J. Membrane Biol. 153, 211–216 (1996)

The Journal of
Membrane
Biology

Mechanosensitive Properties of BK Channels from Embryonic Rat Neuroepithelium

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Received: 1 February 1996/Revised: 25 March 1996

Abstract. The mechanosensitive properties of large-conductance Ca^{2+}-activated K^+ (BK) channels from embryonic rat neuroepithelium were investigated with the cell-attached and inside-out configurations of the patch-clamp technique. The channels were activated in both recording configurations by negative pressures applied to the patch electrode, but reversal of the effect was total and immediate in inside-out patches whereas it was incomplete and delayed in on-cell patches. This mechanosensitivity was not mediated by Ca^{2+} ions or fatty acids, suggesting that it is an intrinsic property of these channels. Cytochalasin B did not affect mechanosensitivity in on-cell patches but increased it in inside-out patches. Kinetic studies showed that stretch increased the mean open time of the channels and decreased the slowest time constant of their closed-time distributions. The present as well as previous results suggest complex interactions between embryonic BK channels and their membranous and submembranous environment.

Key words: Ca^{2+}-activated K^+ channels — Stretch — Mechanosensitivity — Cytoskeleton — Rat Neuroepithelium — Patch clamp

Introduction

Since its original description in chick muscle [3], stretch sensitivity in ion channels has been found to span the entire evolutionary tree (for reviews, *see* [17, 18]), suggesting both an ancient biological origin and a crucial role for cell function. Accordingly, ionic selectivity and conductance of stretch-activated (SA) channels are varied [17]. Further, a number of channels initially classified in terms of their voltage or ligand modulation have been shown to be stretch sensitive, an example being the serotonin-sensitive S channel in *Aplysia* [24]. Consistent with the large variability that characterizes large-conductance Ca^{2+}-activated K^+ (BK) channels [12], stretch sensitivity is absent from skeletal muscle BK channels [3], whereas it is an intrinsic property of these same channels in mammalian cortical collecting tubules [16], vascular smooth muscle [1, 7], and human macrophages [8]. A different physiological significance for each of these cases has been suggested, namely osmoregulation, myogenic tone and cell adherence, respectively.

Our report extends the observation of the mechanosensitive properties of BK channels to the rat embryonic nervous system. In view of the proliferative state of the cells in our preparation, we can speculate that stretch-sensitive BK channels may play a developmental role, as previously suggested for SA channels in snail neurons [22].

Materials and Methods

The methods have been described in detail elsewhere [11]. In brief, the preparation consists of embryonic (E13–14) rat cerebral vesicles laid in a chamber so that the cells that line the ventricles and compose the proliferative neuroepithelium are accessible to *in situ* recording. The superfusion medium has been slightly modified. It now consists of (mM) 120 NaCl, 5 KCl, 1.25 NaH_2PO_4, 4 $MgCl_2$, 26 $NaHCO_3$, 1 Na pyruvate, 10 dextrose, 1 EGTA, (pH = 7.4 when gassed with 95% O_2, 5% CO_2), and is supplemented with essential amino acids and minimum essential medium vitamins (Gibco). The reasons for a Ca^{2+}-free medium are (i) to obviate cell ''cleaning'' [2], (ii) to facilitate inside-out patch formation [4], and (iii) to prevent Ca^{2+}-contamination when appropriate. Patch pipettes were pulled in two steps (Mecanex BB-CH, Geneva, Switzerland) from borosilicate glass tubing (TW150F-4, WPI, Sarasota, FL) and were fire polished (MF-9, Narishige, Japan) to yield resistances of 6–8 MΩ when filled with a solution containing (mM) 135.2 K Gluconate, 10 KCl, 10 HEPES, and 4.8 KOH (pH = 7.4, osmolarity adjusted to 310 mOsm with glucose). In a few experiments (n = 9), 5 mM EGTA was added to this solution to verify that stretch activation in Ca^{2+}-free medium was not due to contaminating Ca^{2+} being released from the pipette. All drugs were from Sigma (St. Louis,

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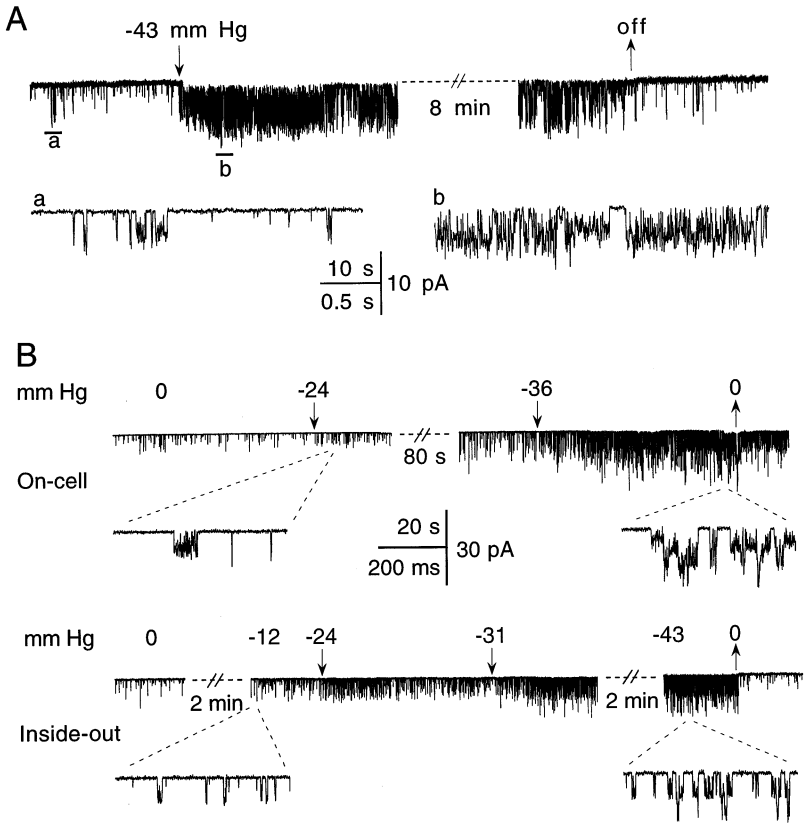

Fig. 1. Stretch activation of BK channels recorded from embryonic rat telencephalic cells. Onset, offset and level of pipette pressure are indicated above the slow-timebase traces. In this and all subsequent figures, unless otherwise noted, values given for filtering (*F*) and sampling (*S*) are those used for display only. (*A*) An inside-out patch was excised directly in the bath medium and held at a pipette potential (V_p) of +30 mV. Channels display buzz-mode activity as indicated by the lower traces expanded from the regions marked *a* and *b* ($F = 0.2$ KHz; $S = 0.5$ KHz). (*B*) In this and all subsequent figures, on-cell and inside-out patches were recorded at a V_p of +30 and +40 mV, respectively. For ''slow'' traces, $F = 0.1$ KHz and $S = 0.2$ KHz. Expanded traces ($F = 1$ KHz; $S = 2$ KHz) show buzz-mode gating in on-cell condition and normal-mode gating after channel conversion with trypsin (*see* Materials and Methods) in inside-out condition. The on-cell activity shown on the top trace returned to control in 32 min. Apparent variations in current amplitude are due either to filter-induced time-average of fast events (inside-out condition) or to a combination of the latter with buzz-mode gating (on-cell condition).

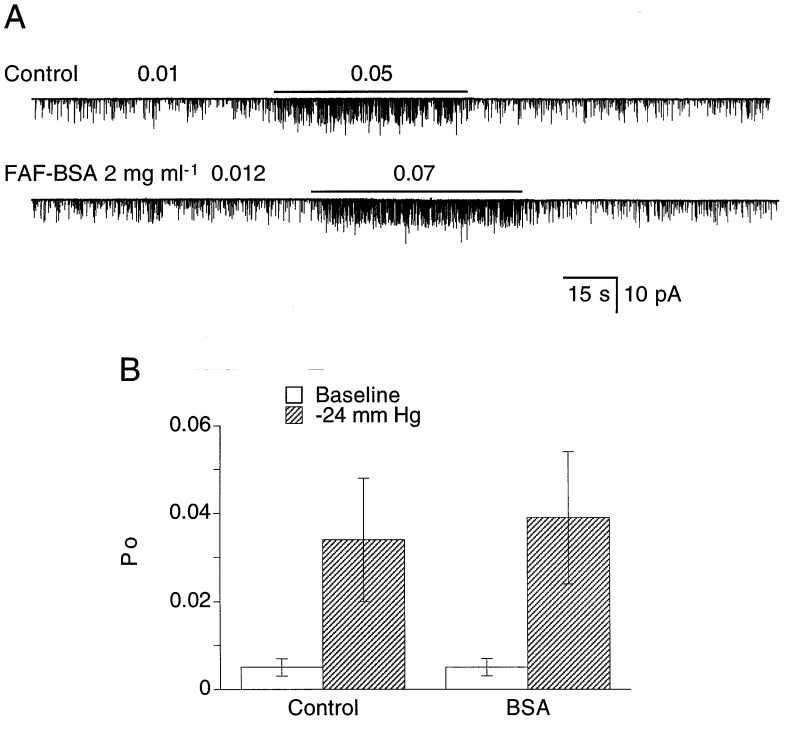

Fig. 2. Lack of effect of fatty-acid-free albumin (FAF-BSA) on the stretch sensitivity of BK channels. (*A*) Continuous traces ($F = 0.1$ KHz; $S = 0.2$ KHz) showing an inside-out patch challenged with −24 mm Hg of pressure (bars above traces) before and after an application of BSA that was started at the offset of the first pressure challenge. Values above traces indicate open probability (P_o). (*B*) P_o was measured in seven patches showing lack or rare occurrences of simultaneous openings (number of unitary steps, i.e., number of channels, $\leqslant 2$) either directly with a standard half-amplitude threshold protocol (*see* ref. 12), or from amplitude histograms as described in Materials and Methods. Both methods gave virtually identical results.

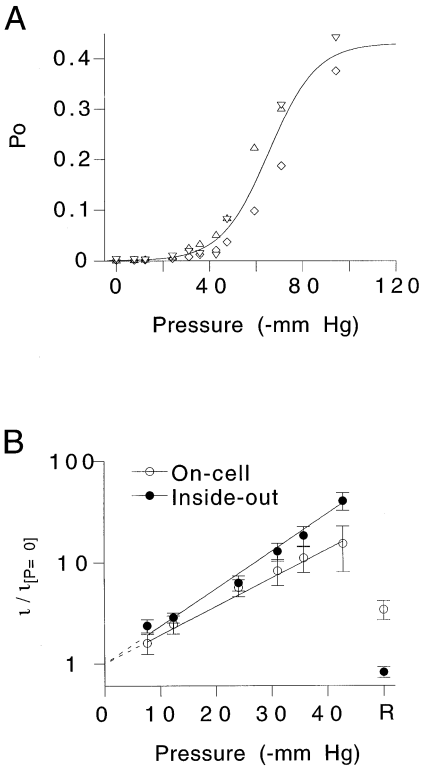

Fig. 3. Pressure-activation relationships for embryonic BK channels recorded from on-cell and inside-out patches in the same conditions as in Fig. 1*B*. (*A*) Pressure *vs.* open probability (P_o*;* calculated from amplitude histograms) data from 3 inside-out patches (*cf.* different symbols) were fitted with a Boltzmann equation of slope parameter = 10.2 mm Hg^{-1} (*see* text for remaining parameters). (*B*) Average current (ι) at a given pressure was normalized to the current in the absence of pressure. $\iota_{[P\ =\ 0]}$ was equal to 0.27 ± 0.06 pA (n = 15) for on-cell condition and 0.12 ± 0.06 pA (n = 15) for inside-out patches. The data were fitted with an exponential constrained to an intercept of 1. Recovery values (*R*) were estimated either immediately (inside-out) or when channel activity had reached a minimum (on-cell), i.e., 111 ± 24 sec after pressure release.

MO) and were dissolved in water or directly in recording medium, except for cytochalasins which were dissolved at 10 mM in DMSO.

The cell-attached (on-cell) and inside-out configurations of the patch-clamp technique [4] were used. It should be noted that ''gentle'' GΩ seals [5] were readily obtained upon release of an initial—and very slight—positive pressure after cell contact. Routine experiments in this laboratory have allowed unambiguous identification of BK channels. A striking feature of these channels is their *buzz* gating mode [9], which prevents both resolution of open amplitude and kinetic analysis, but can be converted to normal mode by perfusion of inside-out patches with 0.03 mg ml^{-1} trypsin [11]. To compare on-cell *vs.* inside-out patch sensitivity, and since channel conversion in on-cell patches is not feasible, we expressed channel activity as average current (indicated by ι). ι was determined from amplitude distributions of records 30–120 sec long and relates to open probability (P_o) according to the formula: $\iota = i \cdot P_o \cdot N$, where i is unitary amplitude and N the number of channels in the patch. During on-cell recording, we made sure that cell resting potential was constant by checking abrupt changes in i. Despite buzz-mode gating, we are confident that we could resolve at least a 30% change in i, which corresponds to a 30% change in driving force, or <30 mV. Given the low voltage sensitivity of these channels (e-fold change in P_o per ~60 mV depolarization; [11, 12]), any variation in resting potential below that range would thus have little impact on the results. To study stretch activation, suction was applied to the patch pipette through a micrometer syringe and pressure was monitored through a manometer (Model PM01D, WPI). Patch configuration within the pipette apparently remained stable throughout any given recording. The recording protocol and kinetic analysis (done with SES-PAT software, courtesy of J. Dempster) were exactly as described before [13]. Current was filtered at 2 KHz (8-pole Bessel) and digitized at either 10 KHz or 20 KHz (the latter for kinetic analysis). Data are reported as mean ± SE.

Results

Stretch activation was studied in cell-attached and inside-out patches during separate experiments. Previous work had revealed a consistent Ca^{2+}- and voltage-independent runup of neuroepithelial BK channel activity toward near-maximal open probability (P_o) following inside-out patch excision [12]. This phenomenon would seriously hamper any study of channel modulation were it not for the property of ''intracellular'' trypsin to downregulate and literally ''titrate'' P_o according to its application time. Consequently, this procedure was applied here whereby inside-out patches were exposed to trypsin for both gating mode conversion (*cf.* Materials and Methods) and P_o downregulation. In both cell-attached and inside-out configurations, channel activity was increased by negative pressures applied to the patch pipette (Fig. 1). Unspecific leak was routinely checked by monitoring seal resistance and by inverting patch potential in inside-out patches (which, due to the K^+ gradient, resulted in a flat baseline if seal integrity was preserved). To check whether buzz-mode gating (*cf.* Materials and Methods) and/or trypsin treatment constituted specific conditions, we examined the effect of stretch on untreated inside-out patches in which channel activity (in the buzz mode) was moderate. In those patches (n = 5), the onset, offset and degree of stretch activation did not appear to be different from that of treated inside-out patches (Fig. 1*A*). Occasionally, some form of adaptation (rundown) during sustained pressure was seen (Fig. 1*A*), but this was not investigated further. Upon release of the negative pressure, stretch-induced activity recovered instantly in inside-out patches (Fig. 1*A* and *B*), whereas on-cell activity did not immediately return to baseline levels (Fig. 1*B*). In fact, recovery on-cell was mostly partial and, in a few cases, absent. This evoked the possibility that a soluble mediator released during membrane stretch remained around in on-cell patches and was responsible for activation. This putative mediator cannot be Ca^{2+}since inside-out patches were activated in Ca^{2+}-free medium (*cf.* Materials and Methods). (On BK channel activation in zero Ca^{2+}, *see* [1, 7, 12]). As an alternative candidate, a recent study on small-conductance SA channels [15] suggested fatty acids by showing that defatted albumin, a fatty acid ''buffer,'' decreased the response to stretch.

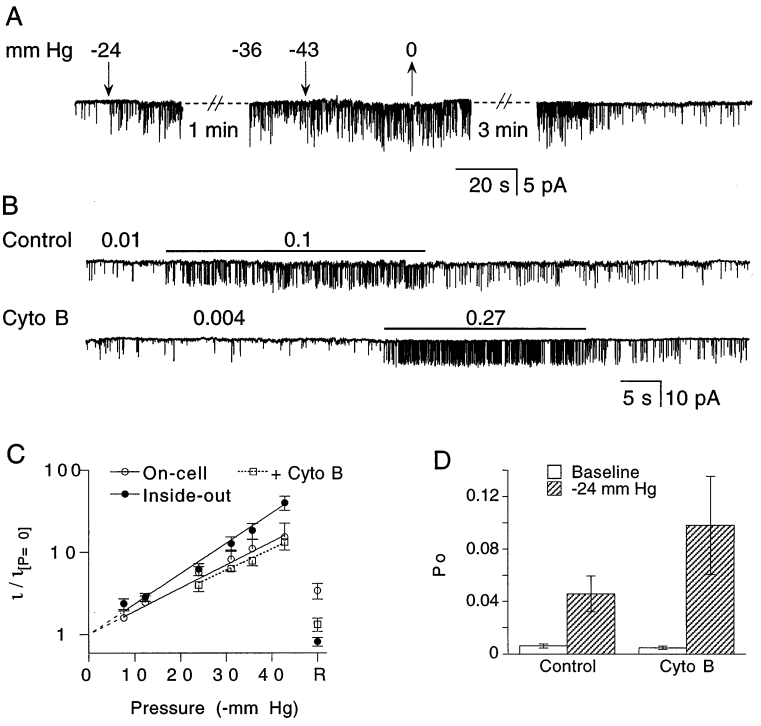

Fig. 4. Effects of cytochalasin B on stretch activation of BK channels. (*A*) On-cell recording from a preparation exposed for 2.25 hr to 20 μM cytochalasin B. The baseline activity shown at the initial part of the trace was increased by consecutive pressure challenges as indicated. Note that it took >3 min after pressure release for channel activity to return to control. (*B*) Recording of an inside-out patch subjected to −24 mm Hg of pressure (bars above traces) before and after an application of 10 μM cytochalasin B that was started at the onset of the first pressure challenge. Seventy seconds elapsed between the two traces. Numbers indicate open probability (P_o). $F = 0.1$ KHz and $S = 0.2$ KHz for both *A* and *B*. (*C*) Figure 3*B* is reproduced here for comparison with data from 4 cell-attached patches recorded after exposure to 20 μM cytochalasin B for 2–5 hours. Recovery in cyto B was measured 79 ± 50 sec after pressure release. (*D*) P_o was measured as in Fig. 2*B*. For each condition (control *vs.* cyto B), baseline P_o was subtracted from pressure-activated P_o and the differences compared (*see* text).

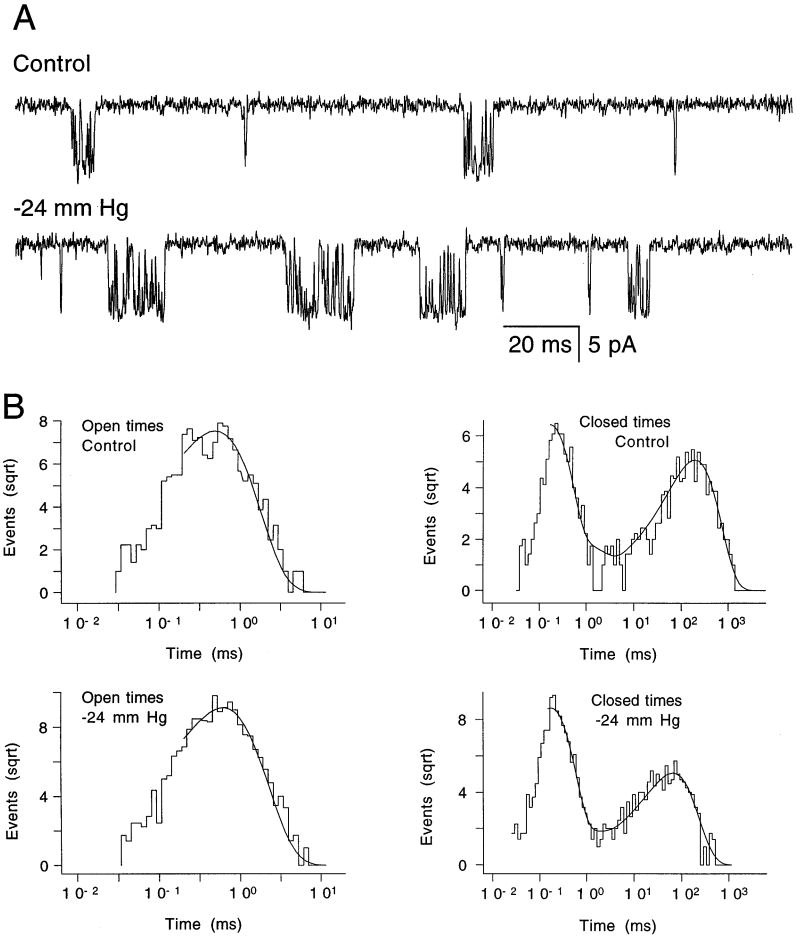

Fig. 5. Effects of stretch activation on BK channel kinetics. (*A*) High-resolution traces from an inside-out patch recorded in control and test conditions are displayed with the settings used for analysis: $F = 2$ KHz; $S = 20$ KHz. (*B*) Open- and closed-time distributions (same patch as in *A*) are displayed on a square-root (variance-stabilizing) *vs.* log-time scale (*see* [13]). Events shorter than twice the dead time of the recording system (2 × 0.1 msec) were excluded from the fit. Control and test open times were fitted with single exponentials of $\tau = 0.46$ and 0.58 msec, respectively. Control closed times were fitted with three τs $= 0.14$, 0.78 and 161.8 msec and having respective weights of 58, 4 and 38%. Under stretch, these values were 0.16 msec (72%), 0.8 msec (3%) and 61.3 msec (25%).

Table. Comparison of BK channel kinetics in control and under application of a negative pressure (τs in msec)

	τ_o	τ_{c1}	%	τ_{c2}	%	τ_{c3}	%
Control	0.48 ± 0.03	0.14 ± 0.01	57 ± 5	0.75 ± 0.03	3.7 ± 0.7	274.6 ± 66.7	39 ± 5
−24 mm Hg	0.61 ± 0.02	0.16 ± 0.01	74 ± 3	0.81 ± 0.02	2.3 ± 0.4	81.8 ± 18.3	24 ± 3
P	0.0002	0.32	0.0007	0.35	0.14	0.008	0.0003

Kinetics were studied in seven inside-out patches from dwell-time distributions yielding one open (τ_o) and three closed time constants (τ_c), the respective contribution of which (%) is also given. The *P* values were obtained from paired *t*-tests.

Using similar protocols, we did not find any effect of defatted albumin (2 mg ml^{-1}) on stretch-activated responses (Fig. 2). Results similar to ours have been reported with regard to three K^+-selective SA channels from rat brain [6].

Saturation of stretch-induced activation could not be obtained due to patch rupture before maximal activation. However, results from three inside-out patches tested with negative pressures up to 94 mm Hg suggested a pressure of half-activation of ~66 mm Hg and a $P_o$max of ~0.43 (Fig. 3*A*). Activation *vs.* pressure data from 15 on-cell and 15 inside-out patches are plotted in Fig. 3*B*. Mean current (ι) increased e-fold per 11.7 ± 0.3 mm Hg in inside-out patches and 15.1 ± 0.7 mm Hg in on-cell patches. As noted above, recovery was markedly different in the two experimental conditions. Furthermore, the dispersion in the degree of activation was larger in the on-cell condition (*cf.* error bars). Differences between the two conditions could arise from an involvement of the cytoskeleton, which led us to test the effects of the microfilament disrupter cytochalasin B. In the cell-attached condition, the drug had no apparent effect on stretch activation (Fig. 4*A* and *C*) nor did it suppress the delay in recovery (Fig. 4*A*), although the *extent* of recovery appeared enhanced (Fig. 4*C*). In the inside-out condition, cytochalasin B appeared to increase the sensitivity to stretch ($p = 0.06$, one-tail paired *t*-test; Fig. 4*B* and *D*). In the same line of investigation, we were interested in extending observations from a previous work that, as mentioned earlier, showed run-up of BK channel activity upon patch excision. Since searching for endogenous soluble inhibitors had yielded negative results [12], we hypothesized that the unaltered cytoskeleton in the intact cell (cell-attached configuration) may somehow exert a tonic inhibition on the channel. We thus tried to provoke on-cell the type of ''spontaneous'' activation that follows patch excision by superfusing the cells with inhibitors of various components of the cytoskeleton (concentration, number of experiments and min-max time of application given in brackets): neither colchicine (0.1–1 mM; $n = 5$; 10–24 min), a microtubule inhibitor, cytochalasins *B* and *D* (0.1 mM; $n = 13$; 10–20 min), two microfilament disrupters, nor acrylamide (10 mM; $n = 5$; 10–15 min), an intermediate filament disrupter [20], modified on-cell activity.

Finally, kinetics were analyzed in inside-out patches containing single channels (no superimposed openings) converted to normal mode by a brief exposure to trypsin (Fig. 5*A*). In low intracellular [K^+] conditions, BK channels display one open and three closed time constants (τ; Fig. 5*B*) [13]. The comparative kinetics are reported in the Table. Under stretch only two kinetic components, the fast and intermediate closed τ (and the latter's area), were *not* affected. The largest contribution to the elevated P_o came from a reduction of the slowest closed τ, but an increase in mean open time also contributed. The effect of stretch on mean open time contrasts with most published results although a close look at others' data suggests a systematic (though not statistically significant) trend toward similar increases (*see* e.g. [3, 22]).

Discussion

In the present study, we have found that BK channels in the immature brain are stretch sensitive. The range of sensitivity (e-fold change in activity per 10–15 mm Hg of pressure) is comparable to that of other SA channels [18], although somewhat on the low side [6]. A clear difference between inside-out and cell-attached patches was the delayed (or lack of) recovery from activation in the latter condition. After having ruled out soluble mediators, we looked at a possible involvement of the cytoskeleton. This was further motivated by the finding that patch excision elicits a runup of channel activity and by the logical inference that channels *in situ* may be repressed by unknown factors [12]. Although cytochalasin B appeared to increase stretch sensitivity in inside-out patches, it failed to modify the basic features of stretch activation on-cell or mimic the effect of patch excision. These observations would be consistent with a failure of the drug to reach its target during on-cell recording. However, we do not believe this is the case; the drug was effective at low concentration and with a fast onset in inside-out patches, which, given the good membrane solubility of the drug, should have been matched by the higher concentrations and longer exposures used in the on-cell condition. Also, in regard to the second (stretch-independent) aspect of our investigation, it has recently been reported that BK channel activity on-cell is not affected by prolonged cytochalasin treatments that modify cell morphology [19]. Therefore, it can be said that the cytoskeleton does have an influence on stretch sensitivity, as indeed found previously by others [3, 23],

but some of the transduction mechanisms involved are still unknown. It might be relevant to note that trypsin did not seem to have any major impact although, given its properties toward the channel's intrinsic function, the most that can be said is that it does not prevent stretch activation. One possible interpretation of our results is that the negative pressures used on-cell to stimulate tonically inhibited channels somehow mimic patch excision, perhaps through mechanical separation of the channel from its inhibitory environment. The latter could be an overriding component of the cytoskeleton that is inaccessible to cytochalasin in on-cell patches, while another cytoskeletal component vulnerable to the drug may be present in inside-out patches. Small & Morris [23] have similarly suggested the involvement of a dual system in mechanotransduction. An alternative explanation is that on-cell patches could preserve cytoskeletal integrity in such a way as to lessen cytochalasin's efficiency, while a cytoskeleton already altered by patch excision would promote drug effects.

These considerations bring up the question of the physiological relevance of our observations. There seems to be some discrepancy (at least one order of magnitude) between the tensions involved in the activation of SA channels (and of our BK channels) and those occurring physiologically [21]. Sachs [18] has suggested that the sensitivity of the channel to stretch may itself be under local regulation. In the same line of thought, Morris & Horn [14] suggested that nonpathological alterations of the cytoskeleton may ''activate'' mechanosensitivity. These ideas are relevant to our case since BK channels *in situ* seem to be under tonic inhibition, and we occasionally observe phasic activations evoking the possibility of some physiologic control (*unpublished observations*). Conjectured functions of mechanosensitive channels include a role in cell growth and division [17], a view born out by strong experimental evidence on K^+-selective SA channels [10]. Such a function would suit BK channels in E13–14 neuroepithelial cells for which such processes as proliferation in a limited space, cytokinesis, growth, migration and neurite formation all call upon tensile forces in the membrane.

Many thanks to Dr. Arnaldo Lasansky for his help and advice.

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