In Situ Characterization of the Ca^{2+} Sensitivity of Large Conductance Ca^{2+}-Activated K^+ Channels: Implications for Their Use as Near-Membrane Ca^{2+} Indicators in Smooth Muscle Cells

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ABSTRACT The Ca^{2+} sensitivity of large conductance Ca^{2+}- and voltage-activated K^+ channels ($BK_{V,Ca}$) has been determined in situ in freshly isolated myocytes from the guinea pig urinary bladder. In this study, in situ denotes that $BK_{V,Ca}$ channel activity was recorded without removing the channels from the cell. By combining patch clamp recording in the cell-attached configuration and microfluorometry of fura-2, we were able to correlate $BK_{V,Ca}$ channel activity with changes in cytoplasmic intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$). The latter were induced by ionomycin, an electroneutral Ca^{2+} ionophore. At 0 mV, the Hill coefficient (n_H) and the $[Ca^{2+}]_i$ to attain half of the maximal $BK_{V,Ca}$ channel activity (Ca_{50}) were 8 and 1 μM, respectively. The data suggest that this large Hill number was not a consequence of the difference between the near-membrane $[Ca^{2+}]$ ($[Ca^{2+}]_s$) and the bulk $[Ca^{2+}]_i$, indicated by fura-2. High Hill numbers in the activation by Ca^{2+} of $BK_{V,Ca}$ channels have been seen by different groups (e.g., filled squares in Fig. 4 of Silberberg, S. D., A. Lagrutta, J. P. Adelman, and K. L. Magleby. 1996. *Biophys. J.* 70:2640–2651). However, such high n_H has always been considered a peculiarity rather than the rule. This work shows that a high Ca^{2+} cooperativity is the normal situation for $BK_{V,Ca}$ channels in myocytes from guinea pig urinary bladder. Furthermore, the Ca_{50} did not display any significant variation among different channels or cells. It was also evident that $BK_{V,Ca}$ channel activity could decrease in elevated $[Ca^{2+}]_i$, either partially or completely. This work implies that the complete activation of $BK_{V,Ca}$ channels occurs with a smaller increment in $[Ca^{2+}]_s$ than previously expected from in vitro characterization of the Ca^{2+} sensitivity of these channels. Additionally, it appears that the activity of $BK_{V,Ca}$ channels in situ does not strictly follow changes in near-membrane $[Ca^{2+}]$.

INTRODUCTION

Variations in Ca^{2+} concentration at the inner face of the plasma membrane ($[Ca^{2+}]_s$) play an essential role in smooth muscle cell physiology. Typical examples are the generation of spontaneous transient outward currents (STOCs; e.g., Nelson et al., 1995), spontaneous transient inward currents (STICs; Wang et al., 1992), the isoproterenol-induced relaxation (Yamaguchi et al., 1995), and the superficial buffer barrier (van Breemen et al., 1995). This diffusion-limited region is a space of ~20 nm formed by the apposition of the plasma membrane and the sarcoplasmic reticulum (Somlyo and Somlyo, 1994). The small dimensions of this area have prevented the direct measurement of the changes in $[Ca^{2+}]_s$ during the aforementioned processes.

Different approaches have been developed in an attempt to measure $[Ca^{2+}]_s$. Fay's group pioneered the use of fluorescent Ca^{2+} indicators that have a hydrophobic tail attached to them. However, the more hydrophobic the tail was made to ensure complete incorporation of the dye into cell membranes, the more difficult it became to use these indicators. Furthermore, these hydrophobic Ca^{2+} indicators are not specifically restricted to the plasma membrane (Etter et al., 1994, 1996). Other groups have targeted aequorin to the plasma membrane to monitor $[Ca^{2+}]_s$ (Marsault et al., 1997; Nakahashi et al., 1997). Although this is certainly a more powerful approach, aequorin does not reject Mg^{2+} very well, its Ca^{2+} stoichiometry is rather complex, and cells do not have the ability to reconstitute the active aequorin after it has been consumed.

A third approach is to use endogenous Ca^{2+} sensors. In this respect, Ca^{2+}- and voltage-activated large conductance K^+ channels ($BK_{V,Ca}$) seem to be an ideal choice in smooth muscle cells. Particularly, they are abundant, have a K_d in the micromolar range, and a Hill number (n_H) between 1 and 3 (McManus, 1991; Carl et al., 1996). This latter feature is crucial for $BK_{V,Ca}$ channels to report a wide range of $[Ca^{2+}]_s$. It has been suggested that these characteristics (obtained in excised patches or with channels incorporated in planar bilayers) are not affected when the channels are separated from the cell (Pallotta et al., 1987). However, the activity of $BK_{V,Ca}$ channels can be influenced by many different factors including Mg^{2+} (Golowasch et al., 1986), phosphorylation (Kume et al., 1989), and G-proteins (Kume et al., 1992), among others. It is conceivable, then, that $BK_{V,Ca}$ channels respond to changes in $[Ca^{2+}]$ differently when they are detached from the cell.

To use $BK_{V,Ca}$ channels as $[Ca^{2+}]_s$ sensors, it is necessary to characterize their Ca^{2+} sensitivity in intact cells. Since it is extremely difficult to equilibrate cytoplasmic intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with external $[Ca^{2+}]$ in intact cells, it was decided to correlate $BK_{V,Ca}$ channel activity

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This work is dedicated to the memory of Fred Fay.

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with ionomycin-induced slow changes in $[Ca^{2+}]_i$. Several groups have reported previously that ionomycin effectively removes internal Ca^{2+} stores and equalizes $[Ca^{2+}]_i$ (Williams et al., 1985; Badminton et al., 1996; Huang and Neher, 1996). Accordingly, the data shown in this study indicate that the myoplasmic $[Ca^{2+}]$, sensed by fura-2, was not different from the submembrane $[Ca^{2+}]$, sensed by $BK_{V,Ca}$ channels, when ionomycin was utilized to change $[Ca^{2+}]_i$.

Based on the results shown in this work, $BK_{V,Ca}$ channels have an extremely high Ca^{2+} cooperativity and, at elevated $[Ca^{2+}]_i$, the relationship between ion channel activity and $[Ca^{2+}]_i$ is not constant. These characteristics of $BK_{V,Ca}$ channels in situ make them more like a Ca^{2+} switch than a Ca^{2+} indicator. A preliminary report of this work has previously been presented (Muñoz et al., 1997).

MATERIALS AND METHODS

Cell isolation and fura-2 loading

Albino male guinea pigs (350–450 g) were sacrificed by cervical dislocation followed immediately by exsanguination. The urinary bladder was quickly removed and placed in dissociation solution. The mucosa and submucosa layers were removed mechanically, and ~250 mg of detrusor muscle was cut into ~20-mg pieces. A combination of collagenase and papain was used to obtain single smooth muscle cells. Collagenase (3.0 mg/ml Type IA, Sigma Chemical, St. Louis, MO) was prepared in dissociation solution with 2 mM $CaCl_2$ (final $[Ca^{2+}]$ was 0.2 mM). Papain (1.5 mg/ml, Sigma Chemical) was pre-activated in dissociation solution with 250 μM each of EDTA and DTT for 20 min. Thereafter, the proteases were combined with the muscle strips in 2.5 ml dissociation solution and kept in a shaking water bath for 90 min at room temperature. The muscle strips were washed and incubated with DNAse for an additional 30 min. Single cells were obtained by gentle trituration of the digested tissue with a plastic pipette. The cell suspension was incubated in dissociation solution with 1–2 μM of fura-2/AM in the dark at room temperature for 1 h. Cells were washed and resuspended in Hepes-buffered saline solution and used immediately, or kept at 4°C before being used the same day.

Simultaneous microfluorometry of fura-2 and single channel recording with the patch clamp technique in single smooth muscle cells

The fura-2-loaded cell suspension (10 or 20 μl) was added to a pretreated recording chamber (to slow cell attachment) containing 1 ml depolarizing solution. This chamber was on the stage of a TMD inverted microscope (Nikon, Japan) coupled to an RF-F3010 microfluorometer for fura-2 measurement (Photon Technology International, South Brunswick, NJ). Fura-2 fluorescence excitation ratios (340/380 nm) were obtained each 50 ms and synchronized with ion channel recording by a TTL pulse. Ion channel currents filtered at 200 Hz were recorded with an Axopatch 1D (Axon Instruments, Foster City, CA) coupled to a Digidata 1200 (Axon Instruments) running Axotape (Axon Instruments) at a sampling rate of 1 KHz. Gigaseals were obtained with TW100F-4 borosilicate micropipettes (WPI, Sarasota, FL) of 6–8 MΩ made with a PP-83 vertical puller (Narishige). In the cell-attached configuration (Hamill et al., 1981), K^+ currents in the membrane patch follow the relationship $i_K = \gamma(V_m - V_h - E_K)$. In our recording conditions, membrane potential in the patch was close to 0 mV. This was because the cells were in a depolarizing solution ($V_m \sim 0$ mV) and V_h was held at 0 mV. Therefore, K^+ currents were driven exclusively by the K^+ gradient and any interference by nonselective cation channels with a 0 mV reversal potential was avoided. Ionomycin (10 μM) was applied to the cell with a borosilicate micropipette (4 MΩ) placed 10 μm from the cell, by pressure ejection (4 psi) with a PV830 PicoPump (WPI) for the time period indicated in the figures.

$[Ca^{2+}]_i$ measurements and ion channel activity analysis

Fura-2 ratios, corrected for background fluorescence, were converted to free $[Ca^{2+}]$ using the Grynkiewicz equation and an external calibration (Guerrero et al., 1994a). The external calibration was corrected for viscosity by reducing maximum (R_{max}) and minimum (R_{min}) 340/380 fluorescence ratios by 25% (Poenie, 1990). $K_d{*}\beta$ was 1740 nM. The measured $[Ca^{2+}]_i$ was correlated with the activity (see below) of $BK_{V,Ca}$ channels to determine their Ca^{2+} sensitivity. Inside a cell, the K_d for fura-2 could be higher than 200 nM (the value used here). However, variations in the fura-2 K_d would not affect the Hill coefficient determined for $BK_{V,Ca}$ channels. Conversely, a higher fura-2 K_d would mean also a higher Ca_{50} ($[Ca^{2+}]$ to reach half the maximal $BK_{V,Ca}$ channel activity). Nevertheless, the Ca_{50} reported here is in the range for other smooth muscle $BK_{V,Ca}$ channels (Carl et al., 1996). This suggests that a K_d of 200 nM for fura-2 is adequate. Imaging of fura-2/AM-loaded cells before and after plasma membrane permeabilization with 0.005% digitonin (Roe et al., 1990) indicated that ~90% of fura-2 was cytoplasmic. Initially, digitonin pores allowed Ca^{2+} to enter the cell and fura-2 saturation was obtained. At this point, the 340/380-nm fluorescence ratio was similar to the viscosity-corrected R_{max} value of the external calibration. In a few seconds, fura-2 left the cell with a time constant of 1 s. Imaging digitonin-permeabilized single smooth muscle cells from guinea pig urinary bladder did not show punctuate localization of fura-2 or hotspots (not shown). These data indicate that the loading procedure generated a Ca^{2+}-sensitive and mostly cytoplasmic-localized fura-2.

Because of the abundance of $BK_{V,Ca}$ channels, ion channel activity was calculated by the following relationship: $NP_o = Q_r/Q_e$, where $Q_r = \int^T (i_r - i_b)dt$ and $Q_e = T\,i_K$. Here, i_r was the recorded ion current, i_b was the baseline current, T was 100 ms, and i_K was the amplitude of the unitary $BK_{V,Ca}$ current. Both i_b and i_K were calculated with all-points amplitude histograms from sections where single channel current transitions were evident. The activity of $BK_{V,Ca}$ channels was calculated at each millisecond with a sliding window of 100 ms. $BK_{V,Ca}$ channel activity was matched with its corresponding $[Ca^{2+}]_i$ every 50 ms or 1 s, depending on the rate of change of $[Ca^{2+}]_i$. The relationship between $[Ca^{2+}]_i$ and the $BK_{V,Ca}$ channel activity was fitted to a modified Hill equation: $NP_o = NP_{omax}/\{1 + (Ca_{50}/[Ca^{2+}])^{nH}\}$. It was important to work with cells detached from the bottom of the chamber, otherwise contraction would disengage cells from the pipette. This implied moving the cell through the air-bath solution interface to obtain excised patches. However, the gigaseal could also be lost by this procedure. In those cases where it was possible to obtain excised patches (see Fig. 2), the initial ion current in the presence of 2 mM Ca^{2+} was similar to what was observed when ionomycin had increased $[Ca^{2+}]_i$ beyond 1.5 μM (in the cell-attached configuration). Data are shown as the mean ± SD and n represents the number of different cells studied.

Solutions and chemicals

The dissociation solution contained (in mM) 55 NaCl, 6 KCl, 5 $MgSO_4$, 10 glucose, 80 NaOH, 80 glutamic acid, and 10 Hepes, pH 7.4 (NaOH). The Hepes-buffered saline solution contained (in mM) 137 NaCl, 5 KCl, 4 $NaHCO_3$, 2 $CaCl_2$, 2 $MgSO_4$, 0.42 KH_2PO_4, 10 glucose, and 10 Hepes, pH 7.4 (NaOH). The depolarizing solution contained (in mM) 8 NaCl, 130 KCl, 2 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, and 10 Hepes, pH 7.4 (KOH). The pipette solution contained (in mM) 140 NaCl, 2 $CaCl_2$, and 10 Hepes, pH 7.4 (NaOH). For recording in symmetric $[K^+]$, the pipette solution contained (in mM) 140 KCl, 2 $CaCl_2$, and 10 Hepes, pH 7.4 (KOH). Fura-2/AM was from Molecular Probes, ionomycin (Ca^{2+} salt) and all other reagents were from Sigma Chemical.

RESULTS

$BK_{V,Ca}$ channels are abundant in smooth muscle cells, and have been very well characterized in a variety of cell types (Carl et al., 1996). Furthermore, both pharmacological (Trivedi et al., 1995) and electrophysiological (Markwardt and Isenberg, 1992; Heppner et al., 1997) properties of these channels have been studied in smooth muscle cells from the guinea pig urinary bladder. In the presence of Na^+ and Ca^{2+} in the recording pipette solution and at 0 mV, the average single channel current amplitude was 4.2 ± 0.9 pA (n = 38; Fig. 1 *A*). The extrapolated reversal potential was more negative than −80 mV, suggesting that this is a K^+ selective channel (Fig. 1 *B*). In high K^+ pipette solution, the reversal potential was very close to 0 mV. Although the single channel conductance was variable, it was in general higher than 200 pS (Fig. 1 *B*). This variability could be due to the recently described blockade of $BK_{V,Ca}$ channels in intact cells, which is lost on excision of the membrane patch (Snetkov et al., 1996). Indeed, a strong rectification was observed beyond +30 mV, particularly with the Na^+, Ca^{2+} pipette solution (not shown). In agreement with the abundance of $BK_{V,Ca}$ channels in smooth muscle cells, the channel depicted in Fig. 1 was seen in all membrane patches and always in high numbers. This channel also showed a clear voltage dependency, with a significant reduction in its activity by lower membrane potentials. A complete characterization of its voltage dependency was not carried out because of the channel abundance and the fact that we could not clamp $[Ca^{2+}]_i$ with ionomycin. Thus, this is a K^+-selective, large conductance ion channel, activated by Ca^{2+} and depolarization ($BK_{V,Ca}$).

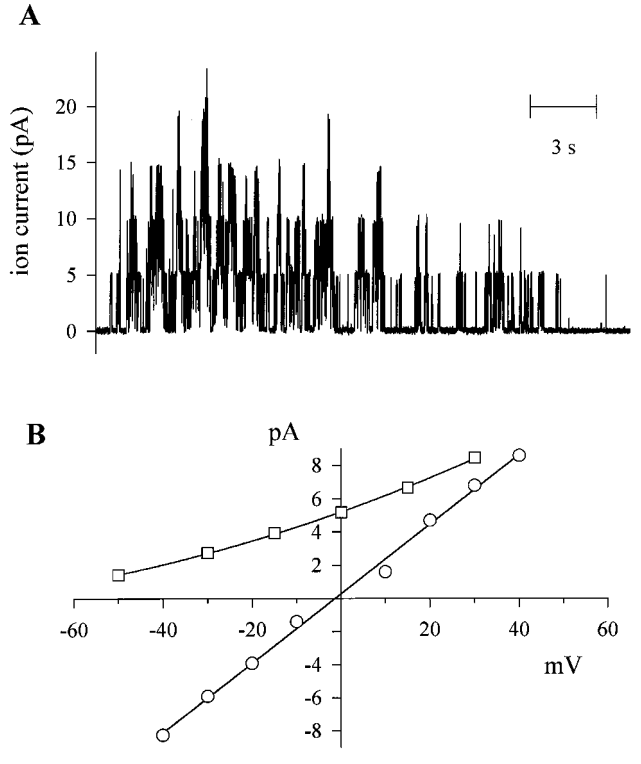

FIGURE 1 $BK_{V,Ca}$ channels in myocytes from guinea pig urinary bladder. (*A*) Single channel current transitions with Na^+, Ca^{2+} pipette solution were close to 5 pA at 0 mV recorded in the cell-attached configuration. This activity was only elicited when ionomycin increased $[Ca^{2+}]_i$ (not shown). Patches always have a large number of channels and in this case $[Ca^{2+}]_i$ did not rise sufficiently high to activate them completely. (*B*) Current-to-voltage curves in the cell-attached configuration showing that the main permeant ion is K^+. The extrapolated reversal potential was lower than −80 mV with Na^+, Ca^{2+} pipette solution (□), and close to 0 mV with symmetric $[K^+]$ (○). The latter had a slope conductance of 210 pS.

Our goal was to characterize the Ca^{2+} sensitivity of $BK_{V,Ca}$ channels in intact cells to use them as $[Ca^{2+}]_s$ reporters. To study the Ca^{2+} sensitivity of $BK_{V,Ca}$ channels in situ, ionomycin, a non-electrogenic Ca^{2+} ionophore, was used to homogeneously increase $[Ca^{2+}]_i$ in single myocytes. These cells were bathed in a depolarizing solution (containing 2 mM Ca^{2+}) to avoid interference by plasma membrane potential changes in the cell-attached recording configuration (Hamill et al., 1981), and to keep internal Ca^{2+} stores full. This depolarizing solution, however, reduced the Ca^{2+} releasing activity of ionomycin, in agreement with previous reports (Fasolato and Pozzan, 1989). Resting $[Ca^{2+}]_i$ was 110 ± 41 nM (n = 38), which was slightly higher compared to myocytes in normal saline solution (94 ± 4 nM, n = 70). At resting $[Ca^{2+}]_i$ and at 0 mV, $BK_{V,Ca}$ channels did not show any activity, as has previously been reported for cell-attached patch recording of $BK_{V,Ca}$ channels from coronary myocytes (Tanaka et al., 1997). Fig. 2 *A* shows that when ionomycin failed to increase $[Ca^{2+}]_i$ beyond 300 nM, there was no significant activation of ion channels (Fig. 2 *A*). This, despite the same patch after excision, showed ion current equivalent to at least 10 channels (Fig. 2 *C*). Further studies (see below) revealed that ionomycin had to increase $[Ca^{2+}]_i$ to a threshold near 500 nM to obtain significant activation of $BK_{V,Ca}$ channels at 0 mV.

It has previously been shown that ionomycin homogeneously increases $[Ca^{2+}]_i$ (Williams et al., 1985; Badminton et al., 1996), which was corroborated by the data shown here. Hence, the activity of $BK_{V,Ca}$ channels, measured as NP_o, was correlated with the changes in $[Ca^{2+}]_i$ (Fig. 3 *B*). It is evident that $BK_{V,Ca}$ channels did not open until $[Ca^{2+}]_i$ had reached a threshold of 500 nM at 0 mV (Fig. 3 *B*). Surprisingly, the activity showed a very steep dependence on $[Ca^{2+}]_i$ and complete saturation beyond 1.5 μM. This relationship was fitted using a modified Hill equation (see Methods). In this case NP_{omax} was 15.1, suggesting that at least 15 channels were present in the patch. Fig. 3 *B* shows that the relationship between the normalized ion channel activity (NP_o/NP_{omax}) and $[Ca^{2+}]_i$ was accurately fitted with an n_H of 8.5, suggesting a very strong cooperativity in the activation by Ca^{2+} of $BK_{V,Ca}$ channels in situ. The same analysis performed in seven more cells gave a Hill coefficient of 8.7 ± 3.2 (n = 8). This implies that the channels are activated by a very small range of $[Ca^{2+}]_i$. Indeed, 10% and 90% of the maximal activity of $BK_{V,Ca}$ channels (NP_{omax}) were obtained with $[Ca^{2+}]_i$ of 0.74 ± 0.10 μM (n = 8) and 1.24 ± 0.24 μM (n = 8), respectively. While this is only a

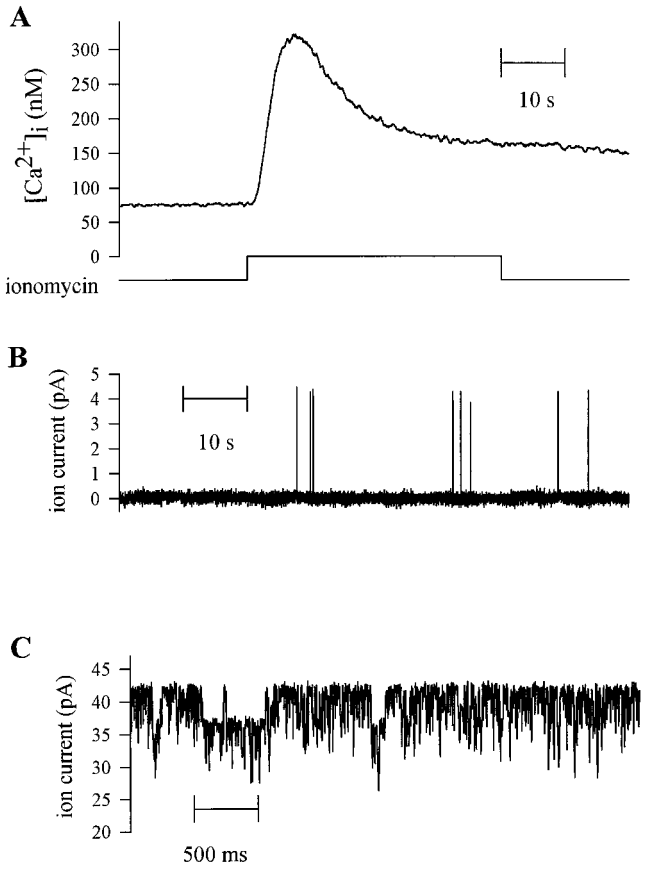

FIGURE 2 $[Ca^{2+}]_i$ threshold for activation of $BK_{V,Ca}$ channels. (*A*) Time course of the ionomycin-induced increase in $[Ca^{2+}]_i$. In this case ionomycin increased $[Ca^{2+}]_i$ to only 300 nM. (*B*) Simultaneous recording of single ion currents in the cell-attached configuration. There was no activity before the rise in $[Ca^{2+}]_i$, and this rise in $[Ca^{2+}]_i$ did not significantly increase the activity of $BK_{V,Ca}$ channels. Single channel currents of 4.2 pA amplitude were observed. (*C*) The same patch showed a 42 pA current after the inside-out configuration was obtained (by moving the cell through the air-solution interface), and the patch was exposed to the bathing solution containing 2 mM Ca^{2+}. This suggests that there were at least 10 $BK_{V,Ca}$ channels in the patch. Note the different scale for ion current and time between (*B*) and (*C*).

0.5 μM difference, an increment 90× higher (45 μM) is required to obtain the same change in activity at 0 mV for $BK_{V,Ca}$ channels in excised patches from guinea pig urinary bladder myocytes (calculated by us from the data reported by Markwardt and Isenberg, 1992). These arguments suggest that the Ca^{2+} sensitivity of BK_{Ca} channels in cells is greater than previously recorded in excised patches or for channels incorporated into planar bilayers (McManus, 1991; Carl et al., 1996).

Interestingly, the Ca_{50} was 0.96 ± 0.11 μM (n = 8) at 0 mV. This is in the range described for $BK_{V,Ca}$ channels from different smooth muscle cells (McManus, 1991; Carl et al., 1996) and close to the 2.3 μM reported by Markwardt and Isenberg (1992).

A high Hill number could be the consequence of Ca^{2+} gradients, where $[Ca^{2+}]_s$ is increasing at a higher rate than the bulk $[Ca^{2+}]_i$. It was evident that fura-2 perceived the rise in $[Ca^{2+}]_i$ induced by ionomycin well before $BK_{V,Ca}$ channels (2.9 ± 1.0 s, n = 4). This implies that when ionomycin is used to increase $[Ca^{2+}]_i$, the bulk $[Ca^{2+}]$ is not necessarily lingering $[Ca^{2+}]_s$. An indirect way of testing whether Ca^{2+} gradients are affecting the Hill coefficient is to compare the numbers obtained with different rates of change in $[Ca^{2+}]_i$. Slow Ca^{2+} removal driven by Ca^{2+} pumps (Guerrero et al., 1994b) is characterized by the absence of Ca^{2+} gradients (Kargacin and Fay, 1991; Blumenfeld et al., 1992). Hence, the same analysis with the Hill equation was applied to those cells where the ionomycin-induced rise in $[Ca^{2+}]_i$ recovered to resting $[Ca^{2+}]_i$. Fig. 4 shows one such case: here NP_o is reported each 50 ms for the rate of rise (Fig. 4 *B*) and only each second for the falling phase (Fig. 4 *C*). Given that the number of points is similar, a 20× difference between both rates of change in $[Ca^{2+}]_i$ is suggested. Nevertheless, the Hill coefficient calculated from the recovery in $[Ca^{2+}]_i$ (n_H = 9.8) was similar to the one obtained for the rise in $[Ca^{2+}]_i$ (n_H = 10.5). Hill coefficients from two more cells gave an n_H = 11.3 ± 3.3 (n = 3) for the slower fall in $[Ca^{2+}]_i$, clearly arguing against the hypothesis that Ca^{2+} gradients were the reason for large Hill numbers in our recording conditions. A similar 0.5 μM range for the $BK_{V,Ca}$ channel activity was calculated from the slow recovery of $[Ca^{2+}]_i$ (10% at 0.89 ± 0.24 μM and 90% at 1.36 ± 0.44 μM; n = 3). The cell shown in Fig. 4 had a slight shift of Ca_{50} to lower $[Ca^{2+}]_i$. However, this difference was neither significant nor reproducible. In the three cells examined, Ca_{50} was 1.10 ± 0.33 μM, which was practically the same as the Ca_{50} obtained from the rise in $[Ca^{2+}]_i$ (0.96 μM). It appears, then, that ionomycin, as has previously been reported (Williams et al., 1985; Badminton et al., 1996), increased $[Ca^{2+}]_i$ in a uniform manner. Thus, $[Ca^{2+}]_i$ is a good approximation of the $[Ca^{2+}]$ seen by $BK_{V,Ca}$ channels when this ionophore is present. Furthermore, $BK_{V,Ca}$ channels consistently showed a high Ca^{2+} cooperativity when recorded in intact cells.

Although very small, a reduction in NP_{omax} can be seen in Fig. 4 (from 15 to 13). A reduction in the maximal activity of $BK_{V,Ca}$ channels was always present, but the extent of this reduction was variable. Fig. 5 shows a cell where a significant drop in NP_{omax} was observed (30 to 20). Interestingly, the Ca^{2+} sensitivity was not affected in those $BK_{V,Ca}$ channels that were still active; specifically, the Ca_{50} was very similar when calculated from either the rise or the fall in $[Ca^{2+}]_i$ (Fig. 5 *B*). A more dramatic and the most frequently observed example of this type of behavior (seen in five cells) is shown in Fig. 6. Here, channel activity dropped almost completely before $[Ca^{2+}]_i$ had returned to 2 μM and again, similarly to the case shown in Fig. 5, the remaining activity ceased only when $[Ca^{2+}]_i$ reached 1 μM. Importantly, in this case there were oscillations of activity on top of the activation by Ca^{2+} (Fig. 6, *arrowheads*).

This reduction in the maximal activity with higher $[Ca^{2+}]_i$, and the apparently Ca^{2+}-independent oscillations in activity, were completely unexpected features. These observations limit the use of $BK_{V,Ca}$ channels as quantita-

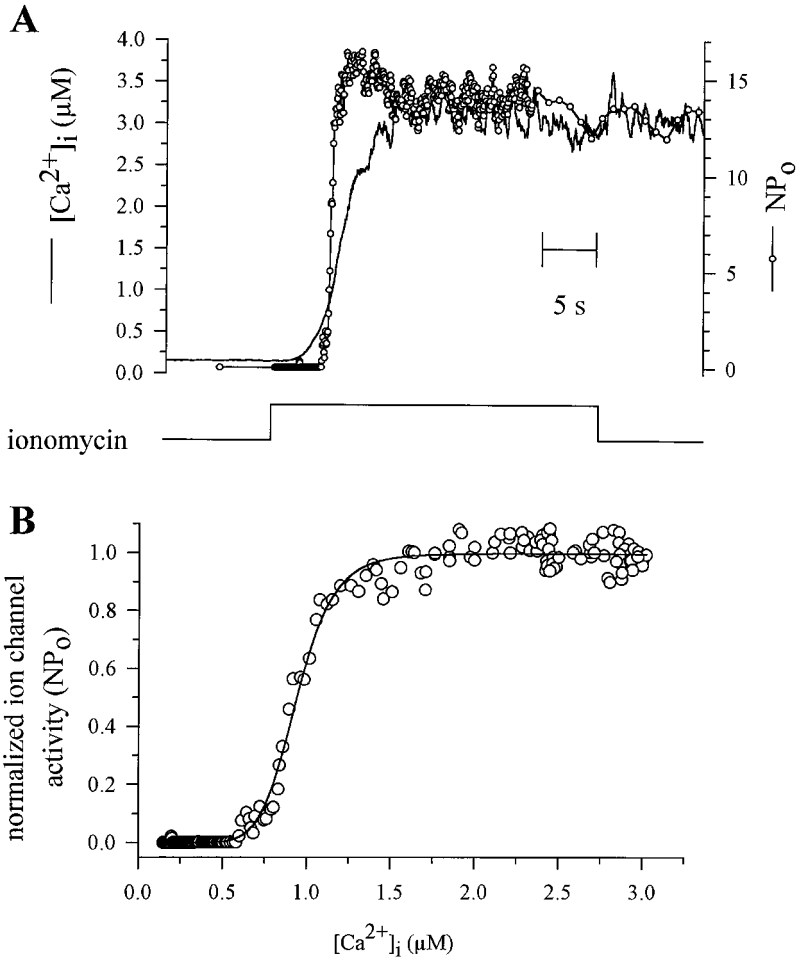

FIGURE 3 Relationship between the activity of $BK_{V,Ca}$ channels and $[Ca^{2+}]_i$ in situ for myocytes from the guinea pig urinary bladder. (*A*) Increment in $[Ca^{2+}]_i$ indicated by fura-2 (*continuous line, left axis*) and the corresponding change in $BK_{V,Ca}$ channel activity (NP_o, *circles, right axis*) in the cell-attached configuration, during application of ionomycin (10 μM in the puffer pipette) for the time indicated. (*B*) The relationship between $[Ca^{2+}]_i$ and the normalized ion channel activity (NP_{omax} 15.1) was fitted to a modified Hill equation with $n_H = 8.5$ and $Ca_{50} = 0.94$ μM (*continuous line*). It is evident that $BK_{V,Ca}$ channels activated when $[Ca^{2+}]_i$ rose to 500 nM, and this activity leveled off completely beyond 1.5 μM $[Ca^{2+}]_i$.

tive near-membrane Ca^{2+} indicators. Furthermore, they suggest that STOCs in guinea pig urinary bladder could be generated by smaller changes in $[Ca^{2+}]_s$ than previously expected from characterizations of $BK_{V,Ca}$ channels in vitro, and that Ca^{2+} acts more like a switch, as has previously been suggested (Golowasch et al., 1986). Additionally, $BK_{V,Ca}$ channel activity can be decreased by a mechanism independent of Ca^{2+} in intact cells.

DISCUSSION

The combination of microfluorometry of fura-2 with single channel recording of $BK_{V,Ca}$ channels in situ in freshly isolated smooth muscle cells has shown two new characteristics of these channels: 1) a very large cooperativity for the activation by Ca^{2+}, and 2) a variable relationship between $[Ca^{2+}]_i$ and $BK_{V,Ca}$ channel activity.

The activity of $BK_{V,Ca}$ channels has been used extensively as a measure of $[Ca^{2+}]_s$ with the assumption that they follow changes in $[Ca^{2+}]$ very closely (Hogg et al., 1993; Ganitkevich and Isenberg, 1996). In general, the effect of different experimental conditions on $[Ca^{2+}]_s$, reflected by changes in $BK_{V,Ca}$ channel activity, is examined in intact cells, and this is correlated with an in vitro calibration of the Ca^{2+} sensitivity of $BK_{V,Ca}$ channels (Roberts, 1993; Allard et al., 1996). Once again, this assumes that these channels behave the same whether they are out of or in the cell (Pallotta et al., 1987). However, it is desirable to examine the Ca^{2+} sensitivity of $BK_{V,Ca}$ channels in the same conditions where they will be used to report subsarcolemmal $[Ca^{2+}]$. To carry this out, ionomycin, a non-electrogenic Ca^{2+} ionophore (Erdhal et al., 1995), was selected to increase $[Ca^{2+}]_i$ in intact single smooth muscle cells while simultaneously recording $BK_{V,Ca}$ channel activity in cell-attached patches. Since the membrane potential is not clamped in the cell-attached configuration, the cells must be kept in a depolarizing solution to avoid changes in the membrane potential by the currents passing through the membrane patch (Hamill et al., 1981). However, this depolarizing solution clearly limited the capacity of ionomycin to increase $[Ca^{2+}]_i$. The longer the cells were maintained in such depolarizing solution the less effective ionomycin became at increasing $[Ca^{2+}]_i$, even when 2 mM Ca^{2+} was present. This agrees with the preference of ionomycin to increase $[Ca^{2+}]_i$ by releasing it from internal Ca^{2+} stores (Albert and Tashjian, 1986; Smith et al., 1989), and that high K^+ solutions prolong the time of recovery of internal Ca^{2+} stores (Sanchez-Fernandez et al., 1993).

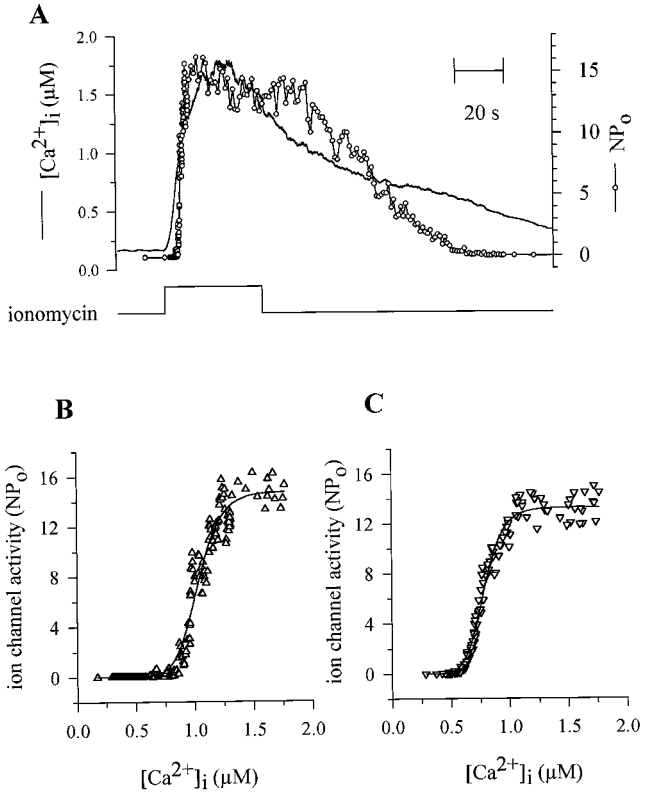

FIGURE 4 High Hill coefficients were not affected by the rate of change in $[Ca^{2+}]_i$. (*A*) Changes in $[Ca^{2+}]_i$ (*continuous line, left axis*) and the corresponding change in $BK_{V,Ca}$ channel activity (NP_o, *circles, right axis*) in the cell-attached configuration, during application of ionomycin for the time indicated. In this cell, $[Ca^{2+}]_i$ recovered to basal levels following application of ionomycin. $BK_{V,Ca}$ channel activity (*circles*) is shown either each 50 ms or each second for the rising and falling phases of the change in $[Ca^{2+}]_i$, respectively. Relationships between $[Ca^{2+}]_i$ and ion channel activity are shown for the rising (*B*, △) and falling (*C*, ▽) changes in $[Ca^{2+}]_i$. They were fitted to the Hill equation obtaining NP_{omax} = 14.8 and 13.3; Ca_{50} = 1.0 and 0.78 μM; n_H = 10.5 and 9.8 for the rise (*B*, △) and fall (*C*, ▽) in $[Ca^{2+}]_i$, respectively. These differences were not significant (particularly n_H) despite the difference between the rates of change in $[Ca^{2+}]_i$. This implies that indeed $BK_{V,Ca}$ channels respond to a very short range of $[Ca^{2+}]_i$ when they are in the cell.

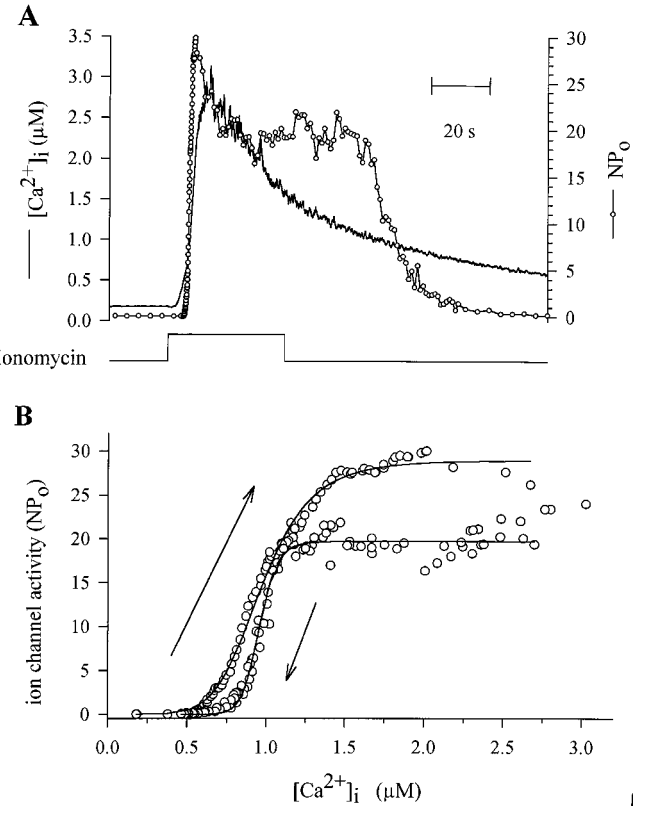

FIGURE 5 Reduction in $BK_{V,Ca}$ channel activity at high $[Ca^{2+}]_i$. (*A*) Changes in $[Ca^{2+}]_i$ (*continuous line, left axis*) and the corresponding change in $BK_{V,Ca}$ channel activity (NP_o, *circles, right axis*), in the cell-attached configuration, during application of ionomycin for the time indicated. In this cell, $BK_{V,Ca}$ channel activity showed an unexpected drop at elevated $[Ca^{2+}]_i$. (*B*) $BK_{V,Ca}$ channel activity (*circles*) as a function of $[Ca^{2+}]_i$ is shown either each 50 ms or each second, for the rise (*upward arrow*) or fall (*downward arrow*) in $[Ca^{2+}]_i$, respectively. There was a significant reduction in NP_{omax} from 29 to 19.7, without a change in the Ca_{50} (from 0.97 to 0.96 μM) for those channels that were still active.

In agreement with previous reports, $BK_{V,Ca}$ channels were abundant in the plasma membrane of guinea pig urinary bladder myocytes (Markwardt and Isenberg, 1992; Heppner et al., 1997). Since membrane patches were obtained with as many as 30 channels, this could conceivably generate local changes in the $[K^+]$. This does not seem to be the case, however, because the amplitude of the current was not significantly changed between the beginning and end of $BK_{V,Ca}$ channel activity. Nevertheless, transient changes in $[K^+]$ at the peak of the ion channel activity, where the single channel current could not be resolved, cannot be excluded. In any event, this would underestimate maximal NP_o, implying that the cooperativity could be even higher than indicated here.

Ionomycin increases $[Ca^{2+}]_i$ homogeneously in other cell types (Williams et al., 1985; Badminton et al., 1996). Although we have no direct evidence that ionomycin is doing this, the following arguments clearly indicate that ionomycin effectively dissipated Ca^{2+} gradients under our recording conditions. These are 1) a slow increase in $[Ca^{2+}]_i$, which together with the presence of mobile Ca^{2+} buffers (e.g., fura-2) is known to reduce the formation of Ca^{2+} gradients (Blumenfeld et al., 1992). Furthermore, it has been shown that Ca^{2+} release from internal stores does not necessarily produce Ca^{2+} gradients (Marsault et al., 1997) or it generates a smaller increment in $[Ca^{2+}]$ at the plasma membrane than the bulk cytoplasm (Nakahashi et al., 1997). Since ionomycin preferentially releases Ca^{2+} from internal stores (Albert and Tashjian, 1986; Smith et al., 1989), the changes in $[Ca^{2+}]_i$ are unlikely to be associated with significant Ca^{2+} gradients. 2) Fura-2 always reported the ionomycin-induced rise in $[Ca^{2+}]_i$ before $BK_{V,Ca}$ channel activity was observed. Ionomycin required almost 3 s to increase $[Ca^{2+}]_i$ to 500 nM (the threshold for activation of $BK_{V,Ca}$ channels). This discards the idea that the 100-ms time window used to calculate $BK_{V,Ca}$ channel activity was too long. Similar values of Ca_{50} and n_H were obtained when the

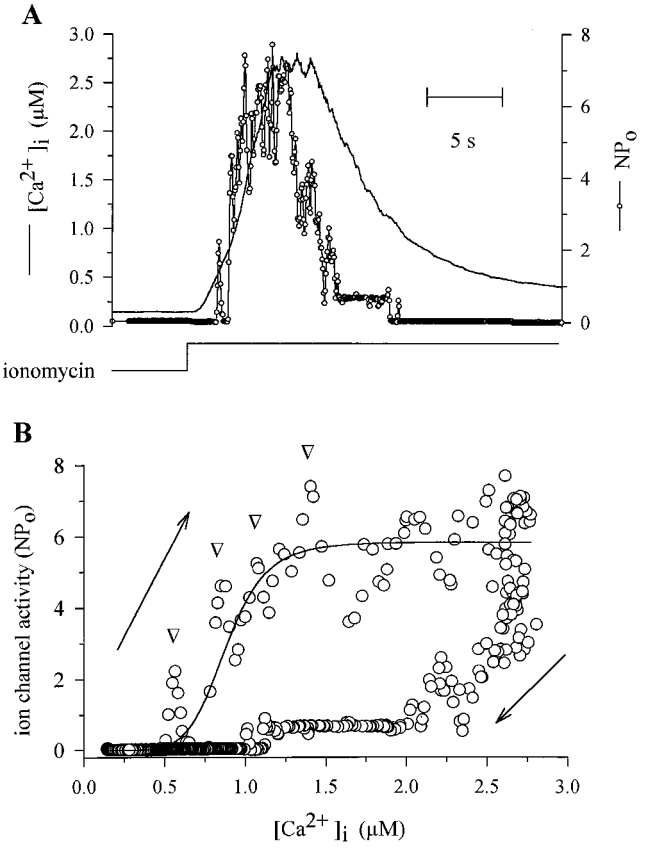

FIGURE 6 Changes in $BK_{V,Ca}$ channel activity independent of $[Ca^{2+}]_i$. (*A*) $[Ca^{2+}]_i$ and $BK_{V,Ca}$ channel activity in response to ionomycin application in a cell where $BK_{V,Ca}$ channels show distinctive oscillations in activity (*arrowheads*, ▽), despite the fact that $[Ca^{2+}]_i$ was increasing. NP_{omax} was 5.8, but clearly there are more than seven channels in the patch, Ca_{50} = 0.88 μM, and n_H = 7.0 for the rise in $[Ca^{2+}]_i$. The ion channel activity diminished so dramatically before the fall in $[Ca^{2+}]_i$ that the Hill equation could not be applied. Note, however, that $BK_{V,Ca}$ channel activity did not cease completely until $[Ca^{2+}]_i$ was near 1 μM.

sliding window used to calculate NP_o was changed to 500 ms. This implies that the delay between $[Ca^{2+}]_i$ and opening of $BK_{V,Ca}$ channels was not due to a slow calculation of NP_o. It also means that diffusion of Ca^{2+}, a very fast Ca^{2+} removal process (Gómez et al., 1996), was faster dissipating Ca^{2+} gradients than ionomycin increasing $[Ca^{2+}]_i$. 3) Most importantly, Hill coefficients were not affected by different rates of change in $[Ca^{2+}]_i$. Ca^{2+} gradients are not present, or are significantly smaller, during the recovery of a rise in $[Ca^{2+}]_i$ due to the slower rate of change in $[Ca^{2+}]$ (Kargacin and Fay, 1991; Blumenfeld et al., 1992). Thus, $BK_{V,Ca}$ channels were effectively activated by a homogeneous increment in $[Ca^{2+}]$. It can be concluded that a high Ca^{2+} cooperativity is a characteristic of $BK_{V,Ca}$ channels in intact cells.

Initially, Hill coefficients for activation by Ca^{2+} of $BK_{V,Ca}$ channels were reported to be between 1 and 3 (McManus, 1991). Nevertheless, Golowasch et al. (1986) proposed that activation by Ca^{2+} of $BK_{V,Ca}$ channels in the cell might have a higher cooperativity than expected from previous reports due to the effect of Mg^{2+}. Although this effect has not been observed consistently at physiological $[Mg^{2+}]$, the idea that $BK_{V,Ca}$ channels could act as calcium switches was put forward. More recently, there have been reports of higher Hill numbers, but they have been considered a peculiarity rather than the rule (Silberberg et al., 1996; Wu et al., 1996). However, one conclusion from this study is that a high Hill coefficient for $BK_{V,Ca}$ channels from guinea pig urinary bladder myocytes was always observed. Furthermore, Carl et al. (1996) have derived the relationship between voltage and Ca^{2+} sensitivities of $BK_{V,Ca}$ channels. The following equation was obtained: $n_H = \Delta V_{1/2}/(k \ln 10)$ [where $\Delta V_{1/2}$ is the shift in the voltage for half-maximal activation and k is the steepness of the activation by voltage from the Boltzmann equation]. This equation shows that $\Delta V_{1/2}$ and $1/k$ are directly related to the Hill coefficient. Based on that, it can be inferred from the data of Meera et al. (1996), obtained with cloned human $BK_{V,Ca}$ channels expressed in oocytes, that there is a window of physiological $[Ca^{2+}]_i$ where the Hill coefficient is the highest. Increasing $[Ca^{2+}]_i$ beyond this window produces a reduction in the Hill coefficient, since a smaller $\Delta V_{1/2}$ was obtained (Fig. 2 of Meera et al., 1996). Similarly, a peak in the steepness of the conductance-voltage curves of *mslo* channels was observed at 1.7 μM $[Ca^{2+}]_i$ (Cui et al., 1997). This higher Ca^{2+} sensitivity at physiological $[Ca^{2+}]_i$ seems to be due to the recently described "calcium bowl" (Schreiber and Salkoff, 1997). Partial deletion of this site inhibits the large shift of $V_{1/2}$ at lower $[Ca^{2+}]_i$, leaving only small shifts at both low and high $[Ca^{2+}]_i$ (Schreiber and Salkoff, 1997). Indeed, it has been reported that a lower Ca_{50} is associated with a high Hill number (Wu et al., 1996) as observed by Golowasch et al. (1986). Although these reports suggest that a high Hill coefficient for $BK_{V,Ca}$ channels is evident only when studied in physiological $[Ca^{2+}]_i$ (which is the range of $[Ca^{2+}]_i$ where this work was carried out), this does not seem to be the sole explanation. This follows from the observation that $BK_{V,Ca}$ channels from urinary bladder smooth muscle cells, when recorded in excised patches and activated by $[Ca^{2+}]_i$ in the range of 100 to 500 nM, showed a Hill coefficient of 1.3 (calculated by us from Fig. 1 of Heppner et al., 1997). Thus, a factor in the intracellular milieu or a posttranslational modification seems to be involved in determining a high Hill coefficient for $BK_{V,Ca}$ channels in situ. Recently, Slob, a new class of cytoplasmic protein that increases dSlo channel activity, was identified for the first time (Schopperle et al., 1998). Conceivably, excised patches would lack the regulation of $BK_{V,Ca}$ channels by this or other types of cytoplasmic proteins.

The value of Ca_{50} depends on the membrane potential (e.g., Markwardt and Isenberg, 1992). However, at the same membrane potential and with $BK_{V,Ca}$ from the same cells, there is a strong variability in the value of Ca_{50} (e.g., Sansom and Stockand, 1994; Wu et al., 1996). Apparently, the presence of accessory proteins, such as the β subunit, can increase Ca^{2+} sensitivity of $BK_{V,Ca}$ channels (e.g.,

Meera et al., 1996). However, important variations in Ca_{50} were also evident even when the α subunit alone was expressed in oocytes (Silberberg et al., 1996). Interestingly, such variability in Ca_{50} was not observed for the $BK_{V,Ca}$ channels reported here. Apparently, all $BK_{V,Ca}$ channel activity leveled off completely for $[Ca^{2+}]_i$ higher than 1.5 μM. This could be explained by considering that all the channels have the same Ca_{50} or, alternatively, that there are channels with a Ca_{50} much higher than 1 μM that were not activated at all by elevating $[Ca^{2+}]_i$ up to 3 μM. The latter explanation is unlikely because excised patches that were exposed to the bathing solution (containing 2 mM Ca^{2+}) did not show any increment in the total ion current. If anything, a small reduction in the $BK_{V,Ca}$ activity was observed after few minutes in the excised patch configuration. Thus, it appears as all $BK_{V,Ca}$ channels have the same Ca_{50} when recorded in intact cells. Nevertheless, an independent measure of the number of channels would be needed to corroborate this observation. This is because elevated $[Ca^{2+}]$ can reduce the activity of $BK_{V,Ca}$ channels (see below).

In general, $BK_{V,Ca}$ currents activated by membrane depolarization at a fixed $[Ca^{2+}]_i$ do not show inactivation in smooth muscle. Nevertheless, Hogg et al. (1993) have reported that STOCs decay much faster than STICs, suggesting that closure of $BK_{V,Ca}$ channels might not necessarily be driven by reductions in $[Ca^{2+}]_s$ only. Interestingly, Rothberg et al. (1996) have demonstrated that high $[Ca^{2+}]$ can induce a low activity mode in $BK_{V,Ca}$ channels. Actually, they found that in asymmetric $[K^+]$ (K^+ being present inside and absent outside, precisely the conditions used in this study), $BK_{V,Ca}$ channels could spend as much as 80% of their time in this low activity mode. Although no kinetic studies were carried out here, it is conceivable that this Ca^{2+}-induced low activity mode underlies the reduction in NP_{omax} seen at higher $[Ca^{2+}]_i$. Interestingly, even when the reduction in NP_{omax} of $BK_{V,Ca}$ channels at high $[Ca^{2+}]_i$ was always observed, the amount of this reduction was variable. This would suggest that the entrance to this mode is not strictly associated with the in situ characterization of $BK_{V,Ca}$ channel activity.

The other aspect revealed by this study is the transient activation of $BK_{V,Ca}$ channels, as shown in Fig. 6. Interestingly, there are oscillations of $BK_{V,Ca}$ channel activity on top of the activation by Ca^{2+}. This suggests that there are other factors (besides Ca^{2+} and voltage) that affect $BK_{V,Ca}$ channel activity as well. The mechanism responsible for the transient activation of $BK_{V,Ca}$ channels is not known in these cells. However, endogenous open-channel blockers (v.gr., polyamines; Snetkov et al., 1996) could be one possibility. Furthermore, a Ca^{2+} sensitivity as high as shown here also implies that a complete activation of $BK_{V,Ca}$ channels can be achieved by smaller changes in $[Ca^{2+}]_s$ than previously expected from in vitro studies.

These characteristics of $BK_{V,Ca}$ channels limit their usefulness as quantitative Ca^{2+} indicators of $[Ca^{2+}]_s$. Nevertheless, $BK_{V,Ca}$ channels appear to be finely tuned to respond to a given $[Ca^{2+}]$ threshold with short, oscillatory K^+ efflux (STOCs). Ultimately, this would avoid K^+ depletion due to their large conductance and a prolonged activation in smooth muscle cells.

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