glass pipettes (resistance 5–8 MΩ when filled with pipette solution), stable recording conditions were achieved in the cell-free mode for up to 120 min.

Preparation of Hippocampal Neurons

Hippocampi of both hemispheres of neonatal rats (P1–P2) were isolated, dissected and minced by using a modification of the procedure described by Jahr and Stevens (1987). A trypsin (0.25%)/EGTA medium was used for tissue disaggregation; dissociation was stopped by the addition of 1 ml fetal calf serum and accomplished by gentle mechanical agitation. After removing cell debris by centrifugation (200 g; 5 min), the cell pellet was resuspended in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% ''Viable AC-2'' medium (Pansystems München) and repeatedly washed before the cells were plated in sonic seal chambers (NUNC, Wiesloch, Germany). Under culture conditions, the medium was renewed after 24 hr and subsequently after three days. The neurons used for the patch clamp experiments were between the third and the fifth day of culture. No attempts were done to systematically relate the presence of the K^+ channels to be described to cellular age or shape.

Preparation of Brain Tissue Extract

According to the procedures described by Just et al. (1996), whole brains of neonatal rats (P1) were collected on ice, subsequently incubated in a lysis buffer (40 μg/ml aprotinin, 0.1 mmol/l phenylmethylsulfonylfluoride, 20 μg/ml leupeptin, 80 μg/ml benzamidine, 50 mmol/l HEPES; pH 7.4), homogenized on ice (3 times for 15 sec at 17000 g with a Polytron homogenizer). After centrifugation for 60 min at 30,000 × g the supernatant was used for the experiments and will be termed cytosol. Proteins and other heat-unstable molecules were denatured by heating the cytosol to 95°C.

Data Analysis

The current records were filtered with an 8-pole Bessel filter at 1 or 3 kHz, stored on tape and digitized with a sampling rate of 5 or 15 kHz, respectively, for subsequent analysis. Analysis included i_{unit}, open time and (in one-channel patches) closed time, and open probability (P_o). The number of functional K^+ channels in an individual patch was estimated from inspecting long-run records (at least 2 min) with high channel activity ($P_o \geqslant 0.5$): the lack of superpositions was taken to be indicative of the presence of only one ($N = 1$) individual K^+ channel. The size of i_{unit} resulted from Gaussian event distributions. Open and closed time analysis was based on the 50% threshold method and, by neglecting the first bin of 0.4 msec, τ_{open} and τ_{closed}, respectively, was obtained from the best weighted fit of probability density functions. P_o (and NP_o in patches where the number of channels could not be determined) was continuously monitored for 30-sec intervals to yield P_o (or NP_o) profiles as reliable measure of channel activity. The relative contribution of short-lasting openings and long-lasting openings to the whole event ensemble was calculated from the area under the biexponentially distributed open time histogram.

Solutions

(*A*) Bathing solution 1 (composition in mmol/l) (used to equilibrate the neurons in the cell-attached experiments and facing the cytosolic membrane surface in the inside-out experiments): KCl 140; $MgCl_2$ 2; glucose 20; EGTA 2; HEPES 10; pH 7.4 Bathing solution 2: KCl 20; K^+ aspartate 120; $MgCl_2$ 2; glucose 20; EGTA 2; HEPES 10; pH 7.4 Bathing solution 3: composition like solution 1 but lacking EGTA. By employing 1 mmol/l EDTA, free Ca^{++} concentrations of 1, 5 or 10 μmol/l were adjusted. Free Mg^{++} concentration was 2 mmol/l. Temperature (controlled by a Peltier element): 19 ± 0.5°C. (*B*) Pipette solution (facing the external membrane surface): KCl 5, NaCl 135; $MgCl_2$ 2; HEPES 10; pH 7.4.

Compounds

All compounds were purchased from Sigma Chemie, München and freshly dissolved before use. Cytochalasin B was dissolved in dimeth-

ylsulfoxide (DMSO), appropriate amounts of this stock solutions were diluted in solution A or in culture medium to give a final cytochalasin B concentration of 25 μmol/l. The DMSO concentration was 10 μmol/l.

Composition of nucleotide cocktail A: cAMP (10 μmol/l), MgATP (4 mmol/l), ADP (100 μmol/l), GTP (100 μmol/l).

Composition of the nucleotide cocktail G: cGMP (20 μmol/l), MgATP (4 mmol/l).

Whenever possible, the data are expressed as mean ± SEM. The statistical significance of differences between means was determined by the *t*-test.

Results

Presence and Activation of a Large Conductance K^+ Channel in Hippocampal Neurons

Cell-attached recordings in depolarized hippocampal neurons identified a large conductance K^+ channel as the most prominent channel type. Openings of this channel could be detected in about 30% of the patches suggesting a considerable density, but openings occurred with a low frequency only. A systematic NP_o analysis for at least 10 min revealed that NP_o fluctuated during the whole observation period between a maximum and a minimal level close to zero. $NP_{o(max)}$ amounted to 0.07 ± 0.03 ($n = 10$; pipette potential (E_p) = 0 mV) and exhibited an only minor voltage sensitivity, if at all. A depolarizing shift of the membrane potential by 40 mV did not significantly increase channel activity, NP_o was 110 ± 5% ($n = 4$) of the initial value. The analysis of the *IV*-relationship at 5 mmol/l external K^+ revealed a slope conductance of 102 ± 3 pS ($n = 4$) and a reversal potential (E_{rev}) near E_p of +70 mV. Open time histograms could be best fitted by two exponentials suggesting two open states, O_1 and O_2 (*see* Fig. 1*A*) while closed time kinetics could not be analyzed because the number of 102 pS K^+ channels in an individual patch could not be determined with reliable accuracy.

Openings of this 102 pS K^+ channel could also be observed in depolarized hippocampal neurons when exposed to bathing solution 2 containing 20 mmol/l Cl^- and 120 mmol/l aspartate instead of 140 mmol/l Cl^- to reduce the cellular volume load. $NP_{o(max)}$ was not systematically analyzed in this particular situation.

This large conductance K^+ channel reacted sensitively to a displacement of the cell-attached patch pipette with a significant activation. Figure 1*A* illustrates such an experiment where the patch pipette was abruptly dislocated by about 3 μm in the horizontal plane. This intervention exerts shear stress on a major part of the adjacent soma and alters its shape considerably. Concomitantly, NP_o increased from 0.016 to 0.40. Removal of shear stress by removal of the patch pipette to its initial position was followed by a slow channel deactivation. It took several ten seconds before NP_o returned to the initial value of close to 0.016. Confirmatory observations come from 5 other experiments of this type with a 20 ± 5-fold $NP_{o(max)}$ increase. Although channel activation is mainly due to a drastic shortening of the nonconducting configuration, open state kinetics can also react to shear stress (*see* Fig. 1*A*, left). For example, in the experiment illustrated in Fig. 1*A*, $\tau_{open(1)}$ increased from 1.6 msec to 2.8 msec and $\tau_{open(2)}$ from 4.6 msec to 5.5 msec, accompanied by an increased frequency of the long-lasting O_2-events. The latter result would be consistent with the notion that the channel spends more time in the O_2-state. Attempts to relate P_o to pipette displacement quantitatively remained unsuccessful and rather suggested that P_o increases in an all-or-none fashion. Some K^+ channels in brain (Kim et al., 1995) and BK_{Ca} channels in artery smooth muscle (Dopico et al., 1994) were shown to be mechanosensitive but the application of pressure or suction (± 20 mm Hg) to the lumen of the cell-attached patch pipette proved ineffective, in the present experiments, to induce channel activation. Channel activation by shear stress may, thus, be related to stretching or a perturbation of the cytoskeleton. Alternative mechanisms such as a mechanically altered membrane permeability for a so-far hypothetical molecule or the possibility that the K^+ channel responds directly to intramembrane physical forces seem less plausible.

To examine the possible influence of the cytoskeleton, cell-attached experiments were performed to study the activity of the 102 pS K^+ channel in neurons pretreated with colchicine (100 μmol/l) for 60 min. The neurons were then exposed to colchicine for the duration of the cell-attached recordings. This colchicine treatment should be sufficient to disrupt microtubules known to spread out radially in the soma and can induce sustained high channel activity. When compared with the control situation in untreated neurons (*see* Fig. 1*A*), P_o was several fold larger and $P_{o(max)}$ attained a level of 0.4 in the colchicine-pretreated neuron illustrated in Fig. 1*B*. Nevertheless, channel activity was occasionally interrupted by silent periods lasting up to 2 sec (*see* Fig. 1*B*). Such episodes are difficult to interpret because of the limiting recording conditions and may either reflect unresolved extreme short openings or a stable nonconducting state. Both possibilities are consistent with the notion that the channel undergoes a P_o shift. Activated 102 pS K^+ channels were similarly observed in hippocampal neurons pretreated for 60 min with cytochalasin B (25 μmol/l). Again, the pretreated neurons were continuously exposed to this nonselective actin depolymerizing agent during the whole cell-attached experiment. The P_o values were significantly increased in neurons exposed to colchicine (0.23 ± 0.07; $n = 5$) and cytochalasin B (0.32 ± 0.09; $n = 5$) when compared with the respective control value as shown in Fig. 1*C*. Channel activation was not accompanied by a significant prolongation of the O_1- and the O_2-state (*see* Table 1).

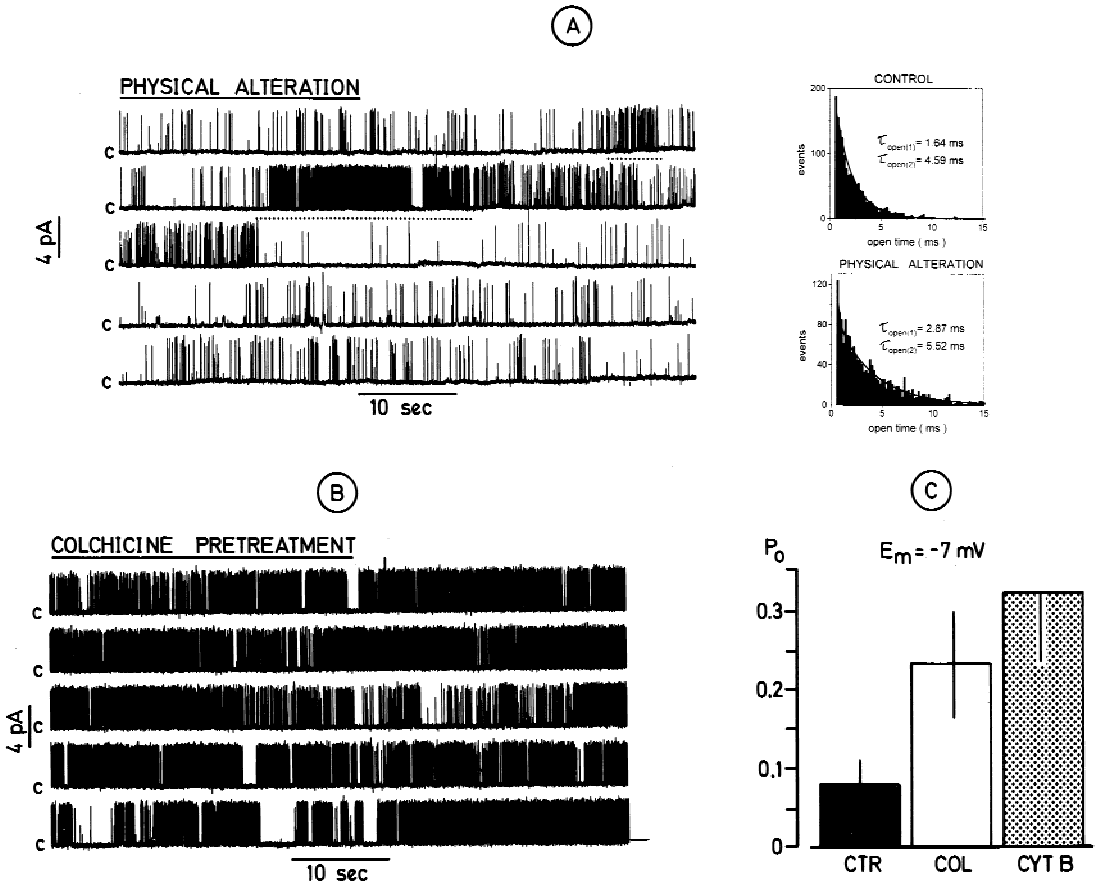

Fig. 1. (*A*) Left: Cell-attached elementary current recordings from a hippocampal large conductance K^+ channel and its response to a pipette displacement as indicated by the dotted lines below the records. C means closed configuration. Right: Open time histograms before (control) and during (physical alteration) pipette displacement. They follow the function $N(t) = 225\exp(-t/0.00164) + 15\exp(-t/0.00459)$ (control) and $N(t) = 116\exp(-t/0.00287) + 84\exp(-t/0.00552)$ (physical alteration). Depolarized hippocampal neuron; pipette potential (E_p) = 0 mV; exp N47. (*B*) Cell-attached elementary current recordings from a hippocampal large conductance K^+ channel in a colchicine (100 μmol/l) pretreated cultured hippocampal neuron. C means closed configuration; E_p = 0 mV; exp N74. (*C*) Open probability of large conductance K^+ channels under cell-attached conditions in normal (CTR, n = 12), colchicine-treated (COL, n = 5) and cytochalasin B (CYT B, n = 5) treated cultured hippocampal neurons. Each column represents mean values, vertical bars are SEM. Depolarized hippocampal neurons; E_p = 0 mV.

Elementary Properties of Ca^{++}-independent BK Channels in Cell-free Conditions

Patch excision and the isolation of the large conductance K^+ channels from the natural cytosolic environment is another intervention capable of inducing channel activation. As shown in Fig. 2, the low P_o mode characteristic for the in-situ situation in normal neurons ($P_o < 0.001$ in this experiment) persisted only for some ten sec after patch excision in EGTA-buffered, nominally Ca^{++}-free solution. Subsequently, P_o gradually increased with time and attained within about 4 min a new steady state close to 0.6. As confirmed in 61 inside-out patches analyzed at −7 mV, activation followed inevitably patch excision and only its time course varied. Channel activation was sometimes rapidly achieved, within a few ten sec, while it took in a few other experiments almost 10 min to be accomplished. P_o was 0.013 ± 0.01 (n = 9) during the first 30 sec after patch excision, increased to 0.063 ± 0.05 (n = 9) during the next 30 sec, and $P_{o(\max)}$ (0.72 ± 0.02; n = 9) was reached 4–9 min after patch excision. Some comparative experiments performed in the absence of EGTA excluded the possibility that EGTA might have an influence on the time course of channel activation. Interestingly, channel activation was prevented when a membrane vesicle had been formed, an occasionally occurring methodological complication. In this particular situation, P_o retained the low level observed prior to patch excision in the cell-attached recording mode. Vesicle opening with the subsequent equilibration of the cytosolic membrane surface to EGTA-buffered, Ca^{++}-free solution allowed the BK channel to activate but usually on a much faster time scale.

Activated BK channels in a nominally Ca^{++}-free cy-

Table 1. Open state kinetics of Ca^{++}-independent BK channels in cultured hippocampal neurons under control conditions and in neurons pretreated with colchicine and cytochalasin B

	$\tau_{open(1)}$ (msec)	*P* (*vs.* Control)	*P* (*vs.* cyt. B)	$\tau_{open(2)}$ (msec)	*P* (*vs.* Control)	*P* (*vs.* cyt. B)
Control	1.93 ± 0.22 (n = 7)			5.10 ± 0.37 (n = 7)		
Colchicine	2.84 ± 0.54 (n = 5)	0.21	0.75	6.75 ± 0.89 (n = 5)	0.10	0.70
Cytochalasin B	2.66 ± 0.22 (n = 5)	0.47		6.12 ± 0.88 (n = 5)	0.73	

For $\tau_{open(1)}$ and $\tau_{open(2)}$, mean values ± SEM are given. *P* indicates the significance level of the differences between means determined by the *t*-test. Pipette potential = 0 mV (membrane potential of the depolarized neurons close to 0 mV).

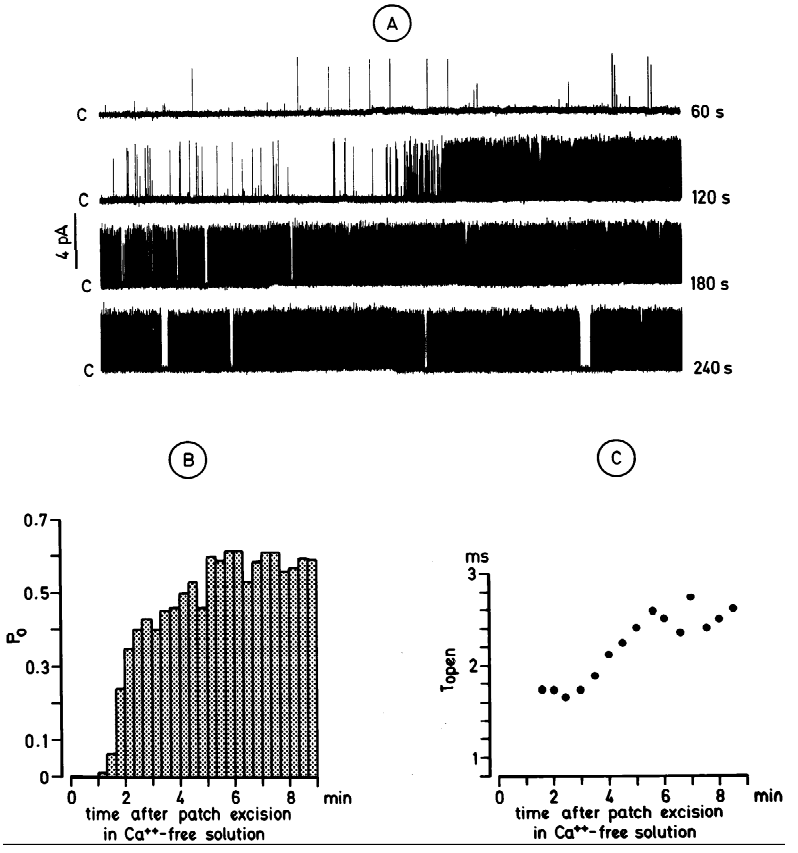

Fig. 2. Activation of Ca^{++}-independent BK channels in cell-free conditions. (*A*) Continuous elementary K^+ current records during the first 4 min after patch excision. P_o during the first 60 sec (*see* uppermost row) corresponds to P_o obtained prior to patch excision in a 10-min cell-attached recording period. C means closed configuration. (*B*) P_o profile of the experiment illustrated in *A*. (*C*) Changes in $\tau_{open(2)}$ during upregulation. EGTA-buffered, nominally Ca^{++}-free cytosolic environment; E_m = −7 mV. Asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration. Exp N9.

tosolic environment exhibited the same elementary properties as the 102 pS K^+ channels found in situ (Fig. 3). At asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration and in the presence of 135 mmol/l external Na^+, permeation was characterized by a slope conductance of 107 ± 3 pS (n = 5) and outward rectification in a potential range near E_{rev}. By extrapolating the *IV*-relationships to the voltage axis, E_{rev} was estimated to be −75 ± 1.9 mV (n = 5) thus deviating by 8 mV from E_K (−83 mV). A ratio $P_{Na}/P_K \sim 0.01$ indicates a K^+-selective pore as expected for BK channels. At symmetrical (140 mmol/l) K^+ concentration, an ohmic *IV*-relationship was found between −40 and +40 mV with a slope conductance of 166 ± 2 pS (n = 4). Occasional subconductance openings reached 50% of the full-sized i_{unit}.

Several Ca^{++}-independent BK channels are characterized by a voltage-dependent open probability (Green

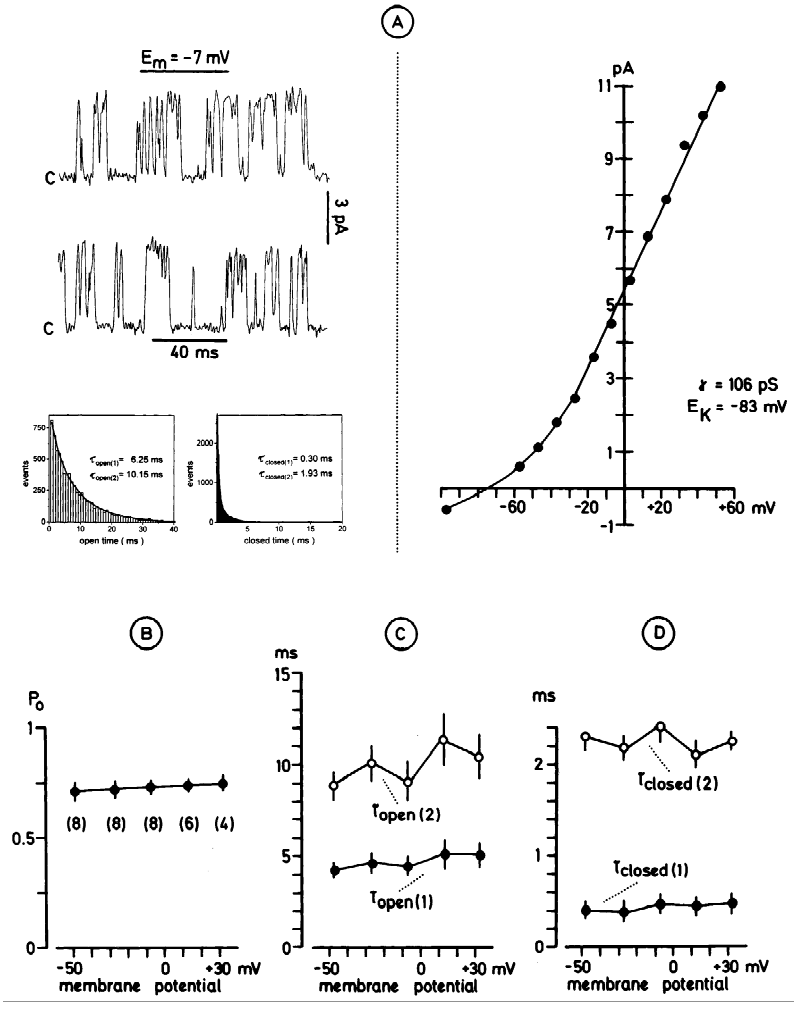

Fig. 3. Elementary properties of activated Ca^{++}-independent BK channels in cell-free conditions. (*A*) Left: Records of elementary K^+ currents (c means closed configuration) and open and closed time histograms; $E_m = -7$ mV. Right: *iv*-relationships. Asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration, 135 mmol/l external Na^+. Exp N76. (*B–D*) Voltage dependence of P_o (B), open-state kinetics (C) and closed state kinetics (D). The data points symbolize the mean values, vertical bars are SEM. The number of experiments in *B* are given in brackets; in *C* and *D*, $n = 3$. Asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration, 135 mmol/l external Na^+.

et al., 1991; Bulan et al., 1994; Ehrhardt et al., 1996). Their hippocampal relatives, however, do not follow this rule. Figure 3*B* presents evidence that P_o of hippocampal Ca^{++}-independent BK channels is voltage-insensitive over a broad potential range, between −47 and +33 mV. To exclude the possibility that a saturated P_o level may be already achieved at −47 mV, P_o was examined in a separate experiment at −47, −107, and −127 mV yielding P_o values of 0.69, 0.68, 0.71, respectively.

Open time probability density functions could be best fitted by two exponentials. Between −47 and +33 mV, $\tau_{open(1)}$ and $\tau_{open(2)}$ did not vary significantly ($P = 0.4$) (Fig. 3*C*). It was excluded that open state kinetics are Ca^{++} sensitive. At 100 μmol/l cytosolic Ca^{++}, $\tau_{open(1)}$ (3.7 ± 0.32 msec; $n = 9$) and $\tau_{open(2)}$ (8.1 ± 1.1 msec; $n = 9$) did not differ from the control values ($\tau_{open(1)}$: 3.3 ± 0.44 msec; $\tau_{open(2)}$: 7.8 ± 1.07 msec; $n = 10$; $E_m = -7$ mV) at nominally Ca^{++}-free conditions and also the ratio O_1-events to O_2-events did not change. Closed state kinetics proved Ca^{++} insensitive, too. At least two closed states (Fig. 3*A*) could be distinguished. Not included in the closed time histograms are prolonged silent periods without detectable channel openings which can also be observed in activated Ca^{++}-independent BK channels in cell-free conditions. Figure 3*D* shows that closed state kinetics are voltage-insensitive between −47 and +33 mV.

Thus, activated Ca^{++}-independent BK channels can be tentatively modelled by a Markovian reaction scheme according to $C_x - C_2 - C_1 - O_1 - O_2$. where C_x refers to possible closed configurations beyond C_2. However, the entrance into the O_2-state seems a critical step. Figure 4 demonstrates a spontaneously occurring, sudden shift of open state kinetics from an apparently single-O-state gating to an apparently two-O-state gating. This resembles modal gating as established in other ionic channels (Hess Lansman & Tsien, 1984; Patlak, Ortiz & Horn, 1986).

Internal TEA treatment (10 mmol/l) reduced unitary current size but left open-state kinetics unchanged (Fig. 5*A*). This is just the response which typically occurs in BK_{Ca} channels (Blatz & Magleby, 1984; Vergara, Moczydlowski & Latorre, 1984; Yellen, 1984) and re-

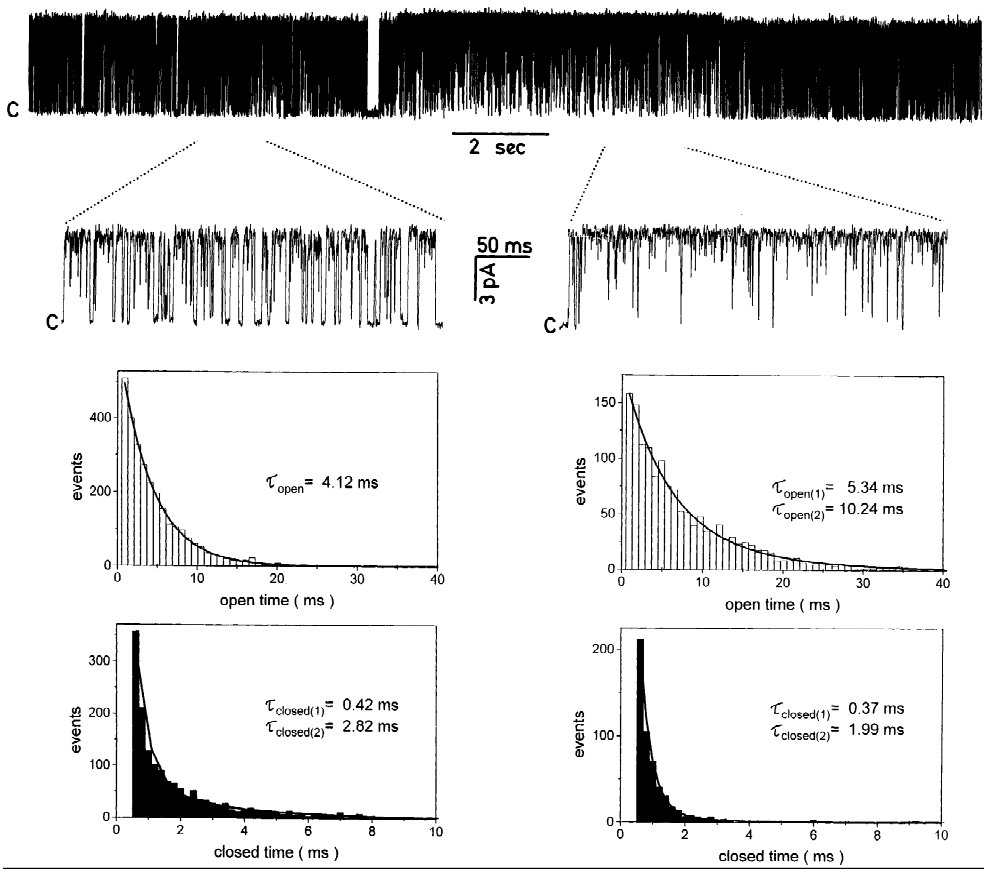

Fig. 4. Spontaneous changes of open state kinetics in isolated Ca^{++}-independent BK channels. *Upper part.* Continuous record of elementary K^+ currents showing a clustering of events with shorter or longer open time. *Lower part.* Open time and closed time histograms from the episode with the shorter (left) and with the longer open time (right). Asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration; E_m = −7 mV; exp N77.

flects fast flicker blockade. As shown in Fig. 5*B*, the TEA action on i_{unit} is voltage-dependent. The apparent $K_{i(TEA)}$ decreases e-fold with 138 mV depolarization which indicates that internal TEA interferes with a site located within the electrical field of the membrane, i.e., distant from the internal mouth within the pore. Interestingly, the obtained $K_{i(TEA)}$ is at least 5 times smaller than the corresponding value reported from BK_{Ca} channels in the literature. Obviously, hippocampal Ca^{++}-independent BK channels possess a higher TEA affinity. It seems worthy to note that the internal TEA treatment had no influence on channel activity; activated Ca^{++}-independent BK channels retained the high P_o mode and, at −7 mV, P_o was 0.75 ± 0.01 prior to and 0.77 ± 0.02 (n = 2) after internal TEA treatment.

Cytosolic acidification depressed the unitary current size. Thus, a pH shift from 7.4 to 6.8 reduced i_{unit} to 70 ± 5% (n = 3; $P < 0.05$) of the control. In contrast, P_o remained unchanged even with a prolonged exposure of Ca^{++}-independent BK channels to acidified internal solutions. Conversely, internal Ba^{++} (5 mmol/l) blocked the channel without a reduction in i_{unit}; P_o decreased within 30–50 sec from 0.64 ± 0.04 to 0.006 ± 0.0001 (n = 2; $P < 0.001$) but the recovery from blockade on washout of Ba^{++} was surprisingly slow as it took 10 min or more.

Comparison of BK_{Ca} Channels with Ca^{++}-independent BK Channels: Differential Pronase Sensitivity

Utilizing inside-out patches in the absence of Ca^{++}-independent BK channels, a first series of experiments was performed to analyze comparatively hippocampal BK_{Ca} channels at −7 mV (*see* Fig. 6*A–D*). The latter were observed in about 30% of the patches when the cytosolic Ca^{++} concentration was increased above 5 μmol/l. At −7 mV, P_o attained almost the saturated level (0.84 ± 0.02; n = 2) at 10 μmol/l internal Ca^{++} and was 0.94 ± 0.01 (n = 9) at 100 μmol/l internal Ca^{++}. As determined in fully activated BK_{Ca} channels, they also

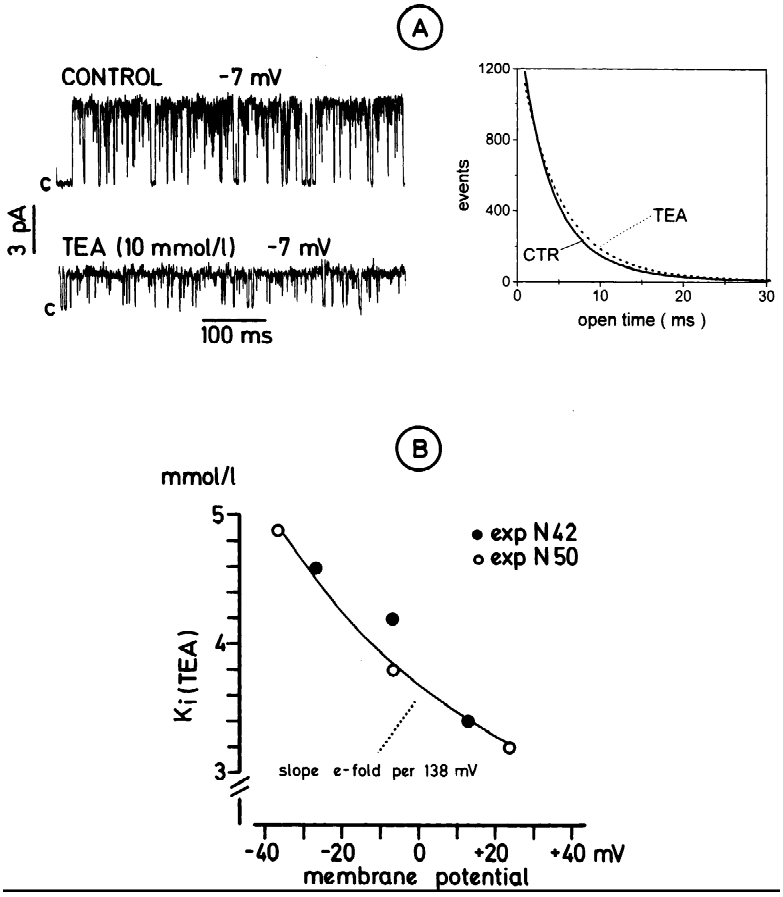

Fig. 5. The effect of internal TEA on isolated Ca^{++}-independent BK channels. (*A*) Records of elementary K^+ currents before and after TEA (10 mmol/l) application. In the right part, superimposed open time histogram fits are shown for control conditions (CTR, solid line) following the function $N(t) = 1256\exp(-t/0.0034) + 239\exp(-t/0.0091)$ and for TEA following the function $N(t) = 1143\exp(-t/0.0043) + 206\exp(-t/0.103)$. $E_m = -7$ mV; exp N42. (*B*) Voltage dependence of the apparent K_i of the depressant TEA action on i_{unit}. Filled and open circles symbolize two individual experiments. The line relating $K_{i(TEA)}$ to membrane potential was drawn by eye.

follow a complicated gating scheme with at least two closed and two open configurations including signs of occasional gating shifts. Episodes with poorly resolved openings (in records filtered at 3 kHz) can last several hundred milliseconds and may be related to the saturating Ca^{++} concentration of 100 μmol/l (Rothberg et al., 1996). Fully activated BK_{Ca} channels have more stable conductive configurations and remain for longer times in both O-states compared with fully activated Ca^{++}-independent BK channels (*see* Fig. 6*C*). This can explain the significantly ($P < 0.005$) higher P_o level of the former, 0.94 ± 0.01 ($n = 9$) *vs.* 0.72 ± 0.04 ($n = 15$; *see* Fig. 6*B*) of the latter. At asymmetrical (5 mmol/l external, 140 mmol/l internal) K^+ concentration, BK_{Ca} channels had a conductance of 130 ± 7 pS which was 1.2 times ($P < 0.05$) the value observed in Ca^{++}-independent BK channels at 5 mmol/l external K^+. However, like Ca^{++}-independent BK channels, BK_{Ca} channels rectify in a potential range near E_{rev} in the presence of 135 mmol/l external Na^+.

The proteolytic enzyme pronase (75 μg/ml) strongly influenced the activity of activated Ca^{++}-independent BK channels when administered cytosolically. In three inside-out experiments, pronase strongly reduced P_o. This inhibitory effect needed about 2–3 min to occur and finally abolished channel activity (Fig. 6*E*) within some ten sec. Washout experiments proved the irreversibility of the pronase effect. In contrast, as found in 5 experiments with BK_{Ca} channels, the latter proved resistant to the same pronase treatment (Fig. 6*F*). Neither a decline in Ca^{++} sensitivity as reported from trypsin-treated BK_{Ca} channels in phospholipid bilayers (Salomao et al., 1992) nor a conversion of the gating mode seen with cytosolic proteolytic enzymes in this channel type (Mienville, 1994) could be detected, i.e., hippocampal BK_{Ca} channels tolerated even a prolonged pronase treatment of up to 15 min without a P_o decline.

The Influence of Brain Tissue Extracts on Activated Ca^{++}-independent BK Channels

Protein phosphorylation acts as a regulatory principle in many K^+ channels (for review *see* Levitan, 1994). Therefore, the influence of several phosphorylating conditions was studied in an attempt to elucidate the reason for the activation of Ca^{++}-independent BK channels in cell-free conditions. The nucleotide cocktails A and G (*see* Materials and Methods) were used to activate PKA and PKG in two experimental protocols, i.e., immedi-

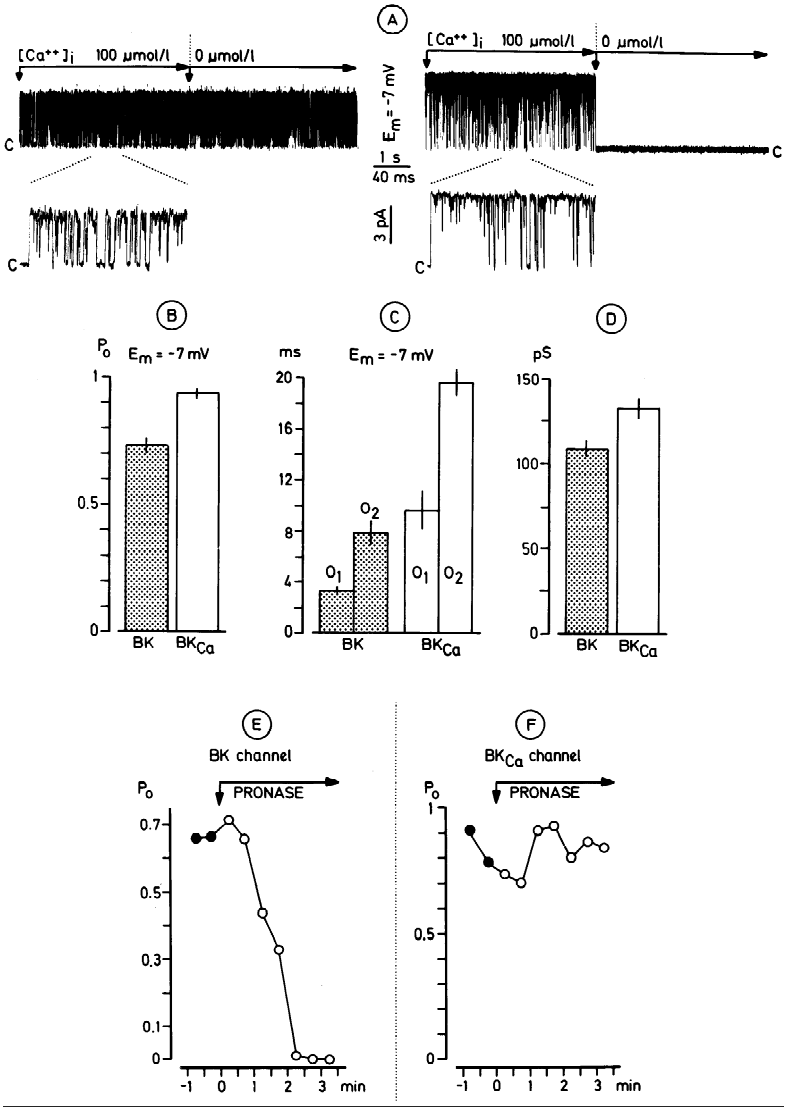

Fig. 6. Comparison of Ca^{++}-independent BK channels with BK_{Ca} channels. (*A*) Records of elementary currents through BK channels (left) and through Ca^{++}-activated BK_{Ca} channels in the inside-out mode at −7 mV. (*B–D*) P_o, open state kinetics (O_1 and O_2 refer to $\tau_{open(1)}$ and $\tau_{open(2)}$, respectively), and slope conductance. Dotted columns symbolize the mean values for BK channels ($n = 9$) and open columns for BK_{Ca} channels ($n = 15$), vertical bars are SEM. (*E* and *F*) P_o profile of a BK channel (*E*, exp N114) and of a BK_{Ca} channel (*F*, exp N115) before (filled circles) and after the cytosolic application of 75 µg/ml pronase (open circles). $E_m = -7$ mV. The data of the BK_{Ca} channel were obtained at a cytosolic Ca^{++} concentration of 100 µmol/l.

ately after patch excision and thus before channel activation or after channel activation had been accomplished. In a total of 9 experiments, the cocktails proved ineffective to prevent channel activation or to depress P_o in activated channels. Likewise, patch excision in MgATP (1 mmol/l) supplemented solutions which contained the unspecific phosphatase inhibitors calyculin A (100 nmol/l) or vanadate (100 µmol/l) did not stabilize the low P_o mode.

However, as shown in Fig. 7*A*, a brain tissue extract (supplemented with 1 mmol/l MgATP) applied cytosolically after channel activation effectively depressed P_o. Control experiments (Fig. 7*B*) excluded the possibility that the protease inhibitor utilized to prevent protein degradation in the cytosol preparation had caused the channel inhibition. Interestingly, the P_o depression proceeded slowly, on a minute time scale, which is reminiscent of the usual kinetics of channel activation after patch excision. The brain tissue extract was equally effective in the absence and the presence of GTP γ S (100 µmol/l), a nucleotide necessary for activating Rho-proteins which are involved in cytoskeletal organization (for review *see* Machesy & Hall, 1996). The extract significantly ($P < 0.001$) reduced P_o from 0.60 ± 0.06 to 0.10 ± 0.05 ($n = 4$). P_o increased to 0.45 ± 0.12 ($n = 4$) on washout but recovery proceeded slowly and needed more than 10 min to restore channel activity. Heated cytosol (Fig. 7*C*) had largely lost an inhibitory activity which is consistent with the assumption that a heat-unstable molecule is involved in the depression of P_o.

Discussion

Neonatal hippocampal neurons can express two distinct types of maxi K^+ channels, the ''classical'' Ca^{++}-

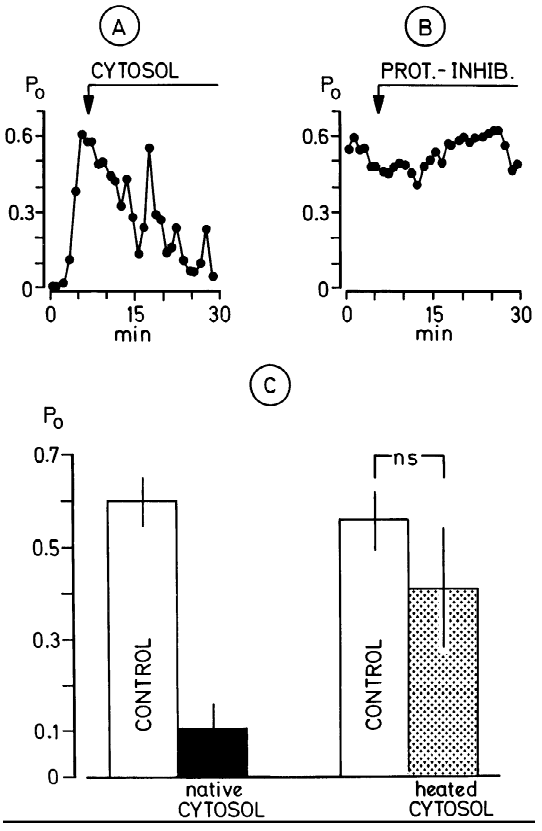

Fig. 7. The influence of brain tissue extracts (cytosol) on activated Ca^{++}-independent BK channels in cell-free conditions. (*A*) Activation of an BK channel upon patch excision and the channel response to cytosol (supplemented with 100 μmol/l GTPγS plus 1 mmol/l MgATP. Zero time refers to the moment of patch excision. E_m = −7 mV; exp N114. (*B*) Failing response of an activated BK channel to protease inhibitor. Zero time refers to the fifth minute after patch excision. E_m = −7 mV; exp N115. (*C*) Differential influence of native brain tissue extract (filled columns, vertical bar is SEM) and denatured brain tissue extract (heated cytosol, dotted column; vertical bar is SEM). E_m = −7 mV; NS means statistically not significant from control (open columns).

dependent BK_{Ca} channel with a conductance of 130 pS (at 5 mmol/l external K^+) and a newly identified 107 pS (at 5 mmol/l external K^+) Ca^{++}-independent BK channel. When compared with the few other Ca^{++}-independent BK channels reported in the literature, the properties of the hippocampal 107 pS BK channel differ mainly in two aspects, namely voltage-independence of P_o and failing TEA sensitivity of P_o. This constellation justifies its classification as a novel member in the Ca^{++}-independent BK channel family.

Hippocampal 107 pS BK channels share with their relatives in dedifferentiated smooth muscle cells (Ehrhardt et al., 1996) and in human pregnant myometrium (Khan et al., 1993) the common property to switch in a high activity mode when isolated from the natural cytosolic environment. Conversion from the typically low activity mode (NP_o close to 0.1 as the uppermost limit) under in-situ conditions to a high P_o mode (P_o close to 0.7) upon patch excision can be most easily explained with the removal of an inhibitory influence which acts in-situ to stabilize the low P_o mode. Interestingly, Ca^{++}-independent BK channels isolated from human pregnant myometrium attain a similar high P_o level (P_o ~ 0.8; Khan et al., 1993). This is a striking argument against the assumption that activation in cell-free conditions represents a peculiarity of BK channels from immature cells. Moreover and as another developmental aspect, Ca^{++}-independent BK channels cannot be considered as the precursor of BK_{Ca} channels as it might be argued from observations in developing spinal neurons of *Xenopus* (Blair & Dionne, 1985). However, it cannot be excluded that the density of 107 pS BK channels varies in hippocampal neurons with maturation, a possibility which has consequences for cellular function.

Circumstantial evidence has been presented in the literature during the last years that the cytoskeleton plays a role in regulating ionic channels. Actin depolymerization by elevated internal Ca^{++} or by cytochalasins and the concomitant structural alteration or breakdown of the cytoskeleton may have opposite effects depending on the species of ionic channels. Neuronal voltage-dependent Na^+ channels (Fukuda, Kameyama & Yamaguchi, 1981), renal low conductance K^+ channels (Wang et al., 1994) and NMDA channels (Rosenmund & Westbrook, 1993), for example, respond with deactivation. Similarly, an accentuated rundown was observed in isolated cardiac ATP-sensitive K^+ channels when cell-free patches were cytosolically exposed to cytochalasins (Furukawa et al., 1996). On the other hand, renal Cl^- channels (Schwiebert, Mills & Stanton, 1994) and amiloride-sensitive Na^+ channels (Cantiello et al., 1991) become activated. Disruption of microtubules by colchicine was reported to be followed by inhibition of neuronal (Johnson & Byerly; 1994) and cardiac (Galli & DeFelice, 1994) Ca^{++} channels and evidence was presented against an influence of colchicine on Ca^{++} channel density (Galli & DeFelice, 1994). Although a number of cytoskeletal and intracellular proteins can associate with ionic channels (for review *see* Sheng & Kim, 1996), the binding domains of ionic channels for cytoskeletal proteins as involved in the control of channel activity must still be defined. It is also not yet clear whether the cytoskeleton plays a physiologically relevant role in the activity control of voltage-dependent and ligand-gated ionic channels.

Ca^{++}-independent BK channels seemingly belong to the category which is under effective cytoskeletal control. Channel activation seen in the cytochalasin B and colchicine-pretreated hippocampal neurons agrees with results in a smooth muscle BK channel (Ehrhardt et al., 1996). Obviously, alterations of the cytoskeleton will

finally activate the channel, most likely by removing an inhibitory influence. More direct evidence for the interference of the cytoskeleton with K^+ channels comes from a human melanoma cell devoid of actin binding protein. In these defective cells, hypotonic stimuli fail to activate K^+ channels (Cantiello et al., 1993). Importantly, cytochalasin B and colchicine treatment could not completely activate the 107 pS BK channel since P_o did not exceed an average level of 0.3. This contrasts to the pronounced channel disinhibition in cell-free conditions with an average P_o of close to 0.7 and indicates that only patch excision eliminates the inhibitory influence completely. Physical detachment of the cytoskeleton from the membrane-associated network seems unlikely to be directly responsible for channel disinhibition because channel activation in cell-free conditions develops slowly, on a minute time scale.

Although cytoskeletal structures still cover isolated membranes as recently demonstrated by scanning force microscopy of inside-out patches (Horber et al., 1995), their equilibrium between polymerization and depolymerization may shift to the latter state when these actin filaments face an artificial environment (Korn & Pantolini, 1987; Stossel, 1993) and a concomitant gradual loss of these or other proteins cannot be excluded. This scenario can explain the quasi-complete disinhibition of the hippocampal Ca^{++}-independent BK channels in cell-free conditions and would also be consistent with the notion that a close physical contact of these proteins with a cytosolic channel domain is involved in channel inhibition. Supporting evidence for an inhibitory molecule that is associated with the internal membrane surface arises from the present experiments with brain tissue extracts. The nature of this molecule still awaits clarification.

The membrane-associated inhibitory principle might have the functional significance to set an upper limit of open probability, at least in 107 pS hippocampal BK channels thereby preventing an excessive increase of K^+ conductance. The cellular cytoskeleton as formed by microtubules and intracellular filaments could be involved in situ to modulate channel activity in a regulatory sense under conditions where hippocampal neurons benefit from variations of K^+ efflux and concomitant fluctuations of membrane potential in controlling cytosolic osmolarity. Such a dualistic activity control may well be based on a common mechanism, i.e., both inhibitory influences interfere with the same cytoplasmic channel domain.

Hippocampal Ca^{++}-independent BK channels possess a cytosolically located, pronase-sensitive domain. This region seems important for maintaining the functional channel state. It is tempting to speculate that this region also provides a target for the postulated membrane-associated inhibitory molecule (or particle) with the consequence that an interference with the latter would finally suppress channel activity. Conversely, removal of the inhibitory molecule allows channel activity in the high P_o mode as long as the pronase-sensitive domain is structurally intact. As predicted from the pronase resistance of 130 pS BK_{Ca} channels, their cytosolic architecture differs in that an equivalent cytosolic domain is either absent, not accessible to proteolytic enzymes, or devoid of proteolytic cleavage sites. To consider Ca^{++}-independent BK channels as a variant of Ca^{++}-sensitive BK_{Ca} channels only lacking Ca^{++} binding sites seems, therefore, an unjustified oversimplification.

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