cAMP accelerates the decay of stretch-activated inward currents in guinea-pig urinary bladder myocytes

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1. Myocytes from the urinary bladder were stretched longitudinally by 5–20%. At −50 mV, stretch induced whole-cell inward currents (I_{in}) between −100 and −600 pA. I_{in} decayed slowly with time to 93 ± 20% (mean ± S.E.M., $n = 6$) of the initial value in 1 min. The mechanisms of this 'adaptation' and its modulation by dibutyryl cAMP (dBcAMP) were analysed with whole-cell and single channel currents.

2. When the cells were internally perfused with 100 μM 8-bromo-cAMP (8BrcAMP), stretch induced an I_{in} of the usual amplitude that decayed completely within 40 ± 13 s. When 200 μM dBcAMP was bath applied 10 s after the start of the stretch, I_{in} decayed to zero within 85 ± 18 s.

3. dBcAMP increased the K^+ current through Ca^{2+}-activated BK channels ($I_{K(Ca)}$) at 0 mV with a time course that correlated well with the decay of I_{in}, and block of $I_{K(Ca)}$ by TEA suppressed the dBcAMP-induced decay of I_{in}. In the presence of intracellular BAPTA, dBcAMP increased the stretch-induced I_{in}. The results suggest that adaptation is caused by superimposition of $I_{K(Ca)}$ which is increased through elevation of near-membrane $[Ca^{2+}]$ and by cAMP-dependent phosphorylation.

4. Single channel analysis was carried out with 140 mM KCl electrode solution and at −50 mV. Stretch-activated channels (SACs) were recorded during pulses of negative pressures between −2 and −5 kPa. Activity (NP_o) of SACs was constant for at least 4 min, e.g. evidence for adaptation was missing. dBcAMP (200 μM) increased NP_o of SACs by 142 ± 35% ($n = 16$).

5. dBcAMP increased NP_o via frequency of openings and channel open time. In five of sixteen patches, dBcAMP induced openings without suction. Similar effects were induced by the catalytic subunit of cAMP-dependent protein kinase (PKA_c), applied to inside-out patches.

6. NP_o, normalized by its maximum, increased with more negative pressure along an S-shaped curve. dBcAMP increased the sensitivity of SACs to stretch by shifting the point of half-maximal activity from −3·2 to −2·6 kPa.

7. The augmentation of NP_o by dBcAMP is attributed to the phosphorylation of SACs promoting their opening. Adaptation of I_{in} is discussed as a 'secondary' effect of stretch-activated channels: Ca^{2+} influx through SACs increases the Ca^{2+} concentration that activates BK channels whose Ca^{2+} sensitivity is increased by cAMP.

During normal filling of the urinary bladder, intravesical pressure rises remarkably little as the bladder distends. If the bladder is artificially filled at faster rates, the pressure rises but falls after filling has ceased, behaviour that is also seen in isolated strips (Brading, 1987). Usually, this mechanical adaptation has been attributed to the passive mechanical properties, modelled with elastic plus plastic elements (Griffiths, 1980). However, it seems likely that in the intact bladder the individual muscle bundles contract spontaneously and thereby contribute an active component to the muscular tone (Brading, 1987). Hence, the response to rapid filling may include a contractile response attributable to the changes in the spontaneous electrical activity (Brading, 1987; see also Wellner & Isenberg, 1994). According to this concept, the fall of muscular tone over time should have a counterpart in the 'adaptation' of the

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electrical activity, i.e. despite the sustained lengthening of the individual myocyte the frequency of the action potentials should fall back to the control level.

The adaptation of urinary bladder tone may be supported by the sympathetic system (Brading, 1987), the β-adrenergic relaxation being mediated by an increase in intracellular [cAMP]. Independent of the sympathetic tone, stretch of the membrane cytoskeleton may stimulate the adenylate cyclase and increase [cAMP], as reported for mouse lymphoma cells during hyposmotic swelling. Since changes in [cAMP] may play a role in the adaptation of muscular tone, we studied the effects of exogenous membrane-permeable cAMP derivatives on the stretch-induced membrane currents. Our results suggest that cAMP-dependent intracellular phosphorylation facilitates adaptation.

This paper studies the possible electrophysiological correlates of adaptation with the voltage-clamp method applied to the isolated smooth muscle cell. The preceding paper (Wellner & Isenberg, 1994) demonstrated that the lengthening-induced increase in action potential frequency can be attributed to a lengthening-induced net inward current (I_{in}). At −50 mV, I_{in} was composed of an inward current through stretch-activated non-selective cation channels (SACs) and an outward current through Ca^{2+}-activated K^+ channels (200 pS BK channels). Hence, adaptation of I_{in} could be due to a diminished activity of SACs or an enhanced activity of BK channels. In this paper, single channel analysis suggests that the activity of SACs does not decay within 5 min, and that it is not reduced but increased upon exogenous application of dBcAMP (dibutyryl cAMP). Measurements of whole-cell currents demonstrate that adaptation of I_{in} is suppressed by blocking the BK channels with 20 mM TEA or by preventing changes in $[Ca^{2+}]_c$ (cytosolic Ca^{2+}) with cell dialysis of 20 mM BAPTA. These results allowed us to put forward the hypothesis that the adaptation of stretch-induced electrical activity is a 'secondary' effect of stretch-activated channels: Ca^{2+} influx through SACs is suggested to increase the Ca^{2+} concentration at the inner side of the membrane resulting in Ca^{2+} activation of cAMP-modulated BK channels. Parts of the results have been presented in abstract form (Wellner & Isenberg, 1993*b*).

METHODS

The methods for cell preparation and whole-cell experiments have been described in a previous paper (Wellner & Isenberg, 1994). In brief, guinea-pigs (*ca* 300 g) were killed by cervical dislocation. Urinary bladder myocytes were enzymatically isolated using the collagenase method (Klöckner & Isenberg, 1985*a*). Until final use, the cells were stored in Kraft-Brühe medium (KB medium; Isenberg & Klöckner, 1982) composed of (mM): 60 KCl, 30 K_2HPO_4, 1 EGTA, 5 $MgCl_2$, 5 creatine, 20 taurine, 2 glucose, 5 succinic acid, 5 glutamic acid and 1 g l^{-1} fatty acid-free albumin, adjusted to pH 7·2 with ~5 mM KOH. Before the beginning of the experiment, the KB medium was replaced by a bath solution containing (mM): 150 NaCl, 5·4 KCl, 3·6 $CaCl_2$, 1·2 $MgCl_2$, 20 glucose, 5 Hepes–NaOH (pH 7·4). In some experiments, 20 mM TEA was added to block K^+ channels. dBcAMP and 8BrcAMP (8-bromo-cAMP) were purchased from Boehringer (Mannheim, Germany) and the catalytic subunit of protein kinase A was from Sigma (St Louis, MO, USA).

Patch pipettes were pulled to 1·5 μm outer diameter (3 MΩ tip resistance). They were filled with (mM): 140 KCl, 0·02 EGTA, 1 $MgCl_2$, 10 Hepes–KOH (pH 7·4). The temperature was between 22 and 25 °C. The currents were recorded with an RK 300 amplifier (Biologic, Echirolles, France), that was connected to a CED-1401 interface (Cambridge Electronic Design, Cambridge, UK) and to an IBM-compatible host. To document the experiment and for illustration purposes, the currents were also recorded on line with a thermo-writer (Tarr 220, Gould Inc., Cleveland, OH, USA). Statistical significance was calculated with Student's t test ($P < 0·05$).

Method of cell stretch

The cell was stretched by means of two patch electrodes. The first electrode, which was also used for intracellular recording, firmly pressed the middle of the cell to the bottom of the experimental chamber. A second electrode was sealed in the cell-attached mode at the end of the cell. An electrical micromanipulator increased the distance between the two electrodes from slack length ($L1$) to $L2$ within 1 s. ΔL defines the extent of stretch by the ratio $(L2 - L1)/L1$. In some experiments the attachment of the second electrode had problems due to debris. Under those conditions, one could observe that the cell returned to slack length during ΔL. In these experiments, stretch induced the appearance of I_{in} which completely decayed within 20–30 s. In other cases, the end of the cell slipped from the electrode and then I_{in} decayed within 10 s. Again, the relaxation of I_{in} was attributed to the decrease of mechanical strain. In the present study on mechanisms of 'adaptation' of I_{in}, we only used data from those experiments where slippage upon stretch did not occur; in these experiments I_{in} remained stable for at least 50–60 s.

Single channel recordings

Bath and pipette solution contained (mM): 140 KCl, 2 $MgCl_2$, 2 EGTA, 10 Hepes–KOH (pH 7·4). The currents were low-pass filtered (1 kHz) and sampled at 2·5 kHz. The negative pressure was generated by a syringe and applied to the patch pipette through a computer command to a magnetic valve (P/N 225PO11-21 NC, NResearch, Maplewood, NJ, USA). It was calibrated using a water-filled manometer and converted into kilopascals. Inward currents (flowing from the pipette into the cell) are shown as downward deflections. The channel activity (NP_o) is the product of the number of channels in the patch (N) and the probability that the channel is open (P_o). NP_o was calculated from the idealized record by division of the time integral by the elapsed time. Depending on N, NP_o can be larger than 1. To avoid confusion, NP_o will always be presented as an absolute number. Changes in NP_o will be given as a percentage of a control NP_o.

RESULTS

Adaptation of stretch-induced inward current I_{in} is accelerated by dBcAMP

A myocyte, voltage-clamped to a holding potential of −50 mV, responds to a longitudinal mechanical stretch with an inward current, I_{in}. As shown in a previous paper

(Wellner & Isenberg, 1994), I_{in} decays slowly during a sustained stretch of several minutes. The slow decay of I_{in} is called 'adaptation'. Figure 1*A* presents an example in which no adaptation of I_{in} is recorded within the initial minute. That is, 2 s after start of the stretch, I_{in} was −688 pA and, after a period of 77 s, I_{in} was −740 pA. When the cell length was returned from $\Delta L = 10\%$ to $\Delta L = 5\%$, I_{in} fell to −480 pA and remained constant for 40 s. Finally, when the cell was returned to slack length, I_{in} fell to zero.

Using ΔL between 10 and 20%, I_{in} decayed to 93 ± 20% ($n = 6$) of the initial value during the first minute. The extent and rate of adaptation were increased when the membrane-permeable dBcAMP (200 μM) was bath applied. An example is shown in Fig. 1*B*. The stretch, when $\Delta L = 20\%$, induced an I_{in} that started at −387 pA. dBcAMP was added to the bath 16 s after the start of the stretch. In the presence of dBcAMP and during constant ΔL, I_{in} completely disappeared within 1 min. On average, in the presence of 200 μM dBcAMP, I_{in} decayed to zero within 85 ± 18 s ($n = 6$).

Decay of I_{in} correlates with an increase in $I_{K(Ca)}$

A previous paper (Wellner & Isenberg, 1994) suggested that I_{in}(−50 mV) is composed of an inward component through stretch-activated non-selective channels (I_{ns}) and an outward current component through Ca^{2+}-activated maxi K^+ channels ($I_{K(Ca)}$). Hence, the decay of I_{in} could be caused

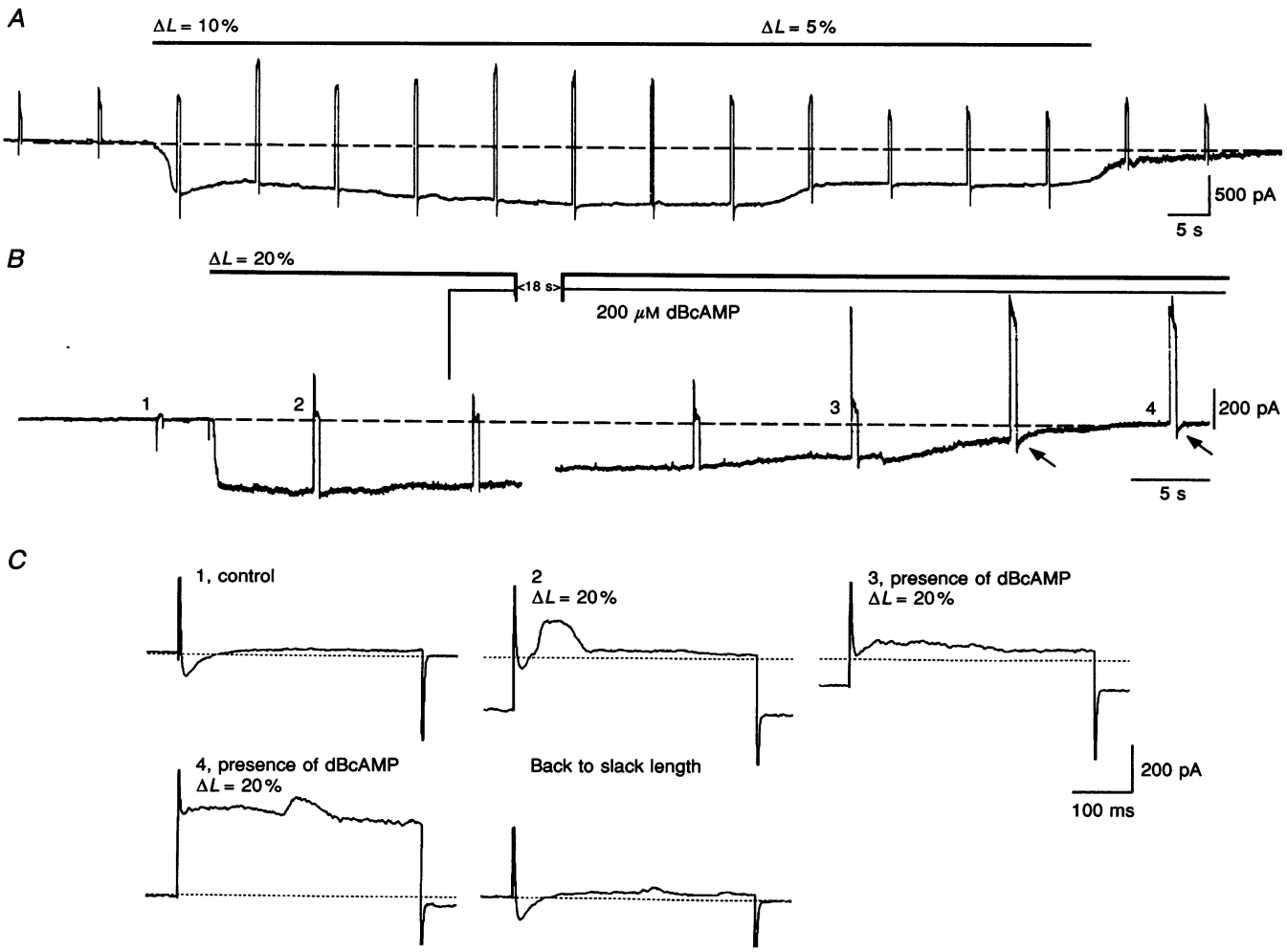

Figure 1. Time course of the decay ('adaptation') of the stretch-induced inward current I_{in}(−50 mV)

Holding potential −50 mV. Pulses of 400 ms to 0 mV were applied at 0·1 Hz. *A*, pen recording in control conditions. Initial 10% stretch (for 77 s) followed by 5% stretch (for 40 s) and return to slack length. All dashed lines mark zero current. Note that I_{in} is stable for the duration of the stretch. *B*, effects of 200 μM dBcAMP, bath applied 16 s after the start of 20% stretch. dBcAMP induces decay of I_{in} and appearance of $I_{K(Ca)}$ and $I_{Cl(Ca)}$ (tail currents through Ca^{2+}-activated Cl^- channels, marked by arrows) within 1 min. *C*, computer playbacks of net currents during the depolarizing clamp steps marked in panel *B*. Note the changes in holding current (I_{in}), the reduction of I_{Ca} and the increase in $I_{K(Ca)}$. Within 1 min of return to slack length and wash-out of dBcAMP, currents were almost identical to control.

by a decay of I_{ns} and/or an increase in $I_{K(Ca)}$. A comparison of the time course of I_{in} and $I_{K(Ca)}$ may be used to distinguish these possibilities. $I_{K(Ca)}$ was estimated from the outward current flowing at the end of the 400 ms clamp pulses to 0 mV.

Under control conditions (Fig. 1*A*, no dBcAMP), the stretch by $\Delta L = 10\%$ nearly doubled $I_{K(Ca)}$ (compare Wellner & Isenberg, 1994). During the stretch both I_{in} and $I_{K(Ca)}$ remained nearly constant. On average, without a significant decay of I_{in}, systematic changes in $I_{K(Ca)}$ were absent ($n = 5$). On re-lengthening to $\Delta L = 5\%$, $I_{K(Ca)}$ returned to near control $I_{K(Ca)}$, and the final return to slack length did not further reduce $I_{K(Ca)}$. Apparently, the change in I_{in} upon re-lengthening does not have a correlate in $I_{K(Ca)}$ (see Discussion).

dBcAMP not only accelerated the adaptation of I_{in}, but also induced an increase in $I_{K(Ca)}$. Typically, this increase started after a delay of 40–60 s. The example in Fig. 1 shows the start of the increase after 40 s (trace 3 in *C*). Within another 10 s the increase amounts to 260% (compare traces 2 and 4 in *C*). Figure 2*A* demonstrates that the time course of the decay of I_{in} and the increase in $I_{K(Ca)}$ are similar. When $I_{K(Ca)}$ was plotted as a function of I_{in}, the correlation coefficient was 0·990 (Fig. 2*B*). On average, the correlation coefficient was 0·956 ± 0·012 ($n = 5$). Although the correlation does not prove that 'adaptation' of I_{in} is caused by the superimposition of $I_{K(Ca)}$, it supports this idea as a working hypothesis.

In approximately 40% of the cells, the combination of stretch and dBcAMP induced not only an increase in $I_{K(Ca)}$ but also the appearance of a negative tail current (Fig. 1*B*, marked by arrows) that flows through Ca^{2+}-activated Cl^- channels ($I_{Cl(Ca)}$; ref. Hogg, Wang, Helliwell & Large, 1993). Figure 2*C* demonstrates a correlation between the time course by which $I_{Cl(Ca)}$ increases and I_{in} decays. Both $I_{K(Ca)}$ and $I_{Cl(Ca)}$ increase with the concentrations of Ca^{2+} and cAMP in the cytosol (BK channels: Sadoshima, Akaike, Kanaide & Nakamura, 1988; Cl^- channels: Klöckner, 1993). However, the cAMP-induced decay of I_{in} cannot be attributed to $I_{Cl(Ca)}$ because this current is negative at −50 mV. Further, the negative tail current decayed completely within approximately 3 s, a property that excludes its contribution to the sustained I_{in}. Therefore, modulation of $I_{Cl(Ca)}$ was not further studied.

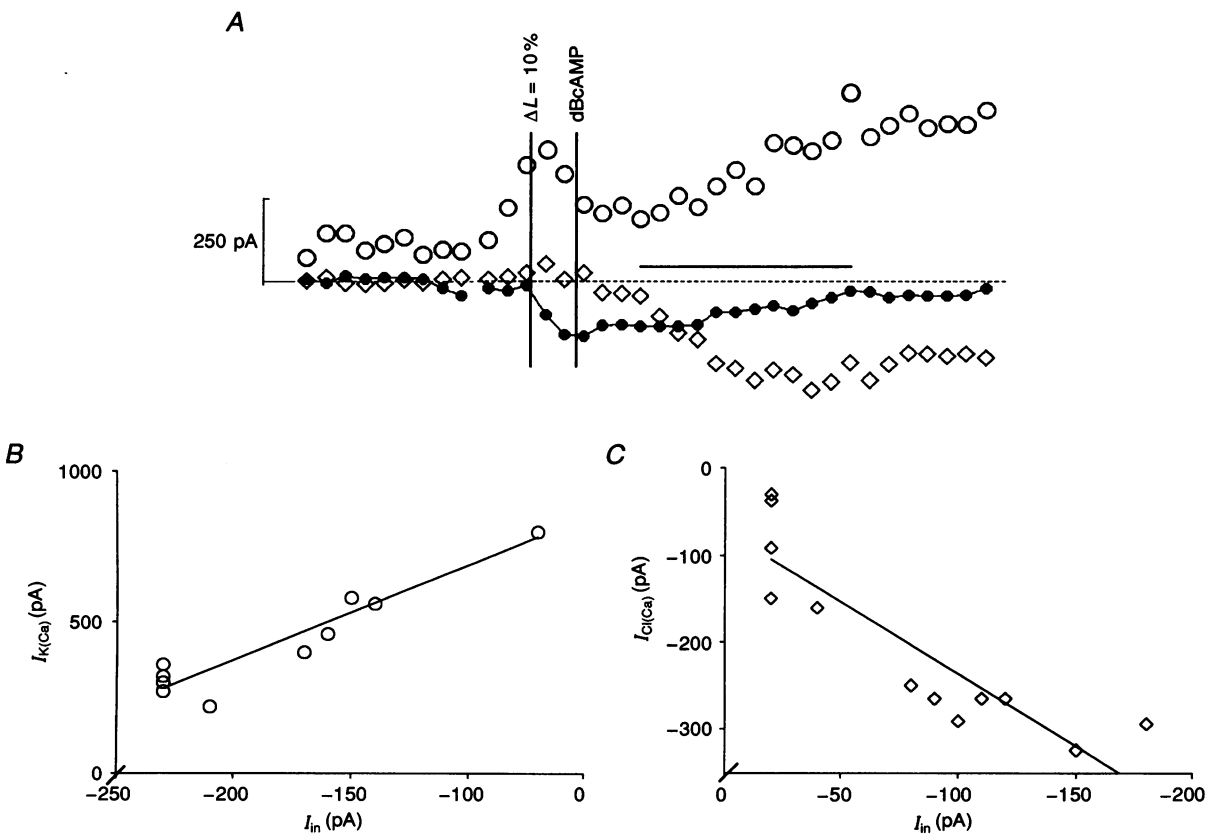

Figure 2. The cAMP-mediated decay of I_{in} (0) correlates with the increase in Ca^{2+}-activated K^+ current ($I_{K(Ca)}$, 1) and Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$, 3)

A, time course of the experiment. Vertical bars mark application of stretch and of dBcAMP. The horizontal line marks the time window for the analysis shown in *B* and *C*. *B*, increase in $I_{K(Ca)}$ (ordinate) correlates with the fall in I_{in} (abscissa). Correlation coefficient $r^2 = 0{\cdot}990$. *C*, increase in the negative peak tail current $I_{Cl(Ca)}$ (ordinate) correlates with fall in I_{in}. $r^2 = 0{\cdot}955$.

The computer playback (Fig. 1) further suggests that stretch reduced the Ca^{2+} current (I_{Ca}). cAMP did not antagonize this reduction or increase control I_{Ca} before the stretch (compare Klöckner & Isenberg, 1985*b*). This finding is supported by similar results in the presence of 20 mM TEA (Fig. 4).

Adaptation of I_{in} in cells internally perfused with 8BrcAMP

In another series of experiments, cells were internally perfused with 8BrcAMP prior to lengthening. In the absence of stretch, the comparison of the currents at the end of the depolarizing pulse (mostly $I_{K(Ca)}$) immediately after rupture of the patch and after 1 min of 8BrcAMP perfusion (Fig. 3, trace 0 in *B*) suggests a more than twofold increase in $I_{K(Ca)}$(0 mV), as expected from the increased Ca^{2+} sensitivity of the BK channel by cAMP-dependent phosphorylation (Sadoshima *et al.* 1988). Intracellular 8BrcAMP did not significantly change the holding current at −50 mV ($n = 4$).

8BrcAMP did not prevent or diminish the effects of stretch in inducing I_{in}. During a sustained stretch of $\Delta L = 10\%$, I_{in} remained constant for approximately 90 s (Fig. 3*A*, −460 pA). After this delay, I_{in} decayed to zero within 20 s while $I_{K(Ca)}$ did not change significantly. Results from four cells dialysed with 100 μM 8BrcAMP indicate that I_{in} completely decayed within a period of 40 ± 13 s. That is, the decay of stretch-induced I_{in} followed a similar time course in cells pre-dialysed with 8BrcAMP and in cells where dBcAMP was added during the stretch. The difference was only the delay after which decay occurred. This was shorter in cells pretreated with 8BrcAMP than in those where dBcAMP was added during the stretch, but the time for permeation of dBcAMP into the cell and for activation of PKA can easily account for this difference.

The result that stimulation of PKA (suggested by enlargement of $I_{K(Ca)}$) does not prevent stretch-induced I_{in} excludes the idea that dBcAMP accelerates the decay of I_{in} by a 'desensitization' process linked to phosphorylation of SACs. The result where I_{in} decayed while I_{Ca} was constant seems to conflict with the above hypothesis that cAMP induces the decay of I_{in} through the Ca^{2+} sensitization of BK channels. $I_{K(Ca)}$(0 mV) that was already increased by the 8BrcAMP pretreatment, did not increase further during adaptation of I_{in} ($n = 4$). In the Discussion this problem will be attributed to the dependence of BK channel activity on voltage, Ca^{2+} and 8BrcAMP; it will be suggested that 8BrcAMP activates the BK channels at

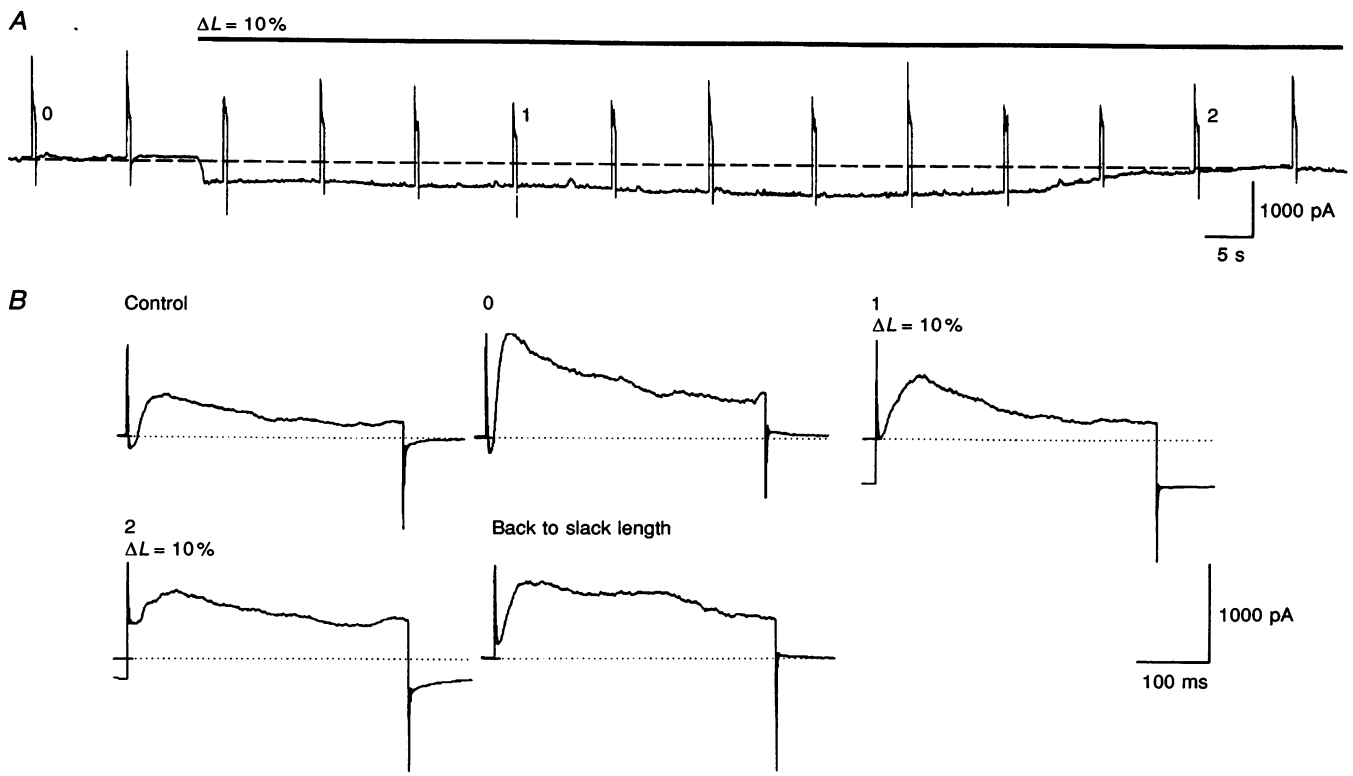

Figure 3. Adaptation of stretch-induced net membrane current in a cell internally perfused with 100 μM 8BrcAMP

A, on-line pen recording. *B*, net currents during 400 ms steps from −50 to 0 mV. Traces were recorded 10 s after breaking the patch, 1 min after dialysis of 8BrcAMP but before stretch (0). Note that $I_{K(Ca)}$, already enlarged by preceding 8BrcAMP perfusion does not further increase during stretch while I_{in}(−50 mV) decays nearly completely after a 1 min delay.

0 mV up to their maximum ('ceiling off') while at −50 mV their activity can still be increased.

Adaptation of I_{in} is suppressed by TEA

$I_{K(Ca)}$ can be blocked by TEA ions (Langton, Nelson, Huang & Standen, 1991). When the experiments were repeated in the presence of 20 mM TEA, ΔL induced I_{in}, reduced I_{Ca} but did not change the current at the end of the 400 ms pulse (not illustrated; see Wellner & Isenberg, 1994). In the constant presence of TEA and during sustained stretch, I_{in} did not decay, and decay was not induced by addition of dBcAMP (Fig. 4). That is, after a dBcAMP treatment of 60 s, I_{in} was 94 ± 7% of the control value immediately after the stretch. This 6% is insignificant and small in comparison with the 100% decay induced by dBcAMP in the absence of TEA. The result that TEA prevented the dBcAMP effects on the decay of I_{in}(−50 mV) strongly supports the idea that the acceleration of adaptation is mediated by a TEA-sensitive K^+ current.

In BAPTA-loaded cells, dBcAMP augments I_{in}

One of the TEA-sensitive K^+ currents flows through activated 200 pS BK channels that are modulated by PKA and activated by an increase in near-membrane cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$). The importance of an increase in $[Ca^{2+}]_c$ for the adaptation of I_{in} was investigated in cells in which the possible increase in $[Ca^{2+}]_c$ was buffered with intracellular 20 mM BAPTA ($n = 4$). In all cells, dialysis of BAPTA from the electrode induced small negative holding currents before the stretch (−17 ± 12 pA). During a stretch of 5%, the holding current became more noisy and negative, i.e. BAPTA did not block the stretch-induced I_{in}. On average, I_{in} was −72 ± 38 pA for ΔL between 5 and 10% (compare Wellner & Isenberg, 1994). In cells dialysed with BAPTA, the application of 400 μM dBcAMP increased I_{in}(−50 mV) by 58% (−114 ± 27 pA, $n = 4$). That is, when the increase in $[Ca^{2+}]_c$ was prevented, dBcAMP did not reduce but increased the stretch-induced I_{in} (Fig. 5). The result strongly suggests that the dBcAMP-induced decay of I_{in} is

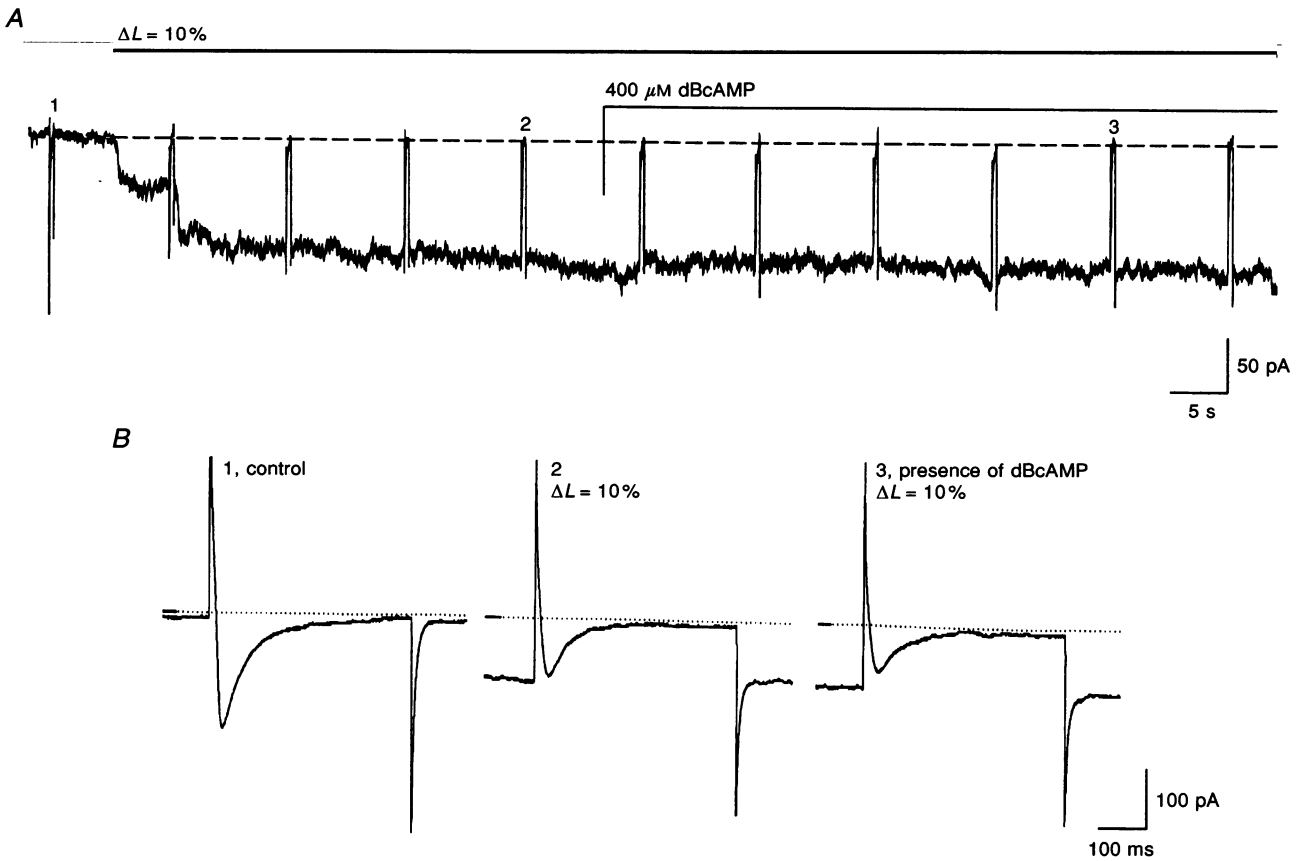

Figure 4. TEA (20 mM; present throughout) blocks the cAMP-mediated adaptation of I_{in}

A, on-line pen recording. Zero current is labelled by the dashed line. Neither I_{in} nor the current at the end of the pulse are significantly changed by 400 μM dBcAMP. *B*, net currents during 400 ms steps from −50 to 0 mV before (1), during stretch (2) and during (3) dBcAMP (cell relaxed to slack length). Dotted lines represent zero current. Note that the late current at 0 mV is not increased by stretch or by dBcAMP. With K^+ channels blocked by TEA, the inward current peak approximates peak I_{Ca}; the stretch-induced 50% reduction of I_{Ca} is not reversed by dBcAMP (75% reduction in trace 3 in *B*).

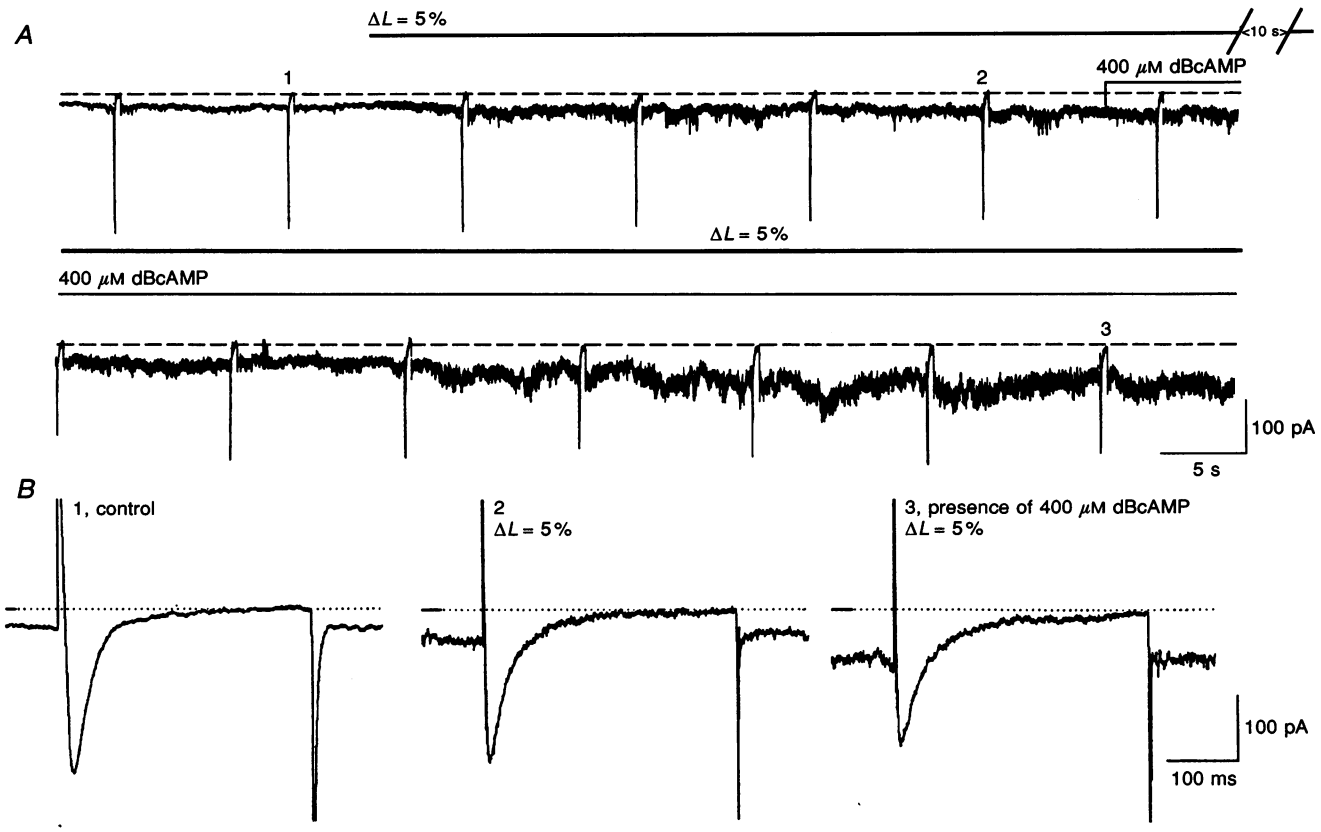

Figure 5. Effects of stretch and dBcAMP on a cell internally perfused with 20 mM BAPTA

A, the two traces show the 140 s pen recording (zero current labelled by dashed line). Note that upon addition of 400 μM dBcAMP, the noisy inward current I_{in} increases. *B*, playbacks of currents before (1), during 5% stretch (2) and during stretch and dBcAMP (3). During dBcAMP, the holding current is more negative; however, neither peak I_{Ca} nor the current at the end of the pulse are significantly changed.

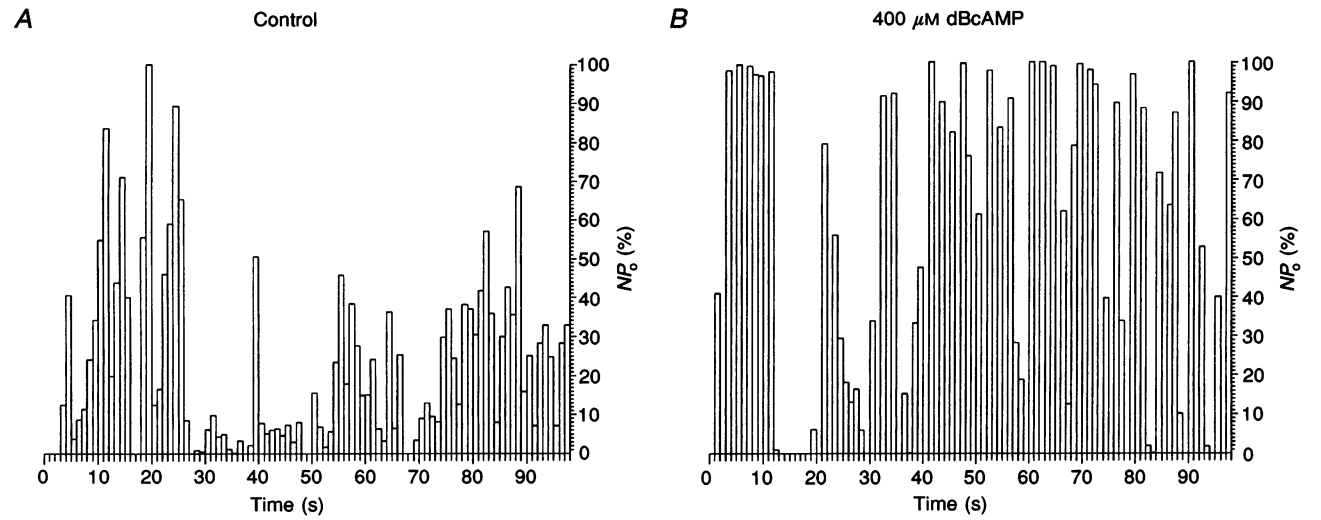

Figure 6. Stability of single channel activity (NP_o) in the absence and presence of dBcAMP

Cell-attached patch, pipette solution containing 140 mM KCl plus 2 mM $MgCl_2$. Holding potential, −50 mV. NP_o during 100 s continuous records is shown in the absence (*A*, −2·9 kPa) and presence of 400 μM dBcAMP in the bathing solution (*B*, −3·4 kPa).

mediated by stretch-induced increments in $[Ca^{2+}]_c$ that, in turn, increase $I_{K(Ca)}$(−50 mV).

Single channel analysis

SAC activity is stable for several minutes

When SACs were activated by a suction to the open end of the patch electrode, NP_o did not systematically decay. Although there were periods of high and low NP_o during a 100 s recording (Fig. 6*A*), mean NP_o was almost constant in twenty-seven of thirty-two patches (recording times up to 5 min). In five patches only, NP_o decayed and finally disappeared within 4 min. Since this phenomenon was rather an exception, it may represent a 'run-down' of NP_o (see Wellner & Isenberg, 1994). Bath application of 200 or 400 μM dBcAMP did not reduce the NP_o. Instead, dBcAMP almostly doubled NP_o within 20 s. After this increase,

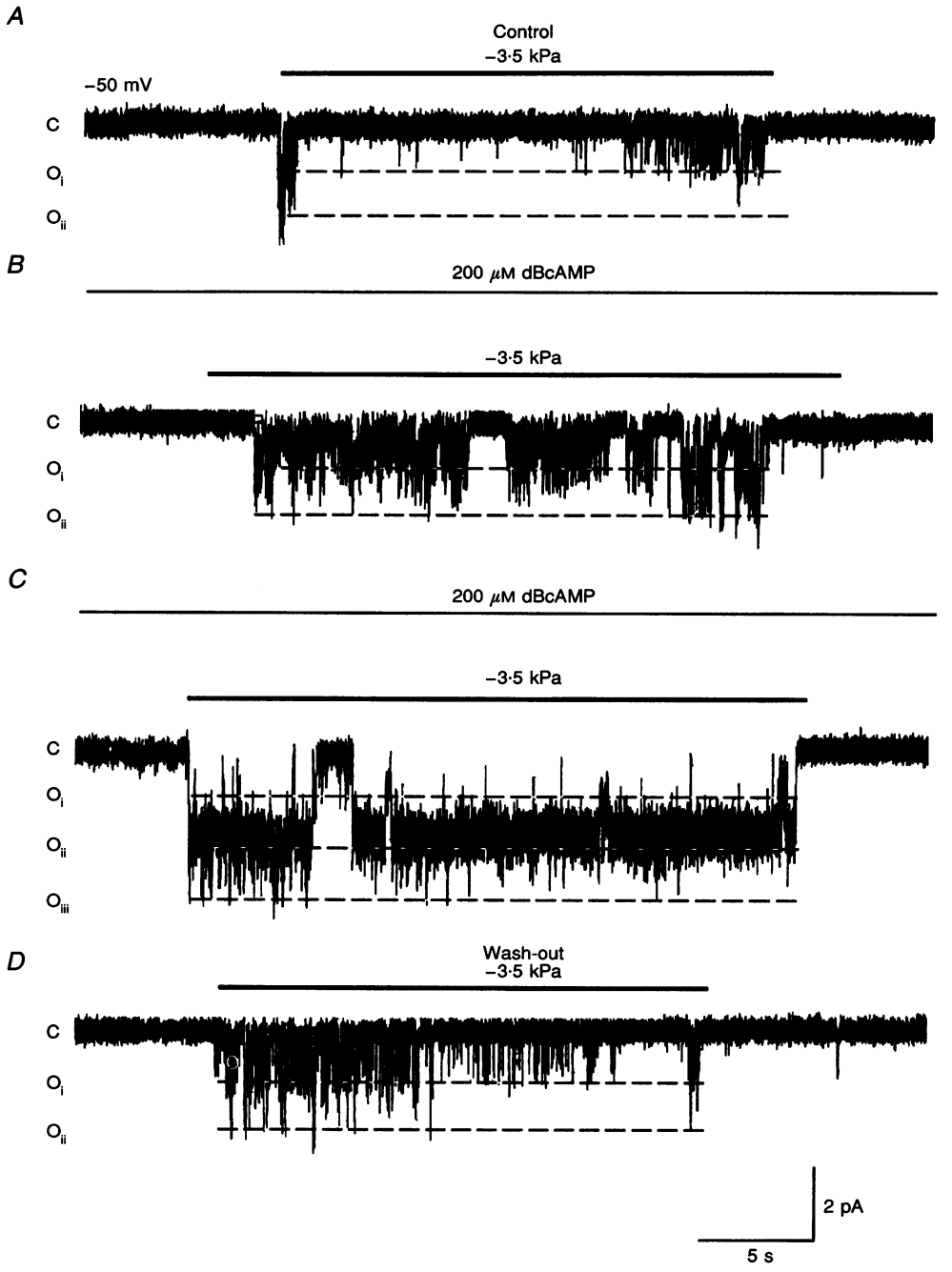

Figure 7. 200 μM dBcAMP reversibly increases the suction-induced channel activity

On-line pen recording before (*A*), 30 s after (*B*) and 90 s after (*C*) bath application of dBcAMP; *D*, 80 s after wash-out of dBcAMP. Superimposition of two current levels is indicated by O_i and O_{ii}. Note that in panel *C*, short and long openings superimpose. Here and in subsequent figures: C, closed; O, open.

NP_o remained constant (Fig. 6*B*). On average, dBcAMP increased NP_o by 142 ± 35% (percentage relative to control before application of dBcAMP, $n = 16$, negative pressures between −2 and −4·5 kPa). The results indicate that the dBcAMP-induced decay of I_{in} is not caused by a reduced NP_o of SACs.

Characterization of the dBcAMP effects on SACs

Details of the cAMP effect on SACs were studied with pulses of negative pressure (20 s, −3·5 kPa) that were applied every minute. Figure 7*A* starts with a control in which suction activated SACs with an NP_o of 0·02. When 200 μM dBcAMP was added 30 s later, the same suction induced an NP_o of 0·08 (Fig. 7*B*). The increase in NP_o was caused by the more frequent appearance of short openings and the occurrence of two simultaneous openings often induced superimposed current levels (marked by O_{II}). After dBcAMP had been present for 90 s, suction-activated NP_o was 0·24 (Fig. 7*C*). In addition to the high frequency of short openings, long openings also occurred. Upon washout of dBcAMP, the effects on NP_o reversed (*D*).

Single channel conductance. Figure 8 compares the currents through SACs before (left) and 2 min after addition of 400 μM dBcAMP (right) for a series of voltage steps. From the open channel current–voltage relation (Fig. 8*B*), a slope conductance of 38 pS was evaluated for the control and 39 pS for currents in the presence of dBcAMP. The mean single channel conductance of 40 ± 1 pS in the presence of dBcAMP ($n = 4$) was not different from the control conductance (39 ± 2 pS; compare Wellner & Isenberg, 1993*a*).

Channel open times. Figure 8*C* shows histograms of the distribution of the open times. Most obviously, open times longer than 10 ms occur only rarely during control conditions (12 ± 7%, $n = 5$) but more frequently in the presence of dBcAMP (21 ± 4%). A double exponential fit, $A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, points to cAMP effects on the slow exponential. dBcAMP increased the amplitude by 60% and the time constant by 100%. On average ($n = 13$), dBcAMP did not change A_1 (control, 71 ± 13%; dBcAMP, 67 ± 10%), τ_1 (control, 1·6 ± 1·0 ms; dBcAMP, 2·9 ± 1·1 ms) or A_2 (control, 29 ± 13%; dBcAMP, 33 ± 10%). Only τ_2 was significantly increased (control, 13·0 ± 4·9 ms; dBcAMP, 27·3 ± 7 ms).

The dBcAMP effect on the size of A_2 remained insignificant because of a high S.E.M. caused by the fact that thirteen of twenty-one patches had long openings (see also Fig. 9*A*) whereas eight did not. Although the long openings always appeared later than the increase in NP_o, in the eight negative experiments the openings remained short for up to 4 min (dBcAMP concentrations of 200 or 400 μM). At the moment, an explanation for the different types of response cannot be offered.

The effects of dBcAMP on the closed-time distribution could be evaluated only in a few patches. This is because the SACs were clustered, i.e. patches with only one active channel were found in only 19% of the trials, and because the 20 s suction time interfered with the analysis. The closed-time distribution was fitted with three exponentials (time constants < 1, 6 and 400 ms). dBcAMP at 200 μM reduced the two long time constants and their contribution to approximately 50%.

Gadolinium-block of cAMP-modulated SACs. To test whether the cAMP-modulated SACs were blocked by Gd^{3+}, a 2 mm layer of solution containing 20 μM Gd^{3+} was added on top of the Gd^{3+}-free solution filling the tip. The seal was formed in the presence of 400 μM dBcAMP and the control response to suction was measured immediately. The current trace at the top of Fig. 9*A* shows frequent short and long openings which is typical for SAC activity in the presence of dBcAMP (see also panel *B*, left). After 2 min, when Gd^{3+} had presumably diffused to the patch of membrane, only short openings appeared (Fig. 9*A*, second trace; Fig. 9*B*, right histogram). After 3 min, suction-induced currents occurred only rarely and their amplitude was reduced (Fig. 9*A*, third trace). After 4 min, suction was unable to induce inward currents (not illustrated). That is, Gd^{3+} blocked both the cAMP-modulated SACs and the control SACs (Yang & Sachs, 1989; Wellner & Isenberg, 1993*a*).

In a small number of patches, the effect of dBcAMP was studied with Na^+ and Cs^+ ions as charge carriers. Electrode solution containing 140 mM NaCl (plus 2 mM $MgCl_2$) yielded a single channel conductance of 37 pS ($n = 2$) in the presence of 200 μM dBcAMP. With dBcAMP and 140 mM CsCl solution (plus 2 mM $MgCl_2$), the single channel conductance was 34 pS ($n = 2$). These conductance values are very similar to that published previously (Wellner & Isenberg, 1993*a*). The results suggest that (a) the cAMP-modulated channel was a non-selective cation channel and (b) the ion selectivity of the channel was not modified by dBcAMP.

Effects of dBcAMP may be mediated by PKA

The suction-induced channel activity survives the excision of the patch into an inside-out configuration for approximately 4 min (Wellner & Isenberg, 1993*b*). During this time, the catalytic subunit of protein kinase A (PKA_c) plus ATP were applied to the cytosolic site of the patch for testing whether PKA can mimic the cAMP modulation of SACs. ATP (0·4 mM) did not change the response to suction; however, the combination of PKA_c plus ATP did. In three patches, SAC-like inward currents appeared spontaneously in the absence of suction (Fig. 10*C*). The activity was increased by application of negative pressure (Fig. 10: from $NP_o = 0{\cdot}015$, shown in panel *C*, to $NP_o = 0{\cdot}06$, shown in panel *D*). That is, in the presence of PKA_c, suction activated an activity that was 215% higher than the response to suction in the control (Fig. 10*A*). PKA_c did not significantly change the amplitude of the single channel current. On average, the single channel conductance in the presence of PKA_c was 39 ± 3 pS which

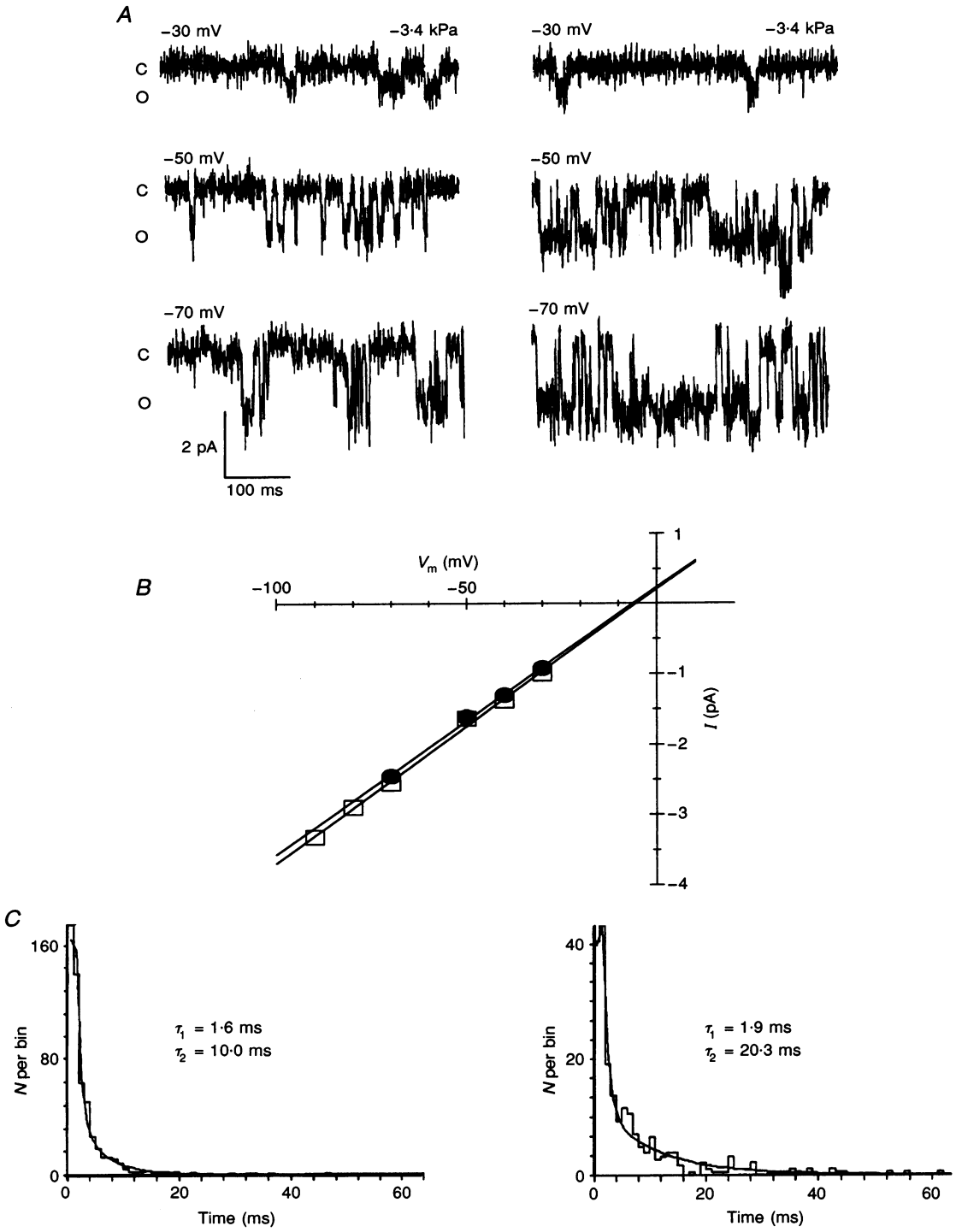

Figure 8. Properties of stretch-activated single channels before (left) and after (right) bath-application of 400 μM dBcAMP (2 min)

A, computer playbacks at −30, −50 and −70 mV. Note that the openings last longer in the presence of dBcAMP. *B*, voltage dependence of the open channel current; single channel conductance is 38 pS before (●) and 39 pS after (□) application of dBcAMP. *C*, lifetime of the open state. Distribution is fitted with two exponentials: control; $0{\cdot}69\exp(-t/1{\cdot}6\text{ ms}) + 0{\cdot}31\exp(-t/10\text{ ms})$. dBcAMP; $0{\cdot}51\exp(-t/1{\cdot}9\text{ ms}) + 0{\cdot}49\exp(-t/20{\cdot}3\text{ ms})$. V_m, membrane potential.

is not different from the control. PKA_c, however, increased the area underneath the Gaussian amplitude distribution from 2·3 pA s (*A*, control suction) to 5 pA s (*B*, PKA, no suction) and to 8·7 pA s (PKA plus suction). On average ($n = 5$), the effects of PKA_c on suction-induced NP_o resembled the effect of dBcAMP. This similarity suggests that the dBcAMP effects may be mediated by a PKA_c-mediated phosphorylation.

When the PKA_c was washed out, NP_o decreased below the control values (Fig. 10*E*). This low NP_o is typical for inside-out patches studied for more than 4 min. In the presence of PKA_c plus ATP, NP_o was stabilized at high values. Since this 'run-down' of NP_o is very much faster in inside-out than in cell-attached patches, it may be caused by the wash-out of cytosolic components (see Discussion).

Spontaneous activity. Three of six patches responded to the application of PKA_c with brief openings of SACs in the absence of suction. Three of six patches did not show this spontaneous activity. In these patches PKA_c increased the contribution of suction-induced long openings (longer than 10 ms) from 4·5 ± 3·5 to 11·5 ± 2·1%. Correspondingly, a longer lifetime of the open state was evaluated from the open-time histograms (increase in the time constant τ_2 from 8·6 to 15·3 ± 0·14 ms). In contrast, the spontaneous openings did not reveal significant changes in the open-time distribution.

Phosphorylation increases the sensitivity of SACs to stretch

The efficacy of negative pressures in inducing SAC activity was studied in the cell-attached configuration. The negative pressure was varied between −2 and −5 kPa, and suction

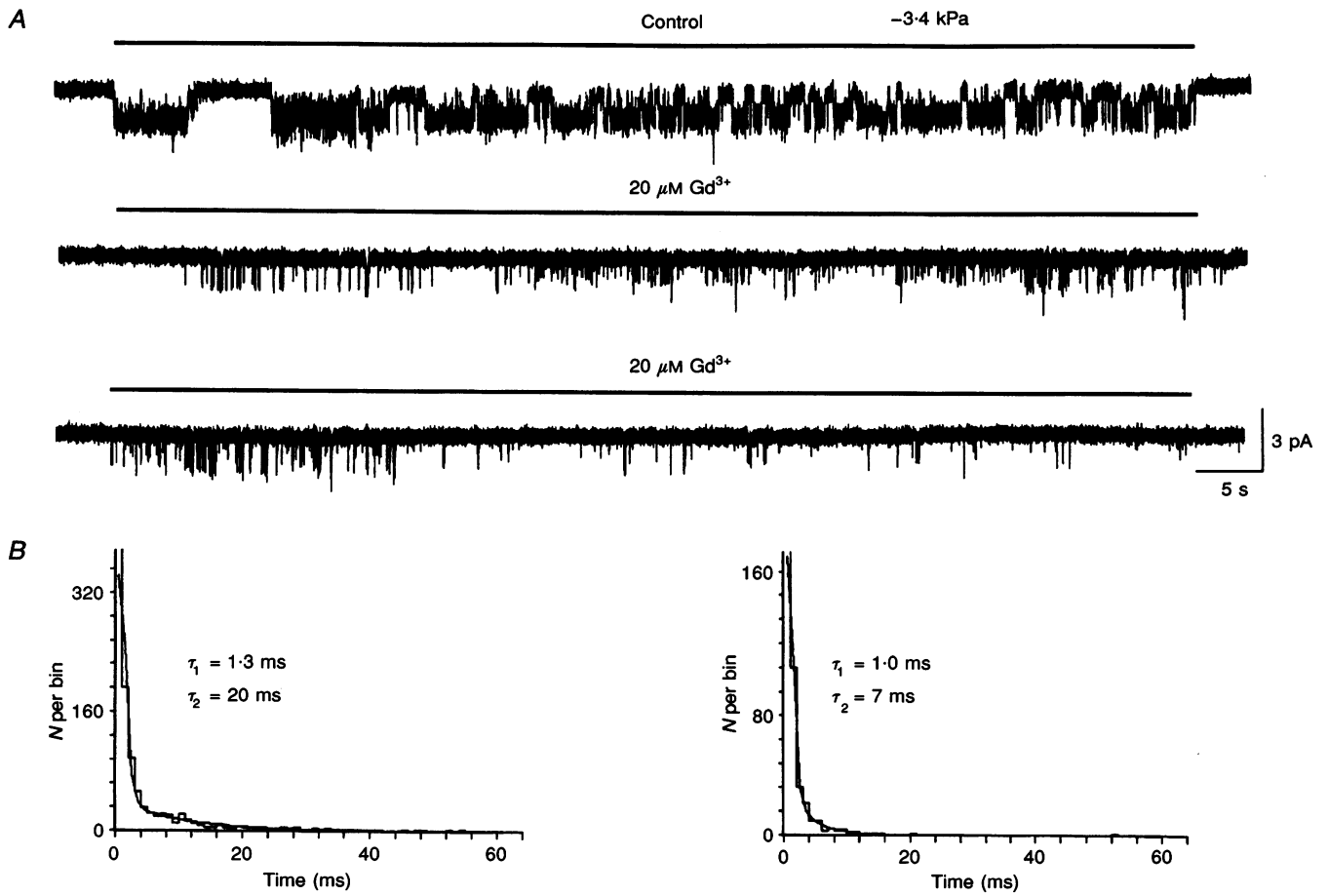

Figure 9. Gd^{3+} sensitivity of the cAMP-modulated stretch-activated single channel currents
Holding potential, −50 mV; suction of −3·4 kPa and 400 μM dBcAMP in the bath. 20 μM Gd^{3+} was layered behind the tip in the pipette. *A*, on-line pen recordings immediately (top trace), 2 min after (middle trace) and 3 min after (bottom trace) forming the patch. *B*, open times. Left: before Gd^{3+} effect, fitted with $0·76 \exp(-t/1·3 \text{ ms}) + 0·24 (-t/20 \text{ ms})$. Right: when Gd^{3+} was effective, fitted with $0·84 \exp(-t/1 \text{ ms}) + 0·16 \exp(-t/7 \text{ ms})$.

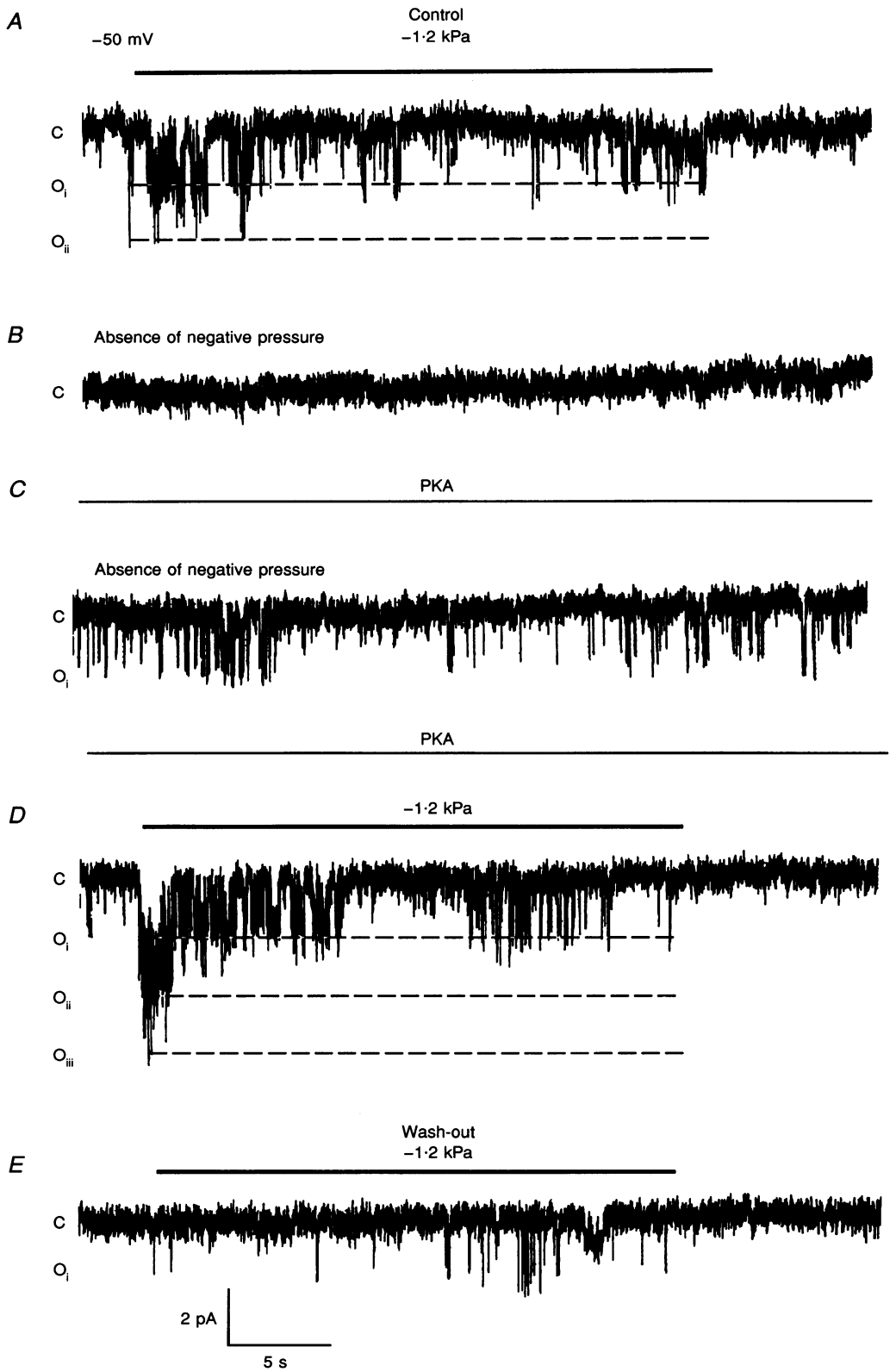

Figure 10. PKA_c (50 units ml^{-1}, plus 0·4 mM ATP) increases NP_o of SACs in inside-out patches
A, suction-induced single channel currents before application of PKA_c, NP_o 0·028%. *B*, control without suction, no channel activity. *C*, 1 min after application of PKA_c, shows spontaneous openings in the absence of suction, $NP_o = 0{\cdot}015$. *D*, PKA_c plus suction, $NP_o = 0{\cdot}06$. *E*, 2 min after wash-out of PKA_c, $NP_o = 0{\cdot}005$.

was applied every minute as a 20 s pulse. Suction of −5 kPa usually destroyed the seal. Since only three to five pulses of suction could be tested in the same cell, the results from several patches were averaged. For this, NP_o measured at a given suction was normalized by the NP_o during the largest suction. The results followed an S-shaped curve. The fit of the mean values with a saturation curve yielded a Hill coefficient close to 2. Without dBcAMP, activation of SACs required a minimal suction of −2·2 kPa, half-maximal NP_o was found at −3·2 kPa, and beyond −4 kPa the NP_o seemed to saturate. In the presence of 400 μM dBcAMP, the curve had the same slope but was shifted to less negative pressures. That is, half-maximal NP_o was already obtained at −2·6 kPa.

Three patches were stable enough for recording the effects of suction in both the absence and presence of dBcAMP. The comparison between these pairs indicated that dBcAMP increased the maximal NP_o by 30% (compare the

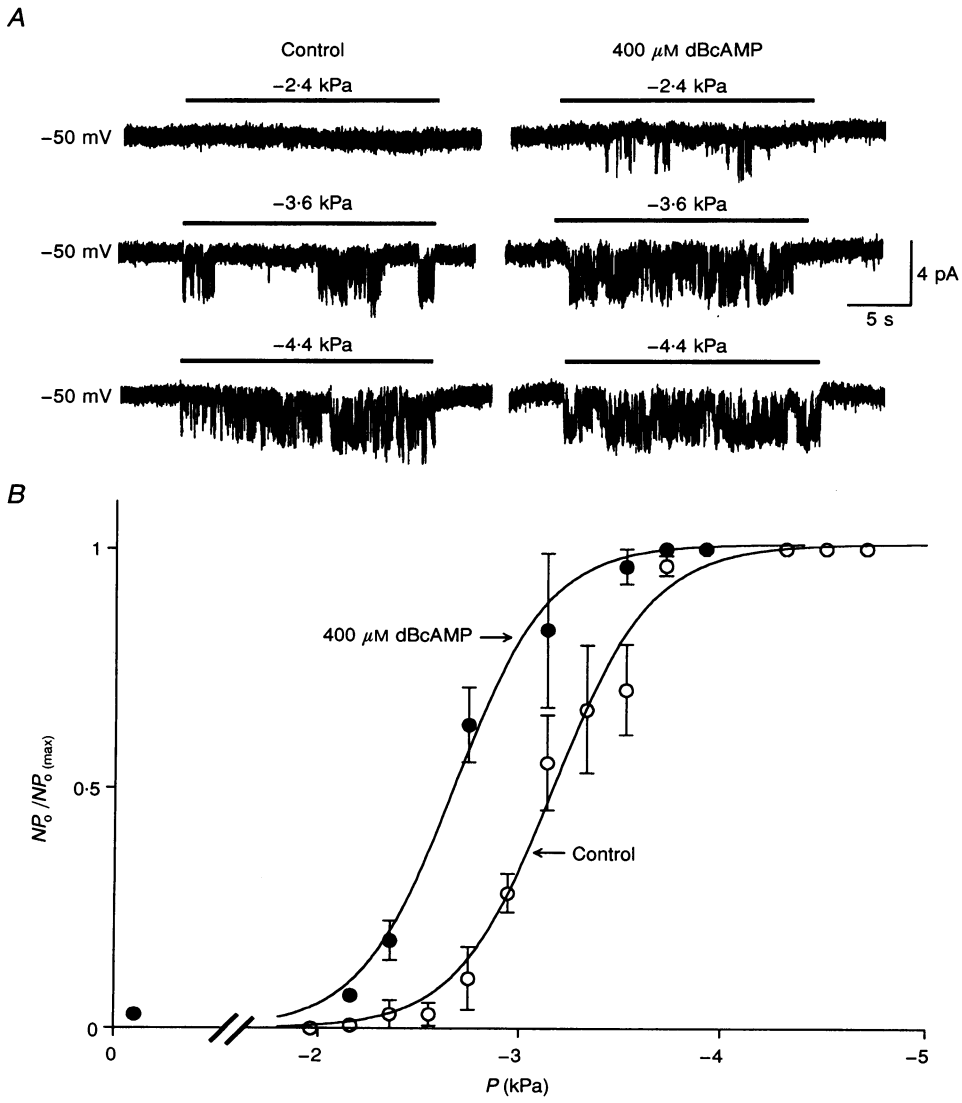

Figure 11. cAMP-dependent phosphorylation increases the efficacy of suction in activating the channels

A, representative original recordings in absence (left) and presence of 400 μM dBcAMP (right). *B*, plot of NP_o/maximal NP_o *versus* negative pressure. Symbols represent the means ± S.E.M. from six patches. Data were fitted by $1/\{1 + (S/S_{0.5})^{-H}\}$. Note: $S_{0.5}$ is the pressure of half-maximal activation. It is −3·2 kPa before and −2·6 kPa after addition of 400 μM dBcAMP. The Hill factor (H) was 1·84 in the absence and 1·98 in the presence of dBcAMP. This fit does not include the spontaneous openings recorded in five of sixteen patches. When $P_o/P_{o\,(max)} = 0{\cdot}03 \pm 0{\cdot}01$ at zero pressure was included, the fit yielded $H = 1{\cdot}44$ and $S_{0.5}$ was −2·9 kPa (curve not shown). Note that in three paired experiments, cAMP also increased maximal NP_o. For the averaged curve, this effect was not considered.

traces −4·4 kPa in Fig. 11*A*). In general, however, the statistical evaluation of the maximal NP_o parameter was hampered by the rupture of the patch. Also, NP_o during a first pulse of suction was usually larger than during a fifth pulse. As a consequence, Fig. 11*B* shows mean values that include not only the pairs but also the responses from different cells. By having normalized measured NP_o by maximal NP_o with and without dBcAMP, the possible increase in maximal NP_o is cancelled.

DISCUSSION

In this study, we investigated the time decay of stretch-induced I_{in}. The results excluded the possibility that 'adaptation' and its enhancement by cAMP-dependent phosphorylation are a direct effect of SACs. Instead, a secondary effect is suggested: Ca^{2+} influx through SACs will increase $[Ca^{2+}]_c$ and this in turn activates K^+ channels (see below). A similar role for K^+ currents in 'adaptation' has been suggested for the adaptation of the stretch-induced electrical activity in crayfish stretch receptors (Swerup, Rydquist & Ottoson, 1983) that was suppressed by TEA (Ottoson & Swerup, 1985) and mediated by Ca^{2+}-activated BK channels (Erxleben, 1993). The resemblance suggests that adaptation of stretch-induced responses due to Ca^{2+} activation of K^+ currents is a general phenomenon.

The chain of molecular events that transduce the stretch into the activation of SACs is not well understood (Sachs, 1987, 1990; Watson, 1991). One may speculate that both longitudinal stretch of the whole cell and deformation of the membrane in the patch pipette can impose a tension on the cytoskeleton which in turn interacts with the membrane proteins in a way that modifies their tertiary structure (Watson, 1991). This hypothesis has been applied to stretch activation not only of channels but also of H^+–Na^+ exchange (Schwartz, Both & Luchene, 1989), adenylyl cyclase (Watson, 1990) and phosphatidylinositol turnover (von Harsdorf, Lang, Fullerton, Smith & Woodcock, 1988). The involvement of the cytoskeleton is suggested by our finding that stretch failed to induce SAC activity in cells that had rounded up.

cAMP facilitation of adaptation

cAMP-dependent phosphorylation is known to modulate the activity of both receptor and voltage-operated ion channels (Hartzell, 1988). In the present study, exogenous cAMP analogues increased NP_o of both BK channels and SACs. While the effect on BK channels has been published (Kume, Takai, Tokuno & Tomita, 1989; Minami, Fukuzawa, Nakaya, Zeng & Inoue, 1993) the cAMP effect on NP_o of SACs is described here for the first time. A similar augmentation of SAC activity was found in cell-attached patches stimulated by dBcAMP and in inside-out patches to which PKA_c was applied. The latter result is compatible with the hypothesis that phosphorylation of the SAC protein augments NP_o.

cAMP and PKA increased the NP_o of SACs in two ways: (1) by increasing the number of short openings and (2) by the induction of long openings, resembling the effects of cAMP on L-type Ca^{2+} channels (e.g. Ono & Fozzard, 1992). In a simple model, one could explain the first effect as being due to the phosphorylation lowering the energy barrier through which the mechanical interaction drives the channel into the open state. The second effect could be attributed to a stabilization of the open channel conformation. dBcAMP augmented the frequency of opening in all experiments while it prolonged the openings in approximately 40% of the trials. In addition, this effect was delayed in relation to the first one. The results could suggest that protein kinase A phosphorylates not only one but several independent sites.

Stretch also activated SACs without stimulation of cAMP-dependent phosphorylation, although at lower efficacy. This could mean that SACs can open even in the absence or at some basal level of phosphorylation. The importance of basal phosphorylation is supported by our finding that the 'run-down' of SACs in inside-out patches is faster in the absence (Wellner & Isenberg, 1993*b*) than in the presence of exogenous ATP, cAMP analogues, and PKA (present paper). However, the possible recovery of SACs from run-down by addition of PKA has not been tested (for L-type Ca^{2+} channels, see Ono & Fozzard, 1992).

In approximately 15% of the patches, dBcAMP or PKA activated SACs in the absence of negative pressure. Since those 'spontaneous' openings occurred rather exceptionally one hesitates to conclude that phosphorylation can activate SACs without interference of the cytoskeleton. Instead, one may speculate that in these patches some deformation of the cytoskeleton remained; this small mechanical force may be sufficient to drive the phosphorylated channel into the open configuration.

Adaptation of I_{in} is caused by Ca^{2+} activation of BK channels

This concept has been established for the crayfish stretch receptor (Erxleben, 1993). In analogy, we have evidence from the smooth muscle cells of the urinary bladder that the decay of I_{in} is not caused by a decay in SAC activity but by the superimposition of increasing currents through BK channels ($I_{K(Ca)}$). Firstly, the adaptation of I_{in} was absent after the BK channels were blocked with TEA. Secondly, the adaptation of I_{in} was prevented by intracellular BAPTA, a result that supports the assumption that stretch activates BK channels indirectly through Ca^{2+} influx through SACs and an increase of $[Ca^{2+}]$ at the inner site of the membrane. In comparison to I_{in} of the crayfish stretch receptor, I_{in} of the smooth muscle cell decayed very slowly; the time course became comparable only in the presence of dBcAMP. One may speculate that in the crayfish stretch receptor the $[Ca^{2+}]$ near the membrane is less well controlled than in the myocytes, or that dBcAMP increases

the Ca^{2+} sensitivity of the smooth muscle BK channels to the same level as in the crayfish stretch receptor.

The Ca^{2+} sensitivity of BK channels in crayfish stretch receptors has not been published. In urinary bladder myocytes, NP_o follows [Ca^{2+}] steeply (Hill coefficient of 3) and with a K_D that depends on membrane potential, e.g. at 0 mV the K_D is 2 μM and at −50 mV it is 7 μM (Markwardt & Isenberg, 1992). In patches attached to resting cells, corresponding to inside-out patches at −50 mV and 0·1 μM [Ca^{2+}], BK channels show an NP_o of approximately 10^{-4} (Trieschmann, Klöckner, Isenberg, Utz & Ullrich, 1991). Clamp steps to 0 mV facilitate whole-cell $I_{K(Ca)}$ by increasing [Ca^{2+}] (via Ca^{2+} influx through L-type Ca^{2+} channels) and by increasing the Ca^{2+} sensitivity. Both cause $I_{K(Ca)}$(0 mV) to increase more rapidly than $I_{K(Ca)}$(−50 mV) during the experiment where sustained stretch was combined with 0·1 Hz pulsing. Stretch increased $I_{K(Ca)}$(0 mV) immediately while I_{in} was initially constant. Usually, I_{in}(−50 mV) started to decay after a delay of 20–60 s, and then the decay of I_{in} correlated with the increase in $I_{K(Ca)}$(0 mV) and with the increase in the tail current $I_{Cl(Ca)}$. The delay could suggest that cAMP-stimulated Ca^{2+}-ATPases can keep the [Ca^{2+}] near the membrane (Low, Darby, Kwan & Daniel, 1993) at a low level for some period of time; thereafter, when the transport capacity is exhausted, Ca^{2+} may accumulate and activate the cAMP-sensitive $I_{K(Ca)}$ and the $I_{Cl(Ca)}$ tail current (compare Klöckner, 1993).

cAMP-dependent phosphorylation does not activate BK channels directly but increases their Ca^{2+} sensitivity (Sadoshima *et al.* 1988; Minami *et al.* 1993). Accordingly, the cAMP effects on adaptation of I_{in} were suppressed by intracellular BAPTA. The combination of exogenous cAMP analogues, sensitizing the BK channels to Ca^{2+}, and Ca^{2+} accumulation from Ca^{2+} influx through stretch-activated channels, induced I_{in} to decay rapidly. In cells with added intracellular 8BrcAMP, stretch induced the decay of I_{in}(−50 mV) at constant $I_{K(Ca)}$(0 mV), suggesting that the pretreatment with 8BrcAMP had already increased the Ca^{2+} activation of $I_{K(Ca)}$(0 mV) to the maximum ('ceiling off' of the effect).

In cell-free patches from smooth muscle cells, stretch-activation of BK channels has also been reported to occur independently of SACs and Ca^{2+} influx (Dopico, Kirber, Singer & Walsh, 1994); it has been suggested that the stretch may activate BK channels through fatty acids as a second messenger (Kirber, Ordway, Clapp, Walsh & Singer, 1992). In patches attached to dividing cells from loach embryos (Medina & Bregestovski, 1991), BK channels were activated during cell division. In these experiments, the sensitivity of BK channels to suction was increased by dBcAMP and PKA_c. Whether and to what extent this type of activation contributes to the adaptation of I_{in} in the present conditions needs to be clarified in the future.

Putative contribution of β-adrenergic stimulation to the myogenic response

In vivo, filling of the urinary bladder causes not only the myogenic response but also a viscero-visceral reflex. The result of this reflex is an enhanced efferent adrenergic activity that is likely to be involved in the mechanical adaptation of the bladder muscle to the intraluminal filling. According to the present results we suggest that the hyperpolarization due to activation of BK channels reduces the Ca^{2+} influx through voltage-gated dihydropyridine-sensitive Ca^{2+} channels. In addition, β-stimulation and an increase in [cAMP] may relax the muscle independently through phosphorylation of the myosin light chain kinase.

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Acknowledgements

We are grateful to Dr A. F. Brading for her comments on this manuscript.

Received 31 March 1994; accepted 7 June 1994.