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Effects of U-37883A on intracellular Ca^{2+}-activated large-conductance K^+ channels in pig proximal urethral myocytes

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Abstract

Kinetic studies of U-37883A (4-morpholinecarboximidine-*N*-1-adamantyl-*N*′ -cyclohexyl-hydrochloride), a vascular ATP-sensitive K^+ channel (K_{ATP} channel) blocker, were performed on pig urethral myocytes to investigate inhibitory effects on large-conductance intracellular Ca^{2+}-sensitive K^+ channels (i.e., BK_{Ca} channels; 225 pS K^+ channels) by use of single-channel recordings (outside-out and inside-out configuration). BK_{Ca} channels in pig urethral smooth muscles showed extracellular iberiotoxin (300 nM) sensitivity and voltage dependency. The α subunit of BK_{Ca} channel proteins was detected in the membrane fraction by use of Western blot technique. Application of U-37883A (≥10 μM) reduced the activity of BK_{Ca} channels in a concentration-dependent manner, not only by decreasing mean openlife time but also by prolonging the mean closed time. These results shows that U-37883A affects channels other than the vascular K_{ATP} channel, and demonstrates how it inhibits the activities of BK_{Ca} channels in urethral smooth muscles.

Keywords: ATP-sensitive K^+ channel; Channel kinetics; Intracellular Ca^{2+}-activated large-conductance K^+ channel; U-37883A

1. Introduction

U-37883A (4-morpholinecarboximidine-*N*-1-adamantyl-*N*′ -cyclohexyl-hydrochloride) has been developed as a nonsulphonylurea drug (Meisheri et al., 1993) and has selective inhibitory effects on vascular ATP-sensitive K^+ channels (K_{ATP} channels) at submicromolar concentrations. High concentrations of U-37883A (≥10 μM) have no inhibitory effect on the activity of pancreatic, cardiac, and skeletal K_{ATP} channels (Meisheri et al., 1993; Wellman et al., 1999); thus, it was generally believed that U-37883A is a selective inhibitor of vascular K_{ATP} channels.

Recently, however, it has been reported that submicromolar concentrations of U-37883A also suppress various other types of K^+ channels in nonsmooth muscle cells (low-conductance apical K^+ channels in the thick ascending limb of the loop of Henle, Wang et al., 1995; dopamine-modulated K^+ channels, Lin et al., 1998; *Shaker* K^+ currents, Surah-Narwal et al., 1999). These results suggest that U-37883A is unlikely to be a selective blocker for vascular K_{ATP} channels. However, in freshly dispersed smooth muscle cells, K^+ channels targeted by U-37883A still remain to be identified by use of single-channel recordings.

In the present study, we have investigated the effects of U-37883A on intracellular Ca^{2+}-activated large-conductance K^+ channels (i.e., BK_{Ca} channels) in pig urethral myocytes using single-channel recordings in order to define the specificity of U-37883A and to study the inhibitory kinetics of U-37883A on BK_{Ca} channels. A preliminary account of the results has been communicated to the 77th annual meeting of the Japanese Pharmacological Society (Tomoda et al., 2004).

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2. Materials and methods

2.1. Cell dispersion and recording procedure of patch-clamp experiments

Pig urethral smooth muscle strips were treated with papain and collagenase in Ca^{2+}-free solution, and myocytes were freshly isolated by gentle tapping method (Teramoto and Brading, 1996). Relaxed spindle-shaped cells, with length varying between 400 and 600 μm, were isolated and stored at 4 °C. The dispersed cells were normally used within 4 h for experiments. Patch-clamp experiments were performed at room temperature (21–23 °C) as described previously (Teramoto et al., 2003). Junction potentials between bath and pipette solutions were measured with a 3 M KCl reference electrode and were <2 mV, so that correction for these potentials was not made. Capacitance noise was kept to a minimum by maintaining the test solution in the electrode as low as possible.

2.2. Drugs and solutions

For recording of BK_{Ca} channels in outside-out patches, the ionic composition of the pipette solution was (mM): K^+ 140, Ca^{2+} 0.75, Cl^- 141.5, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40), estimating the free Ca^{2+} concentration ($[Ca^{2+}]$) as approximately 60 nM; the bath solution was physiological salt solution (PSS) (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, Cl^- 151.4, glucose 10, HEPES 10 titrated to pH 7.35–7.40 with Tris base (sometimes 60 mM K^+ and 140 mM K^+ solution were obtained by replacing 55 mM Na^+ and 135 mM Na^+ with equimolar K^+). To test the intracellular Ca^{2+} sensitivity of BK_{Ca} channels in inside-out patches, the pipette solution was 140 mM K^+ solution and the bath solution was initially (mM): K^+ 140, Cl^- 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40), with increasing free $[Ca^{2+}]$. The concentrations of free $[Ca^{2+}]$ were calculated by use of the commercial software 'EQCAL' (Biosoft, Cambridge, U.K.). Cells were allowed to settle in the small experimental chamber (80 μl in volume). The bath solution was superfused by gravity throughout the experiments at a rate of 2 ml min^{-1}. U-37883A was purchased from BIOMOL Research Laboratories (PA, USA), and prepared daily as a 100-mM stock solution in dimethyl sulphoxide (DMSO). The final concentration of DMSO was less than 0.3% and this concentration did not affect either the membrane currents or the potassium channels. The rest of the drugs were obtained from Sigma Chemical (Tokyo, Japan).

2.3. Data analysis and statistics

For single-channel recordings, the stored data were lowpass-filtered at 2 kHz (−3 dB) and sampled into the

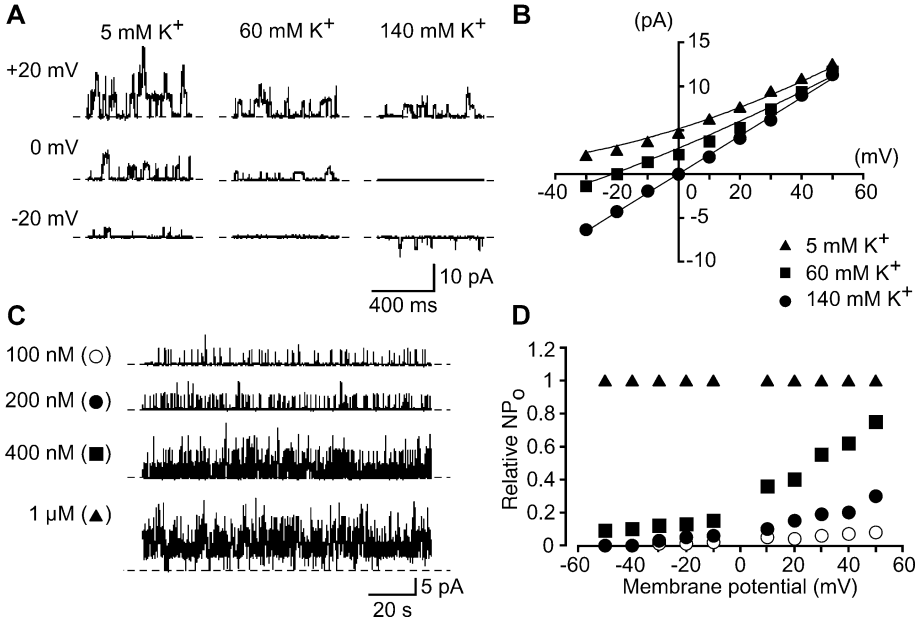

Fig. 1. Properties of BK_{Ca} channels in pig urethra. (A) Single-channel current traces at the indicated membrane potentials (−20, 0, and +20 mV) when $[K^+]_o$ is 5, 60, and 140 mM. The dashed line indicates the current baseline where the channel is not open (digital sampling interval, 1 ms). (B) Experimental current–voltage relationships in the presence of several $[K^+]_o$ solutions (5, 60, and 140 mM) in the same outside-out patches. The pipette solution was 140 mM K^+ containing 60 nM free Ca^{2+}. The channel conductance in 140 mM K^+ condition was 219 pS (225±15, n=40). The curves were fitted by the GHK equation (see the text). (C) BK_{Ca} channel currents recorded with an inside-out patch at a membrane potential of +30 mV in symmetrical 140 mM K^+ conditions (digital sampling interval, 1 ms). When the concentration of Ca^{2+} bathing the intracellular membrane surface was increased, an enhancement of channel activity occurred. (D) Relationships between channel activity (relative NP_o value), membrane potential, and $[Ca^{2+}]_i$ from inside-out configuration. Note that BK_{Ca} channels possess voltage and $[Ca^{2+}]_i$ sensitivity. Each symbol is the same as in (C).

computer with a digitalized interval of 80 μs for detailed single-channel analysis. In Figs. 1 and 2, continuous traces in the figures were obtained from records filtered at 500 Hz just for presentation (digital sampling interval, 25 ms). In Fig. 5, continuous traces in the figures were obtained from records filtered at 2 kHz for presentation (digital sampling interval, 100 μs).

The permeability of a single channel (P_K; cm s^{-1}) was calculated from the following equation:

$$P_K = I_K \frac{RT}{EF^2[K^+]} \quad (1)$$

where E is the membrane potential (V), F is Faraday's constant, R is the gas constant, and T is the absolute temperature (P_K).

The value for the channel permeability calculated from Eq. (1) was put into the Goldman–Hodgkin–Katz (GHK) constant field equation (Goldman, 1943; Hodgkin and Katz, 1949; Eq. (2)) to produce the relationship between the channel current and the patch membrane potential:

$$I_K = P_K \frac{EF^2}{RT} \frac{[K^+]_i - [K^+]_o \exp[-EF/(RT)]}{1 - \exp[-EF/(RT)]} \quad (2)$$

where $[K^+]_o$ and $[K^+]_i$ are extracellular and intracellular K^+ concentrations (mM), respectively.

Values for the channel open state probability (P_{open}) were measured for 1 or 2 min:

$$NP_o = \left(\sum_{j=i}^{N} t_j j \right) / T$$

where t_j is the time spent at each current level corresponding to j=0, 1,2,...N; T is the duration of the recording; and N is taken as the maximum number of channels observed in the patch membrane where P_{open} was relatively high. Data points were fitted using a least squares fitting.

Single-channel currents were analyzed using the half-amplitude threshold technique following the methods detailed by Colquhoun and Sigworth (1995). Lifetime distributions were log-binned using the method of McManus et al. (1987). When the square root of the number of the events in a bin is plotted against the open or closed lifetime, the components of the distribution appear as clear peaks, with their respective time constants falling in the vicinity of the distribution peaks (Sigworth and Sine, 1987). Conditional probability density functions were fitted to the open and closed lifetime distributions by the method of maximum likehood ('PAT' program, kindly provided by Dr J. Dempster, University of Strathclyde, UK). No correction was made for missing events (events briefer than 80 μs). Statistical analyses were performed with analysis of variance (ANOVA) (two-factor, with replication). Changes were considered significant at P<0.01. Data are expressed as mean with standard deviation (S.D.).

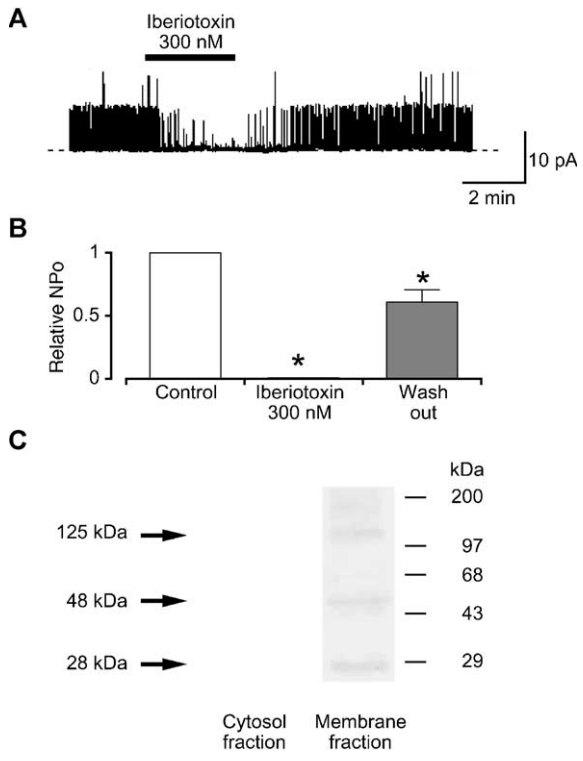

Fig. 2. Characterization of BK_{Ca} channels in pig urethra. (A) In outside-out configuration, 300 nM iberiotoxin reversibly suppressed the channel opening at +30 mV. (pipette solution 140 mM KCl containing 60 nM free Ca^{2+}, bath solution 5 mM K^+ PSS). The dashed line indicates the current baseline where the channel is not open. (B) The relative NP_o value (2 min duration recording) when the control value in each patch was normalized to one (n=5). In the presence of iberiotoxin (300 nM), the relative NP_o value decreased to $0.007 \pm 1.4 \times 10^{-5}$ (n=5, solid column). On removal of iberiotoxin, the relative NP_o value recovered to 0.61 ± 0.1 (n=5, hatched column). *Significantly different from the control (ANOVA, P<0.01). (C) Western blot technique. In the membrane fraction, the full-length α subunit of BK_{Ca} channels (α125), two proteolytic tail fragments with molecular mass of 28 and 48 kDa, were detected. In the cytosolic fraction, no protein was detectable (i.e., negative control).

2.4. Membrane preparation and immunoblotting

To prepare membrane fractions, fresh pig urethral smooth muscle was cut into 5 mm slices and suspended in a solution containing 250 mM sucrose, 1 mM EDTA, 20 mM Tris/HCl (pH 7.4), 0.1 mM phenymethylsulfonyl fluoride (PMSF), 1 μg ml^{-1} leupeptin, and 1 μg ml^{-1} pepstatin A. The tissue was then homogenized and centrifuged at 1000×g for 10 min at 4 °C. The supernatant was further centrifuged at 100,000×g for 30 min at 4 °C. The resultant pellet was resuspended with a solution containing 250 mM sucrose and 20 mM Tris/HCl and used as membrane preparations. Samples of the resuspended membrane preparations were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system (1 h, 15 V). After blocking with 10%

skim milk for 60 min, the membrane was probed with rabbit polyclonal anti-BK_{Ca} channel antibody (Alomone Laboratories, Jerusalem, Israel) for 1 h at room temperature. After washing, the membrane was incubated with horseradish peroxidase-conjugated second antibody and immunoreactive proteins were then visualized by treatment with SuperSignal West Dura Extended Duration substrate (Pierce).

3. Results

3.1. Characterization of BK_{Ca} channel in pig urethra

The physiological and pharmacological properties of BK_{Ca} channels in pig urethra were investigated using single-channel recording. In outside-out configuration, under symmetrical 140-mM K^+ conditions, a high-frequency large amplitude channel was observed (Fig. 1A). The permeability of a single channel (P_K; cm s^{-1}) was estimated to be a value of 4.22 $\times 10^{-13}$ cm s^{-1} from Eq. (1) (see Materials and Methods).

The mean single-channel conductance of this large-conductance channel was 225±15 pS (n=40), with reversal occurring at or very close to 0 mV (0.5±1.2 mV, n=40). When K^+ in the bath was replaced by equimolar Na^+, the current–voltage relationships showed outward rectification and E_K shifted in a manner predicted by the Nernst equation for K^+ to negative potentials. Fig. 1B shows the current–voltage relationships with three different K^+ gradients (extracellular K^+; 140, 60, and 5 mM). The constant field model (Eq. (2); see Materials and Methods) provided a good fit for the current–voltage data for these channels under asymmetrical K^+ gradients, showing a selective K^+ permeability. Similar observations were obtained in seven other patches.

In inside-out patches (symmetrical K^+ conditions), the NP_o of BK_{Ca} channels was both voltage- and Ca^{2+}-sensitive, as shown in Fig. 1C and D. Fig. 1C shows recordings from an excised membrane patch where the inner surface of the membrane was exposed to four different internal Ca^{2+} concentrations ($[Ca^{2+}]_i$) at +30 mV. At 100 nM $[Ca^{2+}]_i$, the NP_o of the channel was low. When $[Ca^{2+}]_i$ was increased to 400 nM, the NP_o was dramatically enhanced. When the holding potential was changed, a steep voltage dependence was exhibited. These features of BK_{Ca} channels are clearly demonstrated in Fig. 1D, which is a plot of the relative NP_o at four different $[Ca^{2+}]_i$ values over a wide range of membrane potentials when the value of NP_o at +50 mV and $[Ca^{2+}]_i$ of 1 μM was normalized as 1.0. These results suggest that voltage may be the primary activating factor on BK_{Ca} channels at intermediate $[Ca^{2+}]_i$—an effect that may reflect a Ca^{2+}-dependent enhancement of voltage sensitivity.

External application of 300 nM iberiotoxin caused a marked inhibition of channel openings, as shown in Fig. 2A (outside-out configuration, 5 mM K^+ PSS, bath/140 mM K^+, pipette at +30 mV). On removal of iberiotoxin, the channel activated again, although it took 2–3 min to wash out the blocking effect even at 2 ml min^{-1} bath perfusion rate and recovery was not complete (Fig. 2B; n=5). As shown in Fig. 2C, immunoreactive protein of 125 kDa was detected in pig urethral smooth muscle membrane fraction but not in the cytosolic fraction. In addition to the full-length α subunit of BK_{Ca} channels (α125), two proteolytic tail fragments with molecular mass of 48 and 28 kDa were also detected as described by Knaus et al. (1995).

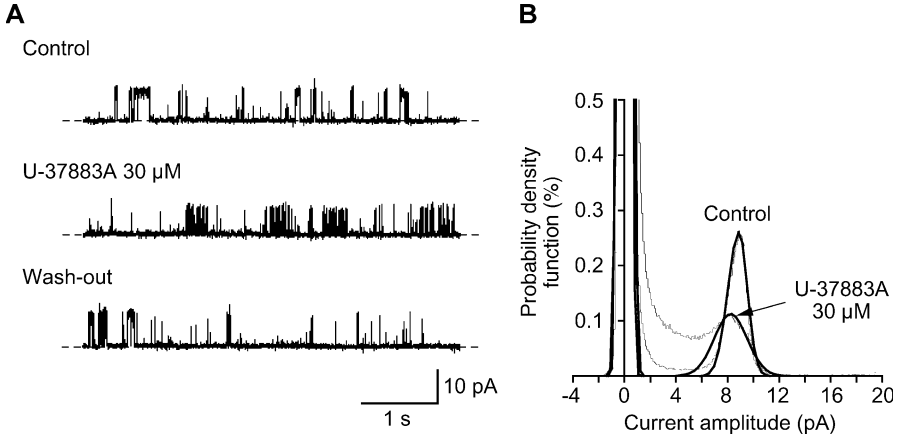

Fig. 3. Effects of U-37883A on the 225 pS K^+ channel at +30 mV in inside-out configuration (pipette solution 5 mM K^+ PSS, bath solution 140 mM KCl containing 60 nM free Ca^{2+}). (A) Current trace of BK_{Ca} channels at each indicated condition (control, 30 μM U-37883A, and washout). The dashed line indicates the current when the channel is not open (digital sampling interval, 1 ms). (B) The all-point amplitude histograms for U-37883A, obtained during the last 4 min in the absence (control, just before the application of U-37883A) or presence of U-37883A (30 μM). Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution by the least squares method. The abscissa shows the amplitude of the current (pA) and the ordinate shows the percentage value of the probability density function (%) for the recording period (4 min).

3.2. Effects of U-37883A on BK_{Ca} channel kinetics

Fig. 3 shows the effects of U-37883A on the activity of the 225 pS K^+ channels (BK_{Ca} channels; NP_o value, 5.4%) at +30 mV in inside-out configuration (pipette solution, 5 mM K^+ PSS; bath solution, 140 mM K^+ solution, $[Ca^{2+}]_i$, 100 nM). U-37883A (≥10 μM) inhibited the unitary amplitude (10 μM, 8.4 pA; 30 μM, 8.2 pA) and the NP_o value (5 μM, 4.2%; 10 μM, 2.9%; 30 μM, 2.4%) in a concentration-dependent manner. Similarly, U-37883A caused an inhibitory effect on channel activity when $[Ca^{2+}]_i$ was increased to 400 nM (data not shown). On removal of U-37883A, the activity of BK_{Ca} channels gradually recovered, but not to the original level (Fig. 3A). Note that U-37883A-sensitive K^+ channels were suppressed by additional application of 300 nM iberiotoxin.

In the present study, we estimated the effects of U-37883A on both the mean openlife time and closed time according to the method described by Colquhoun and Sigworth (1995), although it was not technically feasible to determine the total numbers of the activated channels in these patches. In Fig. 4, the distribution of the openlife times and the closed times was best described by the sum of two exponentials (i.e., fast and slow components).

This is indicated by the solid line in each time distribution. In the control, the time constant of the fast open component (τ_{of}) illustrated was 0.8 ms (0.8±0.2, n=5) and that of the slower open component (τ_{os}) was 6.6 ms (6.7±0.7, n=5). In the closed time distribution, the time constant of the fast component (τ_{cf}) illustrated was 1.5 ms (1.4±0.1, n=5) and that of the slower component (τ_{cs}) was 166 ms (162.7±17.4, n=5). Applying 30 μM U-37883A, the mean openlife time decreased in comparison with that of control [5.2 ms (control) vs. 3.8 ms (30 μM U-37883A)]. In contrast, the mean closed time was prolonged [66 ms (control) vs. 139.9 ms (30 μM U-37883A)]. Fig. 4B shows the distributions of the openlife time and the closed time. Table 1 summarizes the parameters of channel kinetics in the absence and presence of U-37883A. At 5 μM U-37883A, during long openings of BK_{Ca} channels, fast flickering openings with brief closing times were occasionally observed with little change in the unitary amplitude [Fig. 5A; 8.4 pA (control) vs. 8.4 pA (5 μM U-37883A)] and opening kinetics (Fig. 5B).

Table 1
Effects of U-37883A (5 and 30 μM) on the channel kinetics of BK_{Ca} channels. [a] Significantly different from the control (ANOVA, P<0.01)

	Open time		Closed time	
	Time constant (ms)	Mean time (ms)	Time constant (ms)	Mean time (ms)
Control	τ_{of} 0.8±0.2 (n=5) τ_{os} 6.7±0.7 (n=5)	5.1±0.3 (n=5)	τ_{cf} 1.4±0.1 (n=5) τ_{cs} 162.7±17.4 (n=5)	68.1±5.2 (n=5)
U-37883A (5 μM)	τ_{of} 0.7±0.2 (n=5) τ_{os} 7.7±1.7 (n=5)	4.9±0.4 (n=5)	τ_{of} 0.5±0.1 (n=5) τ_{cm} 6.2±1.9 (n=5) τ_{cs} 178.3±21.7 (n=5)	74.5±9.1 (n=5)
U-37883A (30 μM)	τ_{cf} 0.5±0.1 (n=5) τ_{os} 4.9±0.4 (n=5)[a]	3.7±0.2 (n=5)[a]	τ_{cf} 0.6±0.1 (n=5)[a] τ_{cs} 222.1±23.2 (n=5)[a]	145.3±23.3 (n=5)[a]

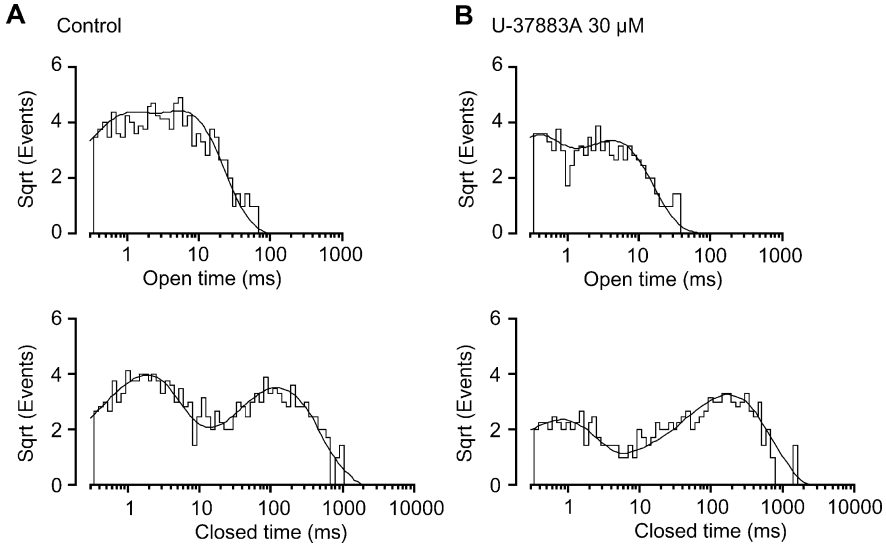

Fig. 4. Effects of U-37883A (30 μM) on BK_{Ca} channel kinetics. All data come from the same patch as in Fig. 3 (inside-out configuration, pipette solution 5 mM K^+ PSS, bath solution 140 mM KCl containing 60 nM free Ca^{2+}). The data were filtered at 2 kHz for analysis. (A) Open and closed time distributions in the absence of U-37883A (i.e., control). (B) Open and closed time distributions in the presence of 30 μM U-37883A.

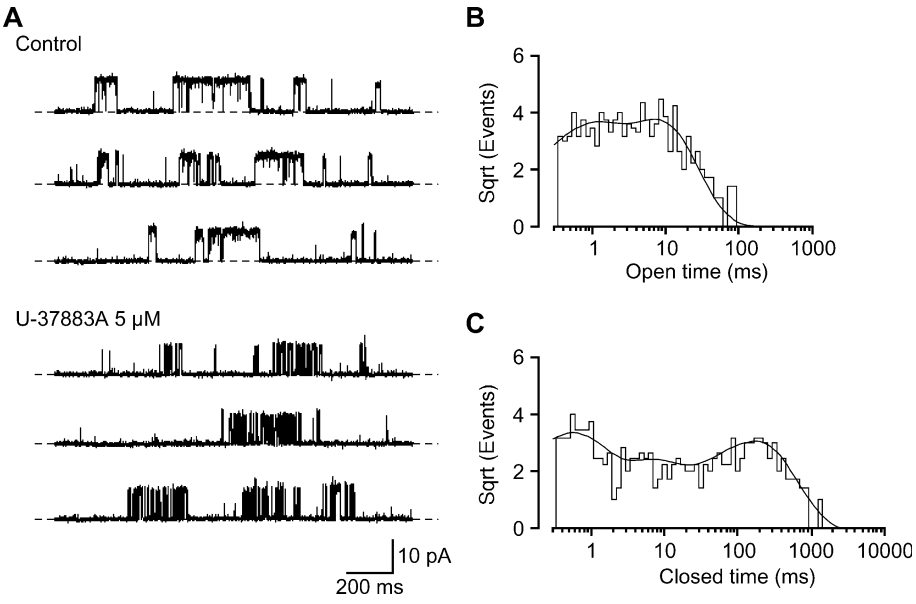

Fig. 5. Kinetic studies of BK_{Ca} channels in the presence of 5 μM U-37883A. All data come from the same patch as in Fig. 3 (inside-out configuration, pipette solution 5 mM K^+ PSS, bath solution 140 mM KCl containing 60 nM free Ca^{2+}). The data were filtered at 2 kHz for analysis. (A) Current trace of BK_{Ca} channels in the absence and presence of U-37883A (5 μM). The dashed line indicates the current when the channel is not open (digital sampling interval, 100 μs). (B and C) Open and closed time distributions in the presence of 5 μM U-37883A.

The additional component of the closed time distribution (τ_{cm}) appeared at only this concentration of U-37883A (Fig. 5C).

4. Discussion

The present study provides the first direct kinetic studies for the inhibitory effects of U-37883A on BK_{Ca} channels in freshly dispersed smooth muscle myocytes using single-channel recordings.

4.1. Pharmacological and electrophysiological properties of BK_{Ca} channels in pig urethra

The BK_{Ca} channel in the pig urethral smooth muscle has a selective K^+ permeability, voltage dependency, and intracellular Ca^{2+} sensitivity very similar to those of the bovine tracheal smooth muscle cells (MacMillan et al., 1995). Iberiotoxin is recognised as a selective BK_{Ca} channel blocker in smooth muscles from binding studies (Garcia and Kaczorowski, 1992), and we have been able to demonstrate an inhibitory effect of iberiotoxin on the channels in smooth muscle cells in outside-out patches. Moreover, we have detected the full-length α subunit of BK_{Ca} channel (α125) by use of Western blot techniques. These direct observations indicate that the iberiotoxin-sensitive K^+ channels in pig urethra belong to the category of BK_{Ca} channels (i.e., maxi K^+ channels, BK, K_{Ca} channels, etc.), which have been widely observed in other smooth muscle cells (reviewed by Latorre et al., 1989; Calderone, 2002; Orio et al., 2002).

4.2. Regulation of BK_{Ca} channels by U-37883A

In the present experiments, U-37883A (5 μM) caused a marked flickering block of BK_{Ca} channels, with no significant change in the unitary amplitude. Furthermore, an additional component of the closed time distribution appeared. This extra closed component represents the nonconductive, open-blocked state, with a time constant reflecting the mean residence time of U-37883A on the channel binding site(s). Thus, we suggest that the inhibitory effects of U-37883A on BK_{Ca} channels seem to be a state-dependent and open-channel block mechanism. Higher concentrations of U-37883A (≥10 μM) reduced the unitary amplitude of BK_{Ca} channels in a concentration-dependent manner, decreasing the channel conductance (Tomoda et al., 2004). It is generally believed that the reduction in the unitary amplitude occurs due to a marked decrease in both the unitary current through the pore of BK_{Ca} channels and the channel activity. Indeed, U-37883A reduced the openlife time and prolonged the closed time. At higher than 30 μM U-37883A, most of the open events did not reach the fully open level, appearing as needle-like events, reducing the mean amplitude of the unitary current (Tomoda et al., 2004). Similarly, in the presence of U-37883A, needle-shaped openings have been observed in other types of K^+ channels (low-conductance apical K^+ channels in the thick ascending limb of the loop of Henle, Wang et al., 1995; dopamine-modulated K^+ channels, Lin et al., 1998). In contrast, U-37883A inhibited K_{ATP} channels not by changing the size of the unitary amplitude but by reducing the NP_o value (Tomoda et al., 2004). Cibenzoline, a K_{ATP} channel pore blocker, caused a similar inhibitory effect on pig urethral

K_{ATP} channels (Teramoto et al., 2002). Thus, the mechanisms by which U-37883A inhibited K^+ channels differed between BK_{Ca} channels and K_{ATP} channels.

4.3. Tissue selectivity of U-37883A for K^+ channels

It was reported that U-37883A has no effect on K_{ATP} currents in the insulinoma-derived RINm5F cells (≤100 μM; Guillemare et al., 1994), cardiac myocytes (≤30 μM; Wellman et al., 1999), and skeletal muscle cells (≤30 μM; Wellman et al., 1999). In contrast, U-37883A inhibited K_{ATP} currents in arterial smooth muscles, thereby indicating that U-37883A possesses a tissue selectivity as a vascular K_{ATP} channel blocker (K_i=3.2 μM; Wellman et al., 1999). In the present experiments, even at submicromolar concentrations, U-37883A potently inhibited the activity of BK_{Ca} channels in pig urethral myocytes, not only decreasing mean openlife time but also prolonging the mean closed time. Furthermore, at concentrations of U-37883A higher than 10 μM, U-37883A reduced the unitary amplitude of BK_{Ca} channels, decreasing the NP_o value. Furthermore, in rat striatal neurons, the dopamine-modulated K^+ channels were blocked by U-37883A (K_i=0.1 μM; Lin et al., 1998). Therefore, it is not feasible to define U-37883A as a vascular K_{ATP} channel blocker since it possesses little ion channel selectivity.

Acknowledgments

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