Cell Membrane Stretch Modulates the High-Conductance Ca^{2+}-Activated K^+ Channel in Bovine Trabecular Meshwork Cells

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Purpose. Anterior chamber structures are subjected to changes in intraocular pressure (IOP). Several studies have pointed out that trabecular meshwork (TM) cells are sensitive to mechanical stretch and that cell-signaling mechanisms are activated in response to elevated pressure. Because membrane stretch has been shown to be a modulator of several ionic conductances, this study was conducted to determine its effects on the high-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels present in TM cells.

Methods Primary cultures of TM cells from bovine eyes were used. Patch-clamp recordings were performed in the cell-attached, inside-out, and whole-cell configurations. To stretch the cell membrane, both suction to the rear end of the patch pipette and hypotonic shock were used. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured in TM cells loaded with fura-2, using an epifluorescence microscope coupled to a charge-coupled device (CCD) camera.

Results Electrophysiological characterization of BK_{Ca} channels was in agreement with previous studies. In cell-attached patches, the open probability of the BK_{Ca} channel (i.e., the amount of time the channel is open) increased consistently when 14- to 45-mm Hg suctions were applied at a constant depolarized voltage. At a constant pressure (25 or 45 mm Hg), channel openings increased when depolarizing pulses were applied to the patch. Stretch activation of the BK_{Ca} channel was not mediated by increases in $[Ca^{2+}]_i$, because it was present in inside-out patches maintained at a constant Ca^{2+} concentration. Nevertheless, it cannot be ruled out that at low suction levels, a minimum Ca^{2+} concentration is necessary for channel activation. Whole-cell currents carried by BK_{Ca} channels increased when the isotonic solution in the bath was exchanged with a hypotonic solution and were selectively blocked by iberiotoxin. In our conditions, the hypotonic shock did not modify $[Ca^{2+}]_i$.

Conclusions The data show that in TM cells, open probability of the BK_{Ca} channel is enhanced by membrane stretching as well as by membrane depolarization and $[Ca^{2+}]_i$. Changes in membrane tension induced by cell volume increase also activated whole-cell BK_{Ca} currents. Homeostatic mechanisms in TM cells may involve BK_{Ca} channel activation in response either to changes in cell volume or changes in IOP. (*Invest Ophthalmol Vis Sci.* 2003;44:706–714) DOI:10.1167/iovs.02-0384

Structures of the eye are subjected to variations in intraocular pressure (IOP) both under physiological and pathologic conditions. As is well known, IOP is maintained by the balance between production of aqueous humor in the ciliary epithelium and its outflow through the trabecular and uveoscleral routes. It is not known whether a sensory mechanism able to detect IOP variations is present in the eye and whether IOP can be continuously modulated. Nerve endings have been identified in the trabecular meshwork (TM), and some fibers located in the scleral spur have characteristics of mechanoreceptors.[1] Electrophysiological techniques have identified mechanoreceptors in the anterior uvea.[2] In addition, in TM and endothelial cells from Schlemm's canal,[3] changes in IOP induce secretion of mediators (such as $PGF_{2\alpha}$) to extracellular medium,[4] increases in intracellular calcium ($[Ca^{2+}]_i$)[5] or cyclic nucleotides,[6] morphologic changes and cytoskeletal rearrangements,[6] and upregulation of several genes.[7] All these findings may be involved in a homeostatic system that regulates TM resistance. In fact, TM cells modulate the pathway's permeability by volume and contractile responses.[8–10] Both actions are likely to induce membrane deformations that activate stretch-activated or mechanosensitive channels, as has been described in many cell types.[11] The main characteristic of this type of ionic channel is the response to membrane stress by changes in the channel's open probability (i.e., the amount of time the channel is open).[12] The high-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel has been well described in TM cells.[13] This type of channel, which is activated by membrane depolarization and by increases in $[Ca^{2+}]_i$, is linked to cell membrane repolarization[14,15] in response to depolarizations induced by different substances. BK_{Ca} channel activity is widely modulated by intracellular mediators, such as PKC or cyclic nucleotides. Also, BK_{Ca} activity can be directly modulated by the extracellular environment, and, for example, nitric oxide has been postulated to mediate cell relaxation in response to vasoactive substances.[14]

BK_{Ca} channels have demonstrated mechanosensitive properties in skeletal muscle,[16] osteoblasts,[17,18] smooth muscle,[19,20] and neuroepithelial cells.[21] Given this published evidence, we decided to study the mechanosensitivity of this channel in TM cells in response to stretching of the cell membrane achieved by two different methods. The present study shows that BK_{Ca} channel activity is modulated by changes in cell membrane tension in addition to its well-known activation by membrane depolarization and increase in $[Ca^{2+}]_i$. Our findings suggest that TM cell membrane deformation, together with membrane depolarization, constitutes a potential mode of regulation of the BK_{Ca} channel's opening. The

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results show that, in addition to other pressure-induced effects in TM cells, BK_{Ca} channel properties provide a possible mechanism to maintain homeostasis of the TM tissue and regulate the outflow pathway in response to changes in IOP.

METHODS

Culture of Bovine TM Cells

Bovine TM cells were cultured using a modification of the technique described by Stamer et al.[22] As described previously,[23] bovine TM strips were digested with 2 mg/mL collagenase (Sigma, Madrid, Spain) and 0.5 mg/mL bovine serum albumin (BSA; Sigma, Madrid, Spain) at 37°C for 2 hours. After mechanical digestion, the supernatant was collected, centrifuged, and resuspended. The resuspended solution was seeded in culture flasks containing Dulbecco's modified Eagle's medium (DMEM; BioWhitaker, Barcelona, Spain) plus 10% fetal bovine serum, 100 mg/mL L-glutamine (Sigma), 100 IU./mL penicillin, 100 μg/mL streptomycin, and 2.5 mg/mL amphotericin-B (BioWhitaker). Cell growth was observed 2 to 4 days after seeding and cells reached confluence 12 to 15 days later. Cell passages were performed using Trypsin-EDTA (BioWhitaker). Cells from passages 1 to 3 were used for electrophysiology and measurements of $[Ca^{2+}]_i$.

Cytosolic Free Ca^{2+} Measurement

Measurement of $[Ca^{2+}]_i$ was performed, as described in detail previously.[23,24] Briefly, bovine TM cells were plated on 25-mm diameter glass coverslips (VWR Scientific Inc., Philadelphia, PA) and then loaded with 5 μM fura-2/AM (Calbiochem, San Diego, CA) for 25 minutes at 37°C in incubation buffer (121 mM NaCl, 4.7 mM KCl, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, and 0.01% BSA [pH 7.4] with NaOH; 287 ± 2 mOsm/Kg; mean ± SD). Coverslips with fura-2–loaded cells were transferred into an open flow chamber (1 mL incubation buffer) mounted on the heated stage of an inverted epifluorescence microscope (Diaphot-300; Nikon, Tokyo, Japan). Fluorescent images were obtained by a charge-coupled device (CCD) camera (CH250; Photometrics, Tucson, AZ) and were digitized, stored, and analyzed on computer (Machintosh 840AV; Apple Computer, Cupertino, CA). After a stabilization period of 10 minutes, image pairs were obtained alternately every 4 seconds, and for a total of 8 minutes, at excitation wavelengths of 340 and 380 nm (10-nm bandwidth filters), to excite the Ca^{2+}-bound and Ca^{2+}-free forms of this ratiometric dye, respectively. The emission wavelength was 510 nm (120-nm bandwidth filter). $[Ca^{2+}]_i$ levels were calculated on a single-cell basis from the 340- to 380-nm fluorescence ratios at each time point.[23,25] After 1 minute in isotonic buffer, bath solution was exchanged by a hypotonic solution modified from the incubation buffer described earlier: 80 mM NaCl, 2.3 mM KCl, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, and 0.01% BSA [pH 7.4] with NaOH; 205 ± 3 mOsm/Kg; mean ± SD). In controls, sham maneuvers were performed by perfusing the cells with isotonic solution (287 ± 2 mOsm/kg; mean ± SD). In both control and experimental groups, Ca^{2+} was recorded for 1 minute in isotonic solution. Subsequently, the solution was exchanged and Ca^{2+} monitored for 7 minutes. Cells were considered to be responders when $[Ca^{2+}]_i$ increased more than 100% above the resting value.

Patch-Clamp Procedures

TM cells were plated onto small glass coverslips and studied 24 to 48 hours thereafter. Coverslips were transferred to a special chamber (0.2 mL) in the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan) to perform the recordings. External solutions were superfused at a rate of 3 to 4 mL/min by gravity. Before the recording session began, culture medium was replaced with the recording solution. Recordings were performed in 2-hour sessions at 21°C to 23°C. Patch pipettes were pulled in an electrode puller (P-97; Sutter Instruments, Novato, CA) and had a filled-tip resistance between 3 and 6 MΩ. Pipette capacitance to ground was neutralized after the seal was formed. An Ag/AgCl wire bath electrode was used. Care was taken to use gentle patches. Before the pipette had entered the bath and until cell contact, positive pressure was applied. Slight suction of far less amplitude than the one applied to induce a detectable stretch activation of channels, was applied to form the gigaseal. After we measured the suction levels used to form the gigaseal in several experiments, we found that they were always less then 10 mm Hg (6 ± 2 mm Hg; mean ± SD, $n = 12$).

Single-channel currents were recorded according to the procedure of Hamill et al.[26] with a patch-clamp amplifier (L/M-EPC7; Heka, Lambrecht, Germany), in the cell-attached and inside-out configurations. Data acquisition and command potentials were controlled by computer (WinWCP 2.1 and PAT 7.4 software developed by John Dempster, University of Strathclyde, Strathclyde, UK) using a commercial interface (CED1401; Cambridge Electronic Design Ltd., Cambridge, UK). Single-channel currents were sampled at 20 kHz, filtered at 3 kHz, and stored on the computer's hard disk. Command potential was set at 0 mV (the cell's resting voltage) and depolarizing or hyperpolarizing pulses were applied. Holding potentials reported in this study were the original values indicated on the amplifier and were not corrected for the liquid junction potential. Currents flowing into the pipette were considered to be positive. Open-channel amplitudes were calculated at each patch potential by use of all-point amplitude histograms or manually when few open channels were seen (e.g., at high hyperpolarized voltages). Open-channel probability (NPo) was calculated as: $NPo = (A_1 + 2A_2 + 3A_3 + \ldots + NA_N)/(A_0 + A_1 + A_2 + A_3 + \ldots + A_N)$ where A_0 is the area under the curve of amplitude histograms corresponding to current in the closed state, and $A_1 \ldots A_N$ represents the histogram's area, which reflects the different open-state current levels for 1 to N channels present in the patch. Histogram parameters were obtained from multiple least-squares Gaussian fits of the data using the PAT 7.4 software.

In single-channel experiments (cell-attached mode), the physiological bathing solution was (mM): NaCl 140, KCl 4.3, $CaCl_2$ 1.3, $MgCl_2$ 1, and HEPES 10 (pH 7.4) with NaOH. Patch pipette solution was (mM): KCl 140, $MgCl_2$ 2, EGTA 2, HEPES 12 (pH 7.4) with KOH. When patches were excised (inside-out mode), bath solution was exchanged by the patch pipette solution in which EGTA was replaced by one of three concentrations of $CaCl_2$ (1, 5, and 20 μM). In some experiments, the patch pipette solution was used as the bath solution. Under all these conditions, K^+ concentration was equal on both sides of the patch.

Whole-cell experiments were performed in the same experimental conditions as for the single-channel configuration. After cells were broken into the whole-cell configuration, they were allowed to stabilize and dialyze for 3 to 4 minutes before the experiment began. Whole-cell currents were recorded at 10 kHz. Leak current and residual capacitative current were subtracted with a *P/N* protocol where *N* is −4. Cells were clamped at −60 mV, and depolarizing pulses were applied in 10-mV steps to evoke outward K^+ currents. In whole-cell recordings, the solution in the pipette was (mM): KCl 140, $MgCl_2$ 2, EGTA 0.1, and HEPES 10 (pH 7.2) with KOH. The bath solution was the physiological solution (301 ± 5.1 mOsm/kg; mean ± SD) used in the cell-attached experiments or a hypotonic solution (213 ± 4.8 mOsm/kg) in which NaCl and KCl concentrations were reduced (mM): NaCl 100, KCl 2, $CaCl_2$ 1.3, $MgCl_2$ 1, and HEPES 10 (pH 7.4) with NaOH. Outward potassium currents were blocked with a specific BK_{Ca} blocker, iberiotoxin (IBTX, 50 nM) obtained from Sigma Chemical Co. (St. Louis, MO).

The patch membrane was stretched by applying negative pressure (suction) to the back end of the patch pipette with a calibrated syringe. Suction was monitored with a pressure transducer (9162-0, Mallinckrodt, Northampton, UK) that was calibrated with a water manometer.

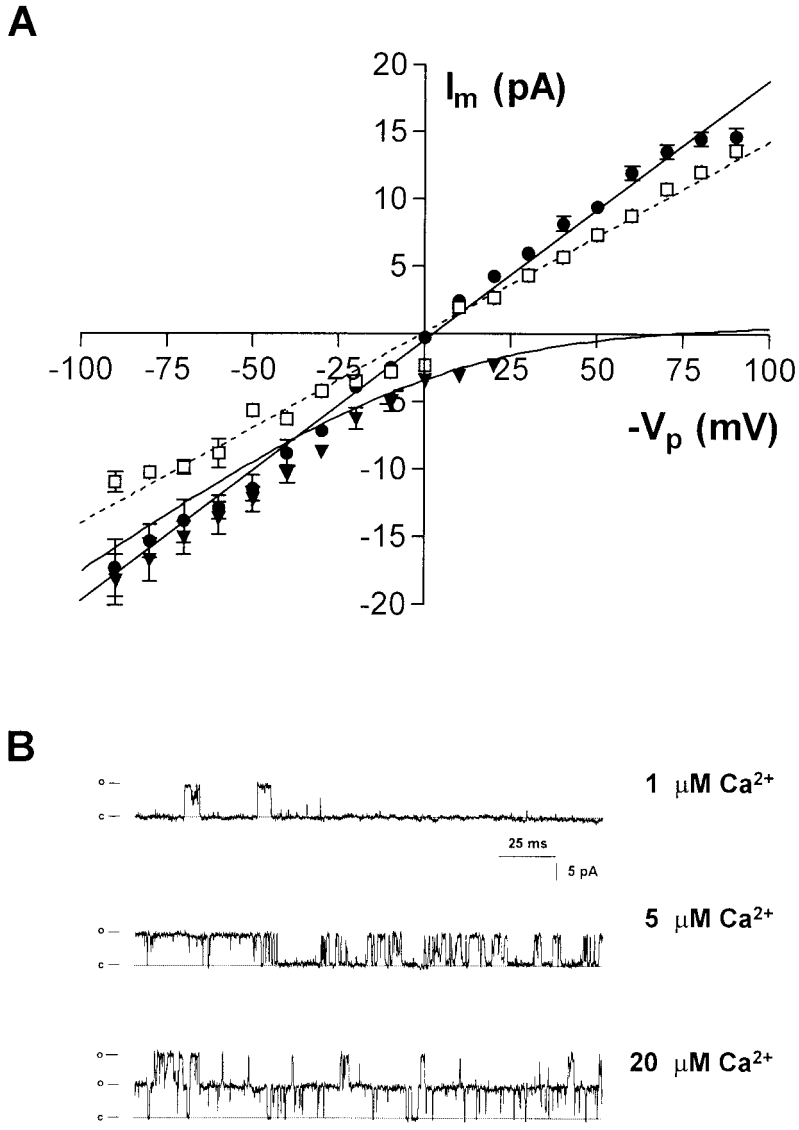

FIGURE 1. Biophysical characteristics of BK_{Ca} channels in TM cells. (**A**) Current–voltage relationship for the BK_{Ca} channel at different levels of $[K^+]$. Experiments using the cell-attached configuration were performed with (□) 140 mM $[K^+]_o$ (n = 31) in the pipette and physiological solution in the bath. *Dotted line*: Linear fitting of cell-attached experiments. Experiments using the inside-out configuration were performed with (●) 140 mM $[K^+]_{o,i}$ (n = 10) or (▼) 4.3 mM $[K^+]_i$ (n = 3). *Solid line*: linear and Goldman-Hodgkin-Katz fitting of inside-out experiments. (**B**) Single-channel BK_{Ca} currents recorded at different $[Ca^{2+}]$ in the inside-out configuration with symmetric 140 mM $[K^+]_{o,i}$. Cells were depolarized +40 mV (−Vp) over the resting potential. Open (o) and closed (c) states of the channel are shown. NPo increased with $[Ca^{2+}]$, showing the typical response of BK_{Ca} channels.

Data Analysis

Results are given as mean ± SEM. Results were statistically analyzed with a paired or unpaired Student's *t*-test. An ANOVA with Bonferroni post hoc test was used to evaluate statistical differences between control recordings and effects of suction application in channel activity and to compare the effects of isotonic and hypotonic solutions. $P < 0.05$ was considered statistically significant.

RESULTS

Patch-clamp experiments in the single-channel configuration performed in TM cells showed outward currents at depolarizing potentials that were identified as the BK_{Ca} channel previously described.[13] Cell-attached experiments with 140 mM K^+ ($[K^+]_o$) in the pipette showed a linear current–voltage relationship with a channel conductance of 142 ± 4 pS (n = 31; Fig. 1A). Because in the cell-attached configuration the K^+ concentration inside the cell is unknown, channel conductance is only approximate. However, in inside-out experiments with 140 mM K^+ at both sides of the patch, channel conductance was 193 ± 3 pS (n = 10; Fig. 1A). The channel had a reversal potential near zero in symmetric K^+ solutions. In inside-out patches, where bath solution was changed to physiological solution (n = 3) in which the ratio of $[K^+]_o$ to $[K^+]_i$ was 140:4.3 mM, the current–voltage curve displayed constant field rectification that fitted with the superimposed curve generated with the Goldman-Hodgkin-Katz equation (Fig. 1A). These data show that the channel was highly selective for K^+ and excluded the participation of other ionic species (e.g., Cl^-). Figure 1A shows the rectification exhibited by the channel when nonsymmetric solutions were used. At depolarizing voltages higher than +20 mV, the channel openings were very small and could not be separated from the recording noise. The channel was activated by increasing $[Ca^{2+}]$ at the intracellular side of the inside-out patch (bath solution in the inside-out configuration). At 1 μM Ca^{2+} and +40 mV, the NPo of the channel was 0.36 ± 0.18 (n = 4; Fig. 1B). NPo increased to 0.57 ± 0.28 and to 1.47 ± 0.73 when $[Ca^{2+}]$ was increased to 5 μM Ca^{2+} and 20 μM Ca^{2+}, respectively ($P < 0.05$ for both vs. 1 μM Ca^{2+}). These results demonstrate the Ca^{2+} dependency of the K^+ channel described (Fig. 1B). All these data are in general agreement with literature results,[13] but a basic characterization was necessary to use in the following experiments. The differences observed in channel conductance were expected, because the experiments performed by Stumpff et al.[13] were performed at 37°C.

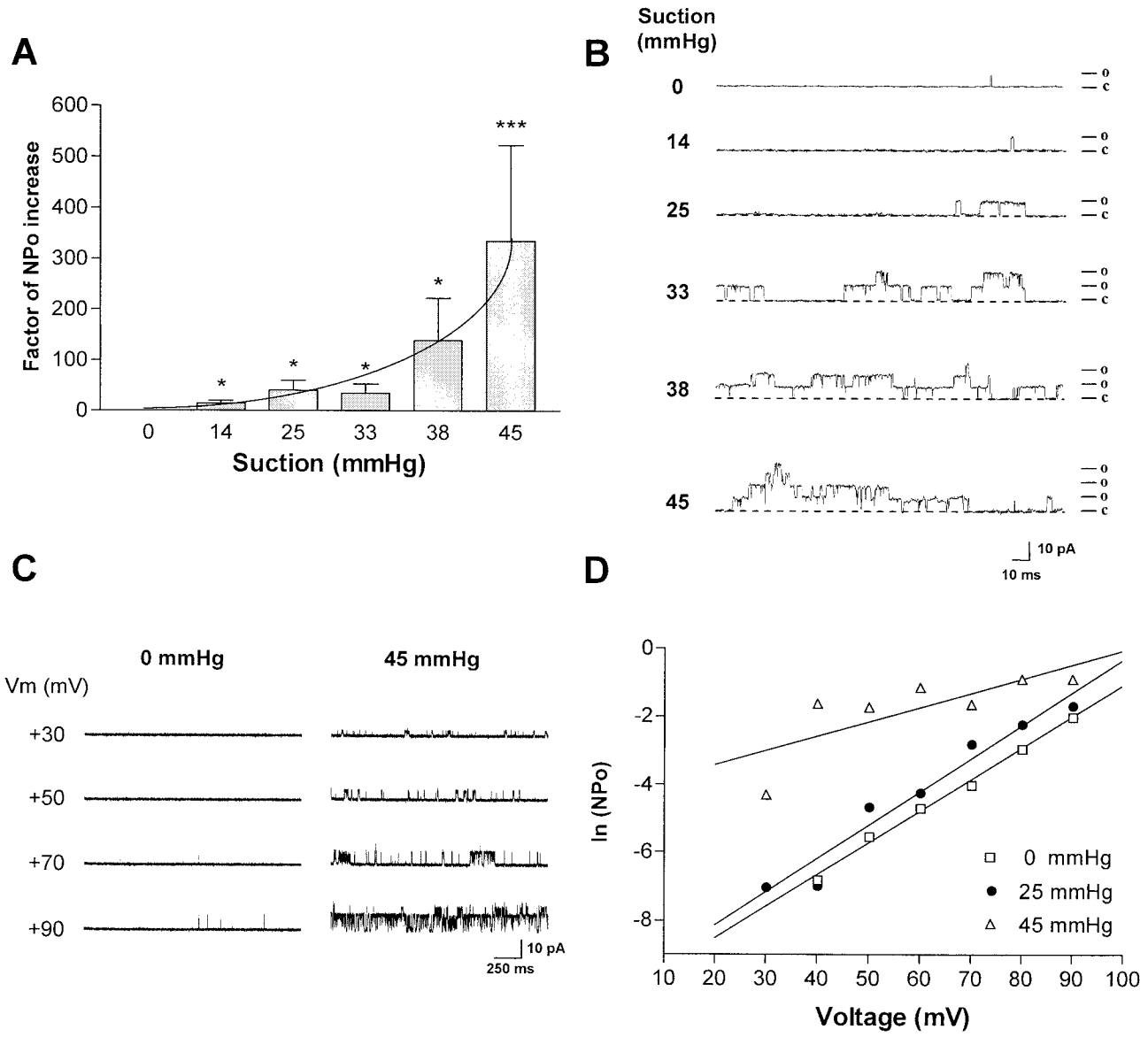

FIGURE 2. Stretch activation of BK_{Ca} channels in the cell-attached configuration in TM cells. (**A**) The ordinate of the plot shows the factor of NPo increase versus the applied suction (mm Hg) at +40 mV (−Vp: pipette voltage) over the resting potential with $[K^+]_o$ 140 mM. All values were normalized with the NPo at 0 mm Hg. Data are the mean ± SEM for 16 different patches (ANOVA, $P < 0.001$). Statistical differences for each group versus the control (0 mm Hg): $^*P < 0.05$ and $^{***}P < 0.001$. (**B**) Recordings obtained from the experiments shown in (**A**). Open (o) and closed (c) states of the channel are shown. (**C**) Four voltage recordings at 0 mm Hg (control) and at 45 mm Hg. Voltages are −Vp. Suction was maintained constant during application of voltage pulses. During recordings at 0 mm Hg, only brief openings of channels were recorded. At 45 mm Hg the number of channel openings increased. (**D**) The natural logarithm of NPo in ordinates versus the voltage applied in abscissa (−Vp). Each point represents the mean of the different recordings performed in each group. (□) $n = 12$; (●) $n = 12$; (△) $n = 6$. Linear fittings are superimposed.

Membrane Stretching

Stretch-induced activation of BK_{Ca} channels was observed in TM cells (Fig. 2). The effect of suction application was tested at +40 mV (Figs. 2A, 2B). Channel NPo increased exponentially with pressure (Fig. 2A). Statistical differences (ANOVA, $P <$ 0.001) were found between control recordings (0 mm Hg) and all groups between 14 and 45 mm Hg (Fig. 2A). In most of the experiments, similar to the example shown in Figure 2B, application of suction increased channel NPo, and frequently more than one active channel was observed simultaneously. To confirm that the channel activated by stretching was the BK_{Ca}, the cell-attached patches were excised at the end of each experiment into physiological solution. Under these conditions, Goldman-Hodgkin-Katz rectification was observed (Fig. 1A). Single-channel current measurements indicated that unitary channel conductance was not modified by stretching the membrane.

The effect of voltage on channel activity in presence or absence of stretching was studied to evaluate the relationship among different channel activators (voltage and stretch; Figs. 2C, 2D). Voltage activation of BK_{Ca} channels is described by a Boltzmann relationship. Thus, the plot of the natural log of the product of the number of channels present in the patch (N) and the probability that a single channel is open (Po) as a function of voltage should be linear at low values of Po.[27] The reciprocal of the slope (a measure of voltage sensitivity) is the potential needed to produce an e-fold change in Po at low Po. We tested two different conditions, 25 and 45 mm Hg of

suction, compared with no suction (0 mm Hg). Activation by suction did not affect the voltage sensitivity of the channels at 25 mm Hg (10.24 and 10.77 mV for an e-fold change in NPo in the presence and absence of 25 mm Hg of suction; Fig. 2D), but it increased channel activity by a constant multiplicative factor. The voltage sensing of the channel was affected by stretching at 45 mm Hg (Fig. 2D), and voltage sensitivity was 23.72 mV for an e-fold change in NPo. These results can be attributed to the high NPo at 45 mm Hg, at which pressure linearity of the NPo versus voltage is low. Figure 2C shows a typical experiment in which number of channel openings increased both by application of suction and depolarization of the cell-attached patch.

Because the BK_{Ca} channel activity is enhanced by Ca^{2+} and because release of Ca^{2+} from intracellular stores or entrance of Ca^{2+} through the plasma membrane by stretch stimulus application remained a possibility, we performed experiments in the inside-out mode to rule out these possibilities.

To study the relation between Ca^{2+} and pressure on channel activity, after obtaining a cell-attached patch, the bath solution was changed to the pipette solution (1 μM Ca^{2+}), with or without EGTA (2 mM). In these conditions, the patch was excised and the inside-out mode was achieved.

At constant Ca^{2+} concentration, application of pressure increased the number of channel openings at both 25 and 45 mm Hg (Fig. 3A). Channel activation induced by stretch was reversible, and release of pressure returned NPo to basal levels (0 mm Hg; Figs. 3). When Ca^{2+} concentration was low, NPo was low, but channel activation still could be induced by stretching the inside-out patch.

Hypotonic Stretching

Hypotonic shock has been used in other cell types to stretch the cell membrane.[17,28,29] This procedure increases cell membrane tension due to cell swelling. We evaluated the effect of an hypotonic stimulus on whole-cell currents mediated by BK_{Ca} channels in TM cells. Because hypotonic shock could increase $[Ca^{2+}]_i$ and therefore activate BK_{Ca} channels, control experiments were conducted to exclude this possibility. $[Ca^{2+}]_i$ was monitored on TM cells loaded with fura-2, as described in the Methods section. After 1 minute of recording on isotonic buffer, perfusion of either isotonic (as the control; 287 ± 2 mOsm/kg; mean ± SD) or hypotonic buffer (205 ± 3 mOsm/kg; mean ± SD) was performed and $[Ca^{2+}]_i$ was monitored for 7 minutes.

In control conditions (isotonic buffer), only 3% of the cells (n = 73) increased $[Ca^{2+}]_i$ from 57 ± 3 to 138 ± 5 nM (mean ± SEM). Perfusion with hypotonic solution induced a $[Ca^{2+}]_i$ increase in 9% of the cells (n = 85) from 58 ± 3 to 147 ± 5 nM, a nonsignificant response compared with the isotonic solution. Therefore, effects of hypotonic shock on BK_{Ca} activity cannot be attributed to Ca^{2+}-mediated activation of the channel.

Assuming that in the experimental conditions used the larger part of the outward K^+ current was mediated by BK_{Ca} channels in TM cells,[13] whole-cell experiments were performed to evaluate outward K^+ currents in response to hypotonic solutions (Fig. 4). Whole-cell currents were first recorded in isotonic medium (control) and afterward, bath solution was replaced by hypotonic medium and hypotonic medium plus IBTX. Cells were clamped at −60 mV and depolarizing pulses to +80 mV were applied in 10-mV steps to evoke outward K^+ currents. In 14 of 17 whole-cell experiments (82% of the cells), the total outward K^+ current increased significantly (ANOVA, $P < 0.001$) in hypotonic medium compared with isotonic medium for the whole voltage range. The Bonferroni post hoc tests showed statistical differences at +70 and +80 mV

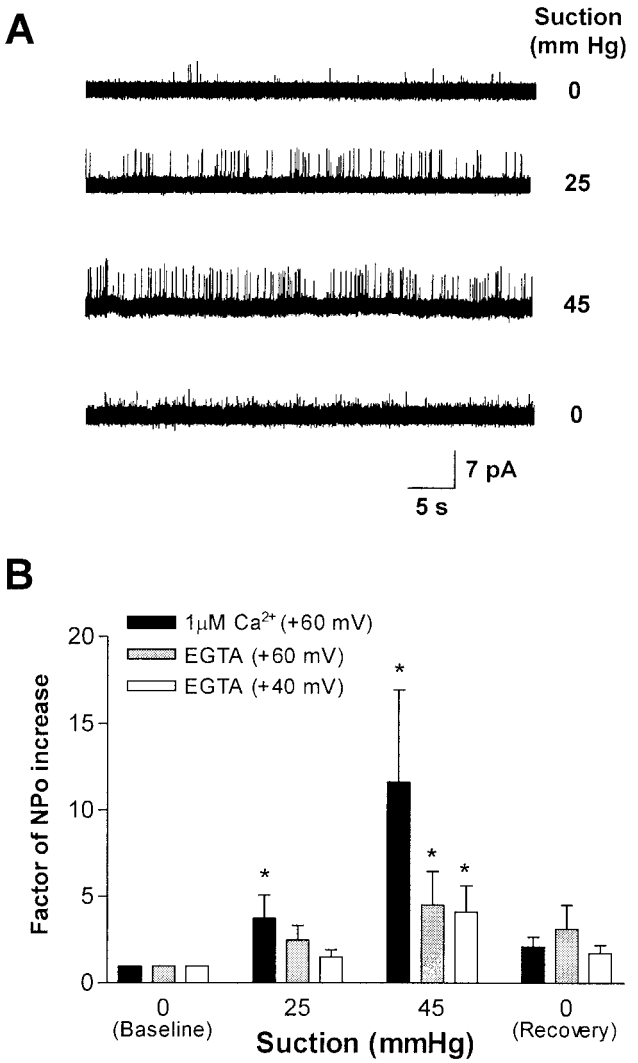

FIGURE 3. Stretch activation of TM cells BK_{Ca} channels in inside-out patches with symmetric 140 mM K^+ at both sides of the patch. (**A**) Recordings were performed at +60 mV over the resting potential (−Vp: pipette voltage) and $[Ca^{2+}]$ was constant (nominally zero Ca^{2+} plus 2 mM EGTA). Channel openings are seen as upward deflections. From the top recording to the bottom, 0, 25, 45, and 0 mm Hg were applied successively. (**B**) The ordinate of the plot shows the factor of increase in NPo versus the applied suction for the different conditions tested: +40 or +60 mV (−Vp) over the resting potential. Each column represents the average (mean ± SEM) of five inside-out patches for each group: 1 μM Ca^{2+} and +60 mV; nominally zero Ca^{2+} plus 2 mM EGTA at +60 mV over the resting potential and nominally zero Ca^{2+} plus 2 mM EGTA at +40 mV over the resting potential. All data were normalized with NPo at 0 mm Hg. Note the different time scale versus the recordings shown in Figures 1 and 2. Under low Ca^{2+} conditions, brief openings of the channel occurred.

($P < 0.05$ and $P < 0.001$, respectively) between the isotonic and hypotonic groups. In hypotonic solution, a 3.21 ± 0.42-fold increase in K^+ current was found. The remaining three cells did not exhibit significant changes. Figure 4A shows the current–voltage relationship in isotonic and hypotonic solutions. It can be noted that in hypotonic conditions, K^+ currents were activated at voltages lower than those in control experiments. Currents normalized with cell membrane capacitance did not show differences with the ones shown in Fig. 4A (raw currents). As described elsewhere,[30,31] two parameters could be used to characterize

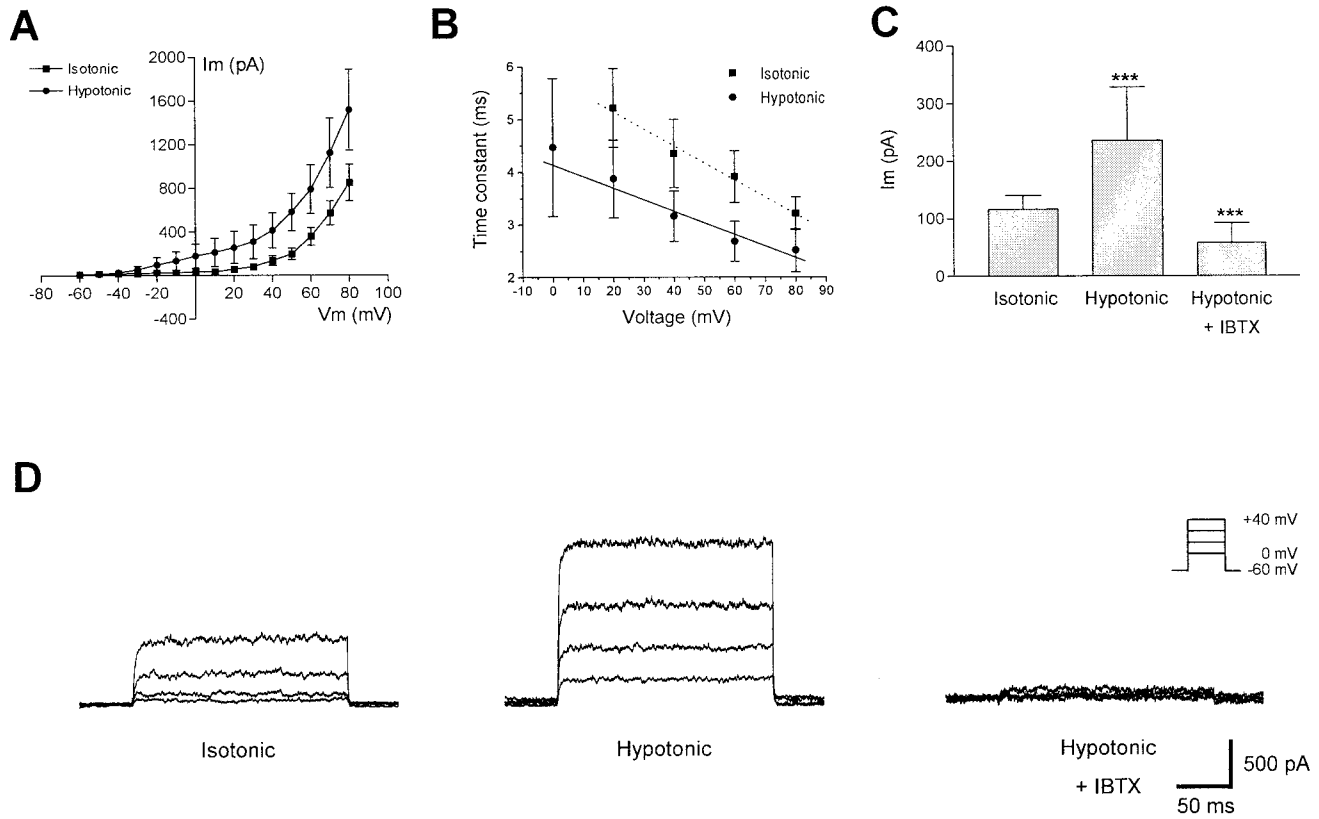

FIGURE 4. **(A)** Current–voltage curves showing outward K^+ currents of TM cells in the whole-cell configuration. The abscissa shows the membrane voltage (Vm), and the ordinate shows the membrane current (Im). Holding potential in the whole-cell configuration was −60 mV (Vm). Experiments were performed with a physiological solution in the bath and high-K^+ solution in the pipette (140 mM K^+). Isotonic medium: 301 mOsm/kg; $n = 11$ cells; hypotonic medium: 213 mOsm/kg; $n = 11$. BK_{Ca} current increased significantly ($P < 0.001$, two-way ANOVA). **(B)** Activation time constants of BK_{Ca} currents in isotonic and hypotonic medium. Data are the mean ± SEM ($n = 15$ cells). Linear regressions are shown as *dotted* (isotonic; $r^2 = 0.99$) and *solid* (hypotonic; $r^2 = 0.96$) *lines.* **(C)** Effects of isotonic ($n = 17$) and hypotonic medium (alone, $n = 17$, or with IBTX; 50 nM, $n = 6$) on whole-cell BK_{Ca} currents at +10 mV. **(D)** Representative experiment showing whole-cell BK_{Ca} current in a TM cell in isotonic, hypotonic, or hypotonic+IBTX medium. Voltage pulses of 200 ms were applied from −60 mV (holding voltage) in 10-mV steps. Pulses from 0 to +40 mV are shown.

BK_{Ca} currents: the fraction of the BK_{Ca} channels in a given population that open at a given potential and the time constants with which they activate and deactivate. Because the total number of BK_{Ca} channels is difficult to estimate, we used the activation time constant (τ_{act}) to measure the effect of hypotonicity on BK_{Ca} currents (Fig. 4B). To obtain τ_{act}, we fit the rising phase of whole-cell currents by a single exponential function. Measurements were performed between 0 and +80 mV in isotonic and hypotonic conditions, and results are plotted in Figure 4B. Linear regressions are also shown as dotted (isotonic; $r^2 = 0.99$) and solid lines (hypotonic; $r^2 = 0.96$). The linear regression for τ_{act} in hypotonic medium was significantly faster than in isotonic medium ($P < 0.01$; two-way ANOVA) at all the measured voltages (+20 to +80 mV). No data were obtained at 0 mV in isotonic medium, because almost no current activation was seen at this voltage. The effect of stretching the membrane by hypotonic shock had the same effect on τ_{act} as an increase in $[Ca^{2+}]_i$ at the cytoplasmic face of the BK_{Ca} channel.[30,31]

Because other currents may be activated by hypotonic solutions (e.g., chloride) and to assure that the outward current increase was due to activation of BK_{Ca} channels, a specific BK_{Ca} channel blocker (IBTX) was added to the hypotonic bath solution. In the presence of 50 nM IBTX, outward currents were reduced significantly ($P < 0.001$; Figs. 4C, 4D).

DISCUSSION

This study suggests that the BK_{Ca} channel is activated by mechanical stretching of TM cells. The results show that this channel was modulated by stretching of the membrane as well as by the membrane voltage (depolarization) and by $[Ca^{2+}]_i$. Similar results have been described in other cell types, such as skeletal muscle,[16] osteoblasts,[17,18] smooth muscle,[19,20] chromaffin,[32] and neuroepithelial cells.[21]

The basic characteristics of the BK_{Ca} channel reported in this study are in agreement with those described by Stumpff et al.[13] in the same cells and in human TM cells.[33] As described, a greater number of channel openings were recorded after depolarization of the cell membrane or increase in $[Ca^{2+}]_i$. Moreover, we showed that membrane tension also modulated the open probability of this BK_{Ca} channel. Both in cell-attached and inside-out patches, the open probability of the channel increased in response to applied suction through the patch pipette, when compared with the control experiments (absence of suction). Pipette suction induced an increase in channel activity in a pressure-dependent manner, and basal levels were recovered after cessation of suction. At low levels of suction, the voltage sensitivity of the channel was unchanged, but the channel activity increased by a constant multiplicative factor. The unitary conductance of BK_{Ca} channels was not modified by suction. We consistently found that stretching

activated the channel in excised inside-out patches exposed to low or no Ca^{2+} solutions. This result shows that stretch activation is not secondary to an increase of internal $[Ca^{2+}]$. A different method of stretching the cell membrane was by using hypotonic solutions. The stretch-induced activation of BK_{Ca} channels found at the single-channel level is confirmed at the whole-cell level through the increase of BK_{Ca} currents in hypotonic solutions. Similar results have been described for other K^+ channels including BK_{Ca}.[17,18,28,29,34]

There is strong evidence that stretching the membrane directly affects the channel protein or some membrane component closely related to the channel, because activation by stretching could be elicited without Ca^{2+} in the external side of the patch (inside-out). Several deformation-sensitive membrane parameters that may influence membrane protein conformational changes have been identified, including membrane dilation, membrane thinning and local changes in membrane curvature or bending (for review see Hamill and Matinac[35]). Nevertheless, to date, the mechanism that confers mechanosensitivity on an ionic channel is still unknown. The possibility that effects on the channel could be mediated by second messengers or cytosolic factors released by the application of suction should be rejected, because little enzymatic machinery is thought to be present in excised patches. It has been suggested that fatty acids released by a stretch-activated membrane-bound phospholipase activates the channel.[20] We cannot rule out this possibility, but it would be difficult in inside-out patches where stretch activation of the channel was also found. It has been postulated that stretch sensitivity of ionic channels is an artifact caused by the membrane deformation induced by suction.[36] Moreover, in the case of the BK_{Ca} channel, increased NPo can be attributed to the influx of calcium through nonselective stretch-activated channels present in the membrane or as a consequence of the deformation of the membrane induced by suction.[12,37,38] We have demonstrated that two different methods of stretching the cell membrane (suction and hypotonic shock) increased BK_{Ca} current. Moreover, this channel can be activated by suction at constant concentrations of Ca^{2+}. That electrophysiological properties of the channel were not affected by suction demonstrates that the BK_{Ca} mechanosensitivity is not attributable to an artifact from the experimental procedure.

BK_{Ca} channels are present in a large number of tissues.[15] In the majority of cells where BK_{Ca} channels are present, cell membrane depolarization is counteracted by a repolarization mediated by these channels.[39] Drug-induced membrane depolarization and increases in $[Ca^{2+}]_i$ activate BK_{Ca} channels, which participate in returning the membrane potential to the resting level. When a cell is at resting membrane potential and $[Ca^{2+}]_i$ is low (as in a relaxed cell), stretch-activation of the channels is not expected. However, in cells exhibiting some degree of contraction, stretch activation of the channel limits this process. Thus, these channels may have a protective role in limiting the contractile tone of the tissue. Membrane stretching can induce different effects, depending on the channels present in a cell. In cells with cationic stretch-activated channels, membrane stretching induces cell contraction.[12] It is thought that in sensory neurons, stretch-activated channels mediate sensory transduction of mechanical stimuli,[12,40] whereas in other cell types, mechanosensitive channels are involved in the regulation of cell volume and growth[12] or cell movement.[41]

In the eye, tissues surrounding the anterior chamber are subjected to tension due to IOP. The ability of cells subjected to pressure variations to detect physiological or pathologic changes in IOP allows these cells to modify their properties to adapt to the new conditions. This may be a physiological function for the stretch sensitivity of ionic channels present in TM cells or in other tissues of the eye, such as the ones identified in rabbit corneal epithelial cells.[42] In physiological conditions, mean IOP is 15 mm Hg, with a diurnal variation of ± 3.7 mm Hg, but this pattern is exaggerated in a glaucoma condition.[43] Moreover, in different types of glaucoma, IOP can increase to 55 to 60 mm Hg.[43] Under our experimental conditions, using cultured cells, we found significant changes in BK_{Ca} activity even with low suction levels (e.g., 14 mm Hg), and therefore IOP changes are likely to modulate BK_{Ca} activity together with other cell functions. Several studies have focused on the involvement of the cytoskeleton in the regulation of aqueous humor outflow, and new drugs active in the cytoskeleton are being studied for use in treating glaucoma.[44] In fact, it has been shown that TM cells adapt to mechanical stretch by altering the cytoskeletal network and signaling cascades.[14] Moreover, TM cells are capable of responding to elevated hydrostatic pressure by increasing enzyme activities[6] or even modifying intracellular calcium.[5] In turn, endothelial cells from the Schlemm's canal synthesize cAMP in response to hydraulic pressure changes.[3] As Gonzalez et al.[7] have demonstrated, the TM is sensitive to changes in IOP, because elevated IOP induces upregulation of several genes in TM cells. The products of those genes, together with the changes induced directly by stretching in the cells, could comprise a homeostatic mechanism.[7]

It has been postulated that the contractile tone of the TM is involved in the regulation of outflow facility.[45] In this sense, it has been shown that relaxation of TM is associated with an enhancement of outflow and contraction with a reduction in outflow facility.[46,47] Recently, tyrosine kinase inhibitors, which activate BK_{Ca} channels, have been suggested as new drugs in the therapy for glaucoma, because of their relaxing effects in the TM.[47] In addition to the modulation of Ca^{2+} and cGMP already described for the BK_{Ca} channel in TM cells,[13] stretch activation could be a modulating mechanism to respond to IOP changes. The TM can contract in a Ca^{2+}-dependent way,[48] but also in a Ca^{2+}-independent way, as has been recently proposed.[49] However, increases in cGMP mediate relaxation of the TM. In these scenarios, the activity of the BK_{Ca} channel could be increased by stretching alone (in low $[Ca^{2+}]_i$) or synergistically with the increase in $[Ca^{2+}]_i$. Finally, the release of paracrine substances as NO stimulates the production of cGMP and therefore causes relaxation of the TM.

All these arguments support a homeostatic role of BK_{Ca} channels on the physiology of the TM. It has been previously described that TM's function is affected by osmolality changes.[9,10] In this study, as well as in others,[50] evidence is provided that hyposmotic solutions increase BK_{Ca} currents. We used IBTX, a well-known specific blocker of BK_{Ca} channels, which selectively inhibited the whole-cell current. These results do not exclude that other currents can be activated by stretching or application of hypotonic solutions (e.g., chloride), as previously reported[51] (Comes N, Gasull X, unpublished observations, 2002). Nevertheless, a chloride component in the hypotonic-induced whole-cell current is unlikely, because we did not use adenosine triphosphate (ATP) in the intracellular solution (pipette). It is known that ATP is necessary for volume-sensitive, outwardly rectifying anion channels to be activated.[52] On osmotic cell swelling, a volume-regulatory KCl efflux is induced as a result of the activation of K^+ and Cl^- channels.[52] Because BK_{Ca} is the main K^+ channel found in TM cells[13] and our results show that BK_{Ca} currents are activated by hypotonic stimuli, we suggest that BK_{Ca} channels may be involved, together with Cl^- currents, in regulation of cell volume.

Considering that two populations of cells may coexist in the TM,[53] it is possible that stretch activation of BK_{Ca} channels have different functions, depending on the TM cell type, coun-

teracting cell contraction (smooth-muscle–like cells) or cell swelling (epithelial-like cells). Finally, the IOP increases that take place in a glaucoma condition definitely would activate BK_{Ca} channels. It would be of great interest to know, in future studies, whether channel activity may be altered in some of these diseases.

In summary, the present data establish that BK_{Ca} channel activity is enhanced by stretching the membrane and suggests that membrane stretching and depolarization and $[Ca^{2+}]_i$ constitute a mode of regulating BK_{Ca} activity. We hypothesize that homeostatic mechanisms in TM cells may involve activation of BK_{Ca} channels in response either to changes in cell volume or changes in IOP.

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