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Glibenclamide-sensitive K^+ channels underlying levcromakalim-induced relaxation in pig urethra

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Abstract

To investigate the possible mechanisms involved in the stable and long-lasting levcromakalim-induced relaxation of the resting urethral tone, we have performed mechanical and voltage-clamp experiments using intact tissue and isolated cells from pig urethra, respectively. At negative membrane potentials, levcromakalim induced time- and voltage-independent membrane currents in whole-cell configurations. In cell-attached patches, levcromakalim not only increased the open-state probability (the NP_o value) of the glibenclamide-sensitive 43 pS K^+ channel (K_{GS}) in a concentration-dependent manner, but also activated K_{GS} with a time- and voltage-independence. During long burst-like channel activity, neither the mean open lifetime nor the mean closed time of K_{GS} exhibited voltage-dependency between −100 and −40 mV. It is concluded that levcromakalim causes a stable and potent relaxation of pig urethra through opening of K_{GS} which possesses time- and voltage-independent activating mechanisms.

Keywords: Burst channel opening; Glibenclamide; K^+ channel; Levcromakalim; Long lasting effect; Urethral resting tone; Voltage-independent activation

1. Introduction

K^+ channel openers are a class of drugs with a wide variety of chemical structures originally characterized by their ability to enhance the channel opening probability of adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K_{ATP}) (reviewed by Edwards and Weston (1993)). Recently, we have reported that levcromakalim, one of the most potent K_{ATP} openers, induces stable and long-lasting relaxation of pig urethra (Teramoto and Brading, 1996). In addition, we have demonstrated that levcromakalim induces a concentration-dependent membrane hyperpolarization which is selectively inhibited by additional application of glibenclamide, and shown the presence of a glibenclamide-sensitive 43 pS K^+ channel (K_{GS}) in this tissue (Teramoto and Brading, 1996, 1997; Teramoto et al., 1997b). Moreover, there is evidence that K_{GS} may play an important role in maintaining the resting membrane potential of pig proximal urethra under quasi-physiological conditions (Teramoto et al., 1997a).

In the present experiments, in order to elucidate further the mechanisms involved in the stable and long-lasting relaxation of pig urethra by levcromakalim, we have investigated the effects of levcromakalim on the activity of the K_{GS} by use of patch-clamp techniques and have studied the voltage- and time-dependency of their opening and closing kinetics for the first time in a smooth muscle. In addition, we have characterized the levcromakalim-induced membrane currents by use of pharmacological tools, including glibenclamide and tolbutamide. It is known that sulphonylureas inhibit not only K_{ATP} openers-induced membrane hyperpolarization but also K_{ATP} openers-induced muscle relaxation and therefore it is of interest to characterize the levcromakalim-induced channels by these pharmacological tools to understand the potent and stable relaxing actions of levcromakalim in lower urinary tract.

2. Material and methods

Fresh female pig urethras were obtained from a local slaughter house and a segment of the proximal urethra was excised from a region 1–2 cm from the bladder neck.

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2.1. Tension measurement

The isometric tension recordings were essentially the same as previously described (Teramoto and Brading, 1996, 1997). To prevent both noradrenaline outflow from sympathetic nerve terminals and β-adrenoceptor stimulation, 3 μM guanethidine and 0.3 μM propanolol were present throughout (at 37°C). A modified Krebs was used (in mM): Na^+ 137, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.0, Cl^- 132.7, HCO_3^- 15.4, $H_2PO_4^-$ 1.2 and glucose 11.5 bubbled with 97% O_2 and 3% CO_2.

2.2. Preparation of dispersed cells and patch-clamp experiments recording procedure

Both the cell preparation and the patch-clamp experimental system used were essentially the same as described previously (the gentle tapping method; Teramoto and Brading, 1997, 1998; Teramoto et al., 1997a,b). Junction potentials between bath and pipette solutions were measured using a 3 M KCl reference electrode and were < 2 mV, so that correction for these potentials was not made. Experiments were carried out at 21–23°C.

2.3. Solutions and drugs

The following solutions were used for whole-cell recordings. Physiological salt solution (PSS) had the following composition (in 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. The pipette solution contained (in mM): K^+ 140, Cl^- 140, EGTA 5, HEPES 10/Tris (pH 7.35–7.40). For single-channel recordings, the composition of both pipette and bath solution was the same (in mM): K^+ 140, Cl^- 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40). Cells were allowed to settle in the small experimental chamber (80 μl in volume) which was superfused by gravity throughout the experiments at a rate of 2 ml min^{-1} bath solution. All drugs were purchased from Sigma (St. Louis, USA). Both levcromakalim (kindly provided by SmithKline Beecham, Harlow, UK) and glibenclamide were prepared daily as 100 or 250 mM stock solutions in dimethylsulphoxide (DMSO). Tolbutamide was dissolved to 1 M in DMSO. The final concentration of DMSO was less than 0.3% and this concentration did not affect either the membrane currents or the K^+ channels. The final concentration of DMSO (less than 0.01%) did not affect the resting tone, either. Thus, we utilized 10 μM levcromakalim as a maximal concentration in the isometric tension recordings. The nystatin-perforated patch technique used was the same procedure described previously (Teramoto and Brading, 1996; Teramoto et al., 1997b).

2.4. Data analysis

The whole-cell current data were low-pass filtered at 500 Hz (continuous traces) by an eight pole Bessel filter and analyzed on a computer (Power Macintosh G3, Apple Computer, Japan, Tokyo, Japan) with the commercial software 'Mac Lab 3.5.2' (AD Instruments, Castle Hill, Australia) and leakage current was not subtracted. For single-channel recording, the stored data were filtered at 2 kHz (-3 dB) and sampled into the computer with an interval of 80 μs using 'PAT' program (kindly provided by Dr. Dempster, The University of Strathclyde, UK). Single-channel events were detected using a half amplitude criterion (Colquhoun and Sigworth, 1995) and inspected manually. Because the total number of functioning channels in each patch was not able to be determined, both the opening kinetics and the closed kinetics were only examined within long bursts in which only one channel was clearly opened. The time distribution of 30 s recording was log-binned using the method of McManus et al. (1987). The conditional probability density function was fitted to the time distribution by the method of non-linear regression fitting (Sigworth and Sine, 1987; Teramoto and Brading, 1997). At every membrane potential, both the open lifetime histogram and the closed time histogram were fitted by a single exponential. Although a small number of events which were longer than 3 ms were sometimes included in the closed time histogram (such as a channel closed time between two bursts), these events were not taken into consideration for the fitting in the present experiments since the total number of these events was negligible when compared to those obtained during the burst channel openings. Events briefer than 200 μs were not included in the evaluation and no correction was made for missed events. The channel activity was calculated using the following equation from an all-point amplitude histogram fitted with the Gaussian equation using least-squares fitting and expressed as an NP_o value,

$$NP_o = \left(\sum_{j=1}^{N} t_j j \right) / T$$

where N is the number of channels, P_o is the open-state probability, t_j is the time spent at each current level corresponding to $j = 0, 1, 2, \ldots N$ and T is the duration of the recording (2 min).

2.5. Statistics

Statistical analyses were performed with a two-paired *t*-test. Changes were considered significant at $P < 0.01$. Data are expressed as the mean ± S.D.

3. Results

3.1. The time course of the effects of levcromakalim on the muscle tone of pig proximal urethra

Levcromakalim (10 μM) caused a rapid and stable relaxation of urethral tone, which was insensitive to additional application of 300 nM iberiotoxin (Fig. 1A). After washing-out of levcromakalim, the time course of recovery of the muscle tone was slow. Namely, the relaxation remained at the peak value for approximately 5 min (4 min and 52 s ± 1 min and 10 s, $n = 11$) and it then took approximately 17 min (16 min and 44 s ± 3 min and 16 s, $n = 11$) for full recovery to the original level. In contrast,

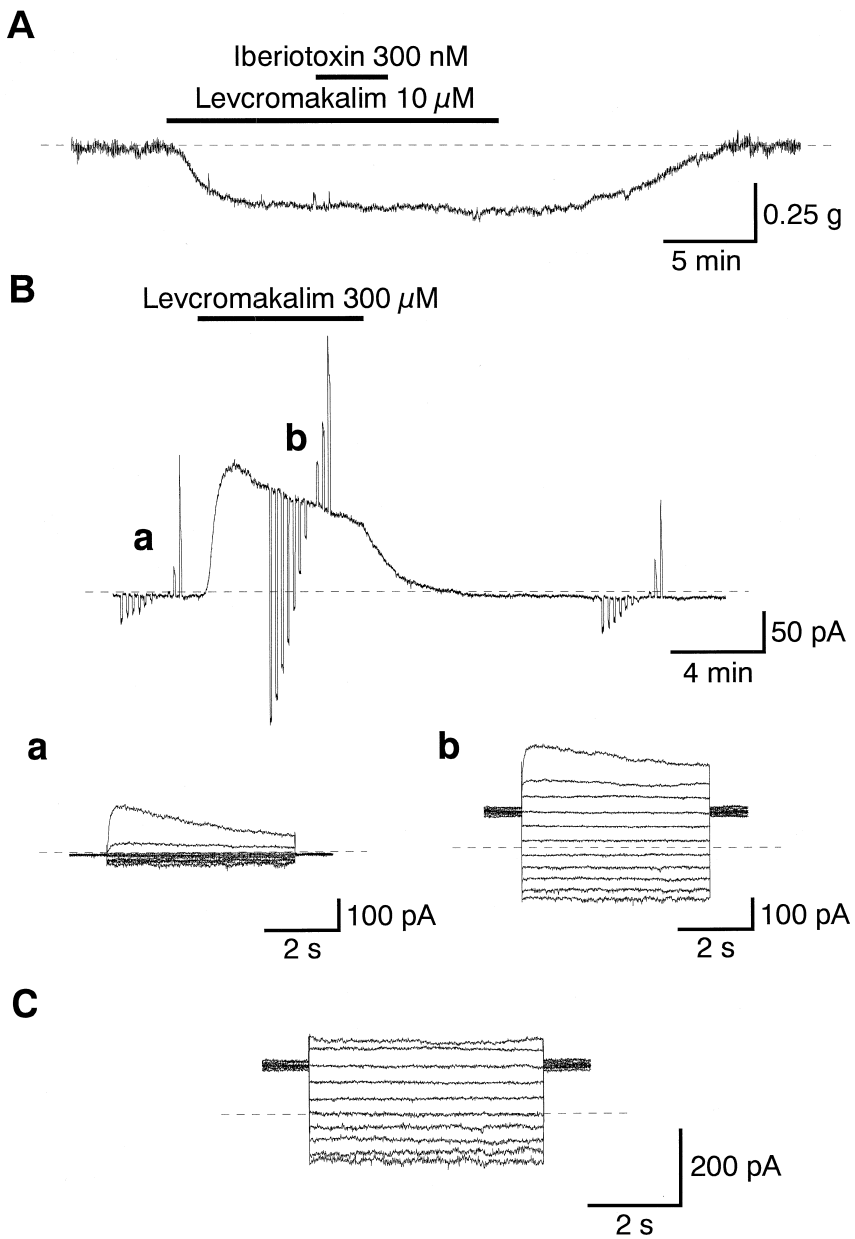

Fig. 1. The effects of levcromakalim on pig proximal urethra. (A) Levcromakalim (10 μM) causes a stable, long-lasting urethral relaxation which was unaffected by application of iberiotoxin (300 nM). Note that the relaxation lasted for several minutes after removal of levcromakalim. The dashed line indicates the mean basal tone level. Guanethidine (3 μM) and propranolol (0.3 μM) were present in the bath solution throughout the experiments. (B) Conventional whole-cell recording from a dispersed smooth muscle cell at −50 mV. Levcromakalim (300 μM) induced an outward current which exhibited a gradual decay in amplitude after the peak at a holding potential of −50 mV (bath solution, PSS; pipette solution 140 mM KCl containing 5 mM EGTA). Rectangular voltage steps (duration 5 s) were applied from −120 to −30 mV at 10-s interval in the absence (control, (a)) and presence of 300 μM levcromakalim (b). The vertical deflections indicate step potential pulses. On removal of levcromakalim, the outward current declined gradually. The dashed line indicates the zero current level. (C) Time- and voltage-independent current recorded in the presence of 300 μM levcromakalim. The membrane current was obtained by subtraction of the current trace in the presence of 300 μM levcromakalim from the control at each membrane potential. The currents show little time- or voltage-dependency.

the levcromakalim-induced outward current recorded in single dispersed cells showed progressive run-down (Fig. 1B). However, levcromakalim-induced currents showed a time- and voltage-independent activation, as shown in Fig. 1C, where levcromakalim-induced currents were obtained by subtraction of the currents induced by step changes of

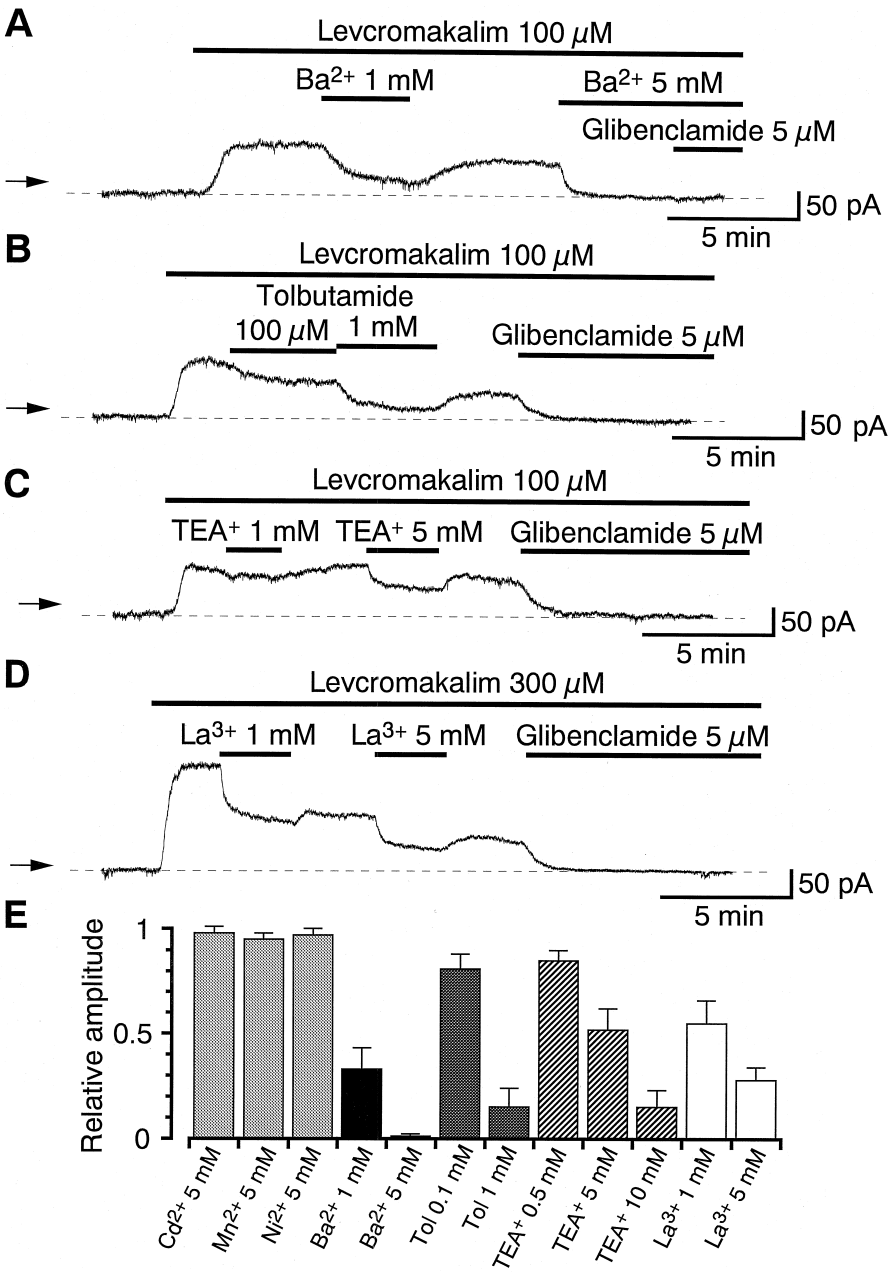

Fig. 2. Pharmacological characterization of the levcromakalim-induced outward current. Whole-cell recording using nystatin-perforated patches. The bath solution was PSS and the pipette solution was 140 mM KCl containing 5 mM EGTA. Bath application of levcromakalim caused a sustained outward current. The arrows indicate the zero current level and the dashed lines the control current level at a holding potential of −50 mV. Levcromakalim and the blocking agents were applied in the bath solution. (A) Effects of Ba^{2+}. Ba^{2+} (5 mM) completely suppressed the 100 μM levcromakalim-induced outward current and additional application of 5 μM glibenclamide had no further inhibitory effect on the membrane current. (B) Effects of tolbutamide. Note that even 1 mM tolbutamide did not completely block the current. (C) Tetraethylammonium^{+} had a weak inhibitory effect on the 100 μM levcromakalim-induced outward current. (D) The inhibitory effects of La^{3+} on the 300 μM levcromakalim-induced outward current. (E) Histogram summarizing the relative inhibitory effects of various K^{+} channel blockers on the levcromakalim-induced outward currents when the peak amplitude of the levcromakalim-induced outward current was normalized as 1.0. Divalent cations (Cd^{2+}, Mn^{2+}, Ni^{2+}, light grey column), Ba^{2+} (filled column), tolbutamide (dark grey column), tetraethylammonium^{+} (hatched column), La^{3+} (open column). Each column shows the relative amplitude of the levcromakalim-induced outward currents at −50 mV (mean value + S.D., $n = 4$).

potential (from −120 to −30 mV) in the presence and absence of 300 μM levcromakalim. On removal of levcromakalim, the outward current at −50 mV declined gradually to the control level, taking several minutes for full recovery (Fig. 1B).

3.2. Pharmacological characterization of the levcromakalim-induced outward current in pig urethra

We then observed the effects of divalent cations (Ba^{2+}, Cd^{2+}, Mn^{2+}, Ni^{2+}), La^{3+} and K^+ channel blockers (tetraethylammonium chloride, tolbutamide, glibenclamide) on the levcromakalim-induced outward current using nystatin-perforated whole-cell configuration to minimize the run-down phenomena. External application of Ba^{2+} (1–5 mM) inhibited the 100 μM levcromakalim-induced outward current in a concentration-dependent manner, and 5 mM Ba^{2+} completely suppressed the current. Additional application of 5 μM glibenclamide did not further suppress the current (Fig. 2A). In contrast, extracellular application of other divalent cations, such as Cd^{2+}, Mn^{2+}, Ni^{2+} (1–5 mM) had little inhibitory effect on the 100 μM-levcromakalim-induced current (traces not shown). Extracellular application of tolbutamide (0.1–1 mM), one of the well-known sulphonylureas, inhibited the 100 μM levcromakalim-induced outward current in a concentration-dependent manner. However, 1 mM tolbutamide did not completely suppress the current, although it was com-

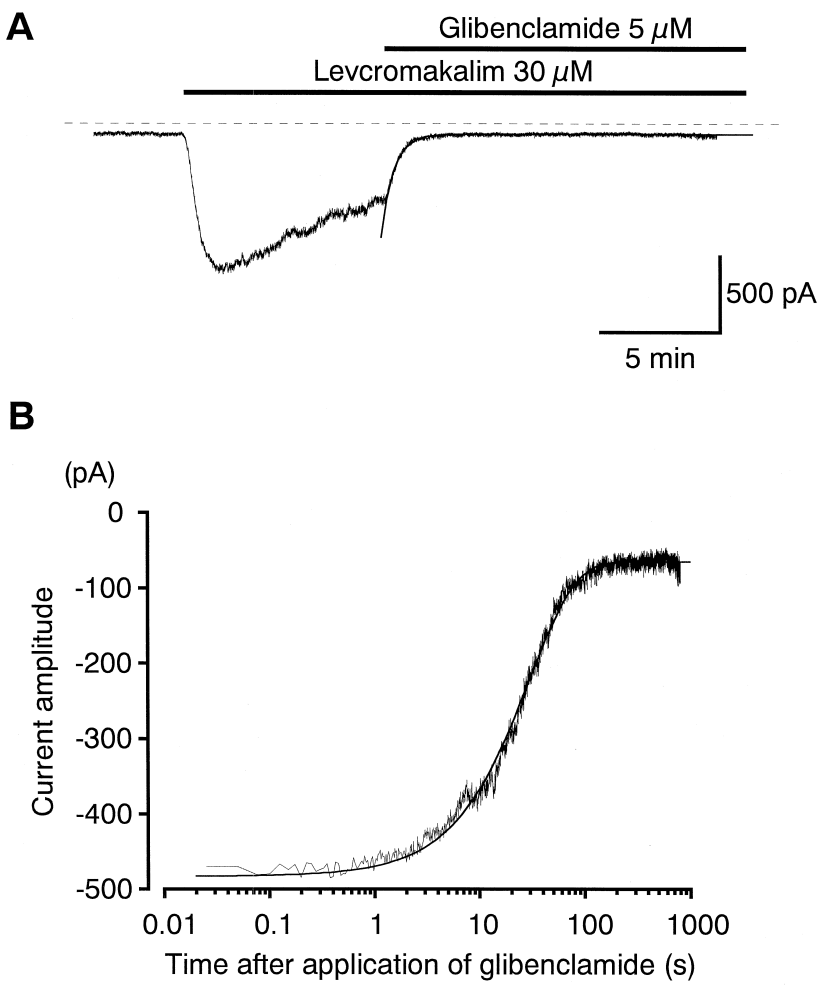

Fig. 3. (A) Inhibitory effects of 5 μM glibenclamide on the 30 μM levcromakalim-induced inward current at a holding membrane potential of −50 mV. The bath contained 140 mM K^+ PSS and the pipette 140 mM KCl containing 5 mM EGTA (i.e., symmetrical 140 mM K^+ conditions). The dashed line indicates the zero current level. (B) Inhibitory effects of 5 μM glibenclamide on the 30 μM levcromakalim-induced inward current. A single exponential (solid line) was fitted to the current decay between time zero (start of application of glibenclamide) and time = 840 s,

$I(t) = I_0 + I_a \exp(-t/\tau_a)$

$I_0 = -65.4$ pA, $I_a = -418$ pA and $\tau_a = 30.7$ s, respectively. The dashed line indicates the zero current level. The expanded fitting trace of the glibenclamide-induced inhibitory time course. The solid line indicates the exponential fitting. The time 0 indicates the time when 5 μM glibenclamide was applied by a concentration jump method. The abscissa scale shows the amplitude of the inward current (pA) and the ordinate scale shows the duration of application of glibenclamide (s) in a logarithmic scale. The membrane current was filtered through 2 kHz and the duration of the sampling time was 25 ms.

pletely abolished by 5 μM glibenclamide (Fig. 2B). Tetraethylammonium$^+$ ($\geq$ 500 μM) showed a reversible inhibitory effect on the 100 μM levcromakalim-induced outward current reducing the current by about 50% (52 ± 10%, $n = 4$) at a concentration of 5 mM (Fig. 2C). On the other hand, La^{3+} caused a concentration-dependent, but partially irreversible inhibition of the levcromakalim-induced current, as shown in Fig. 2D. Fig. 2E summarizes the effects of various K^+ channel blockers on levcromakalim-induced outward currents at a holding potential of −50 mV ($n = 4$).

3.3. The time course of the inhibitory effects of glibenclamide on the levcromakalim-induced membrane currents at −50 mV

To investigate further the inhibitory effects of glibenclamide on the levcromakalim-induced membrane currents, voltage-clamp experiments were performed in symmetrical 140 mM K^+ conditions (bath solution 140 mM K^+ PSS, pipette solution 140 mM KCl containing 5 mM EGTA, $E_K = 0$ mV) at a holding potential of −50 mV in order to enhance the peak amplitude of the levcromakalim-induced

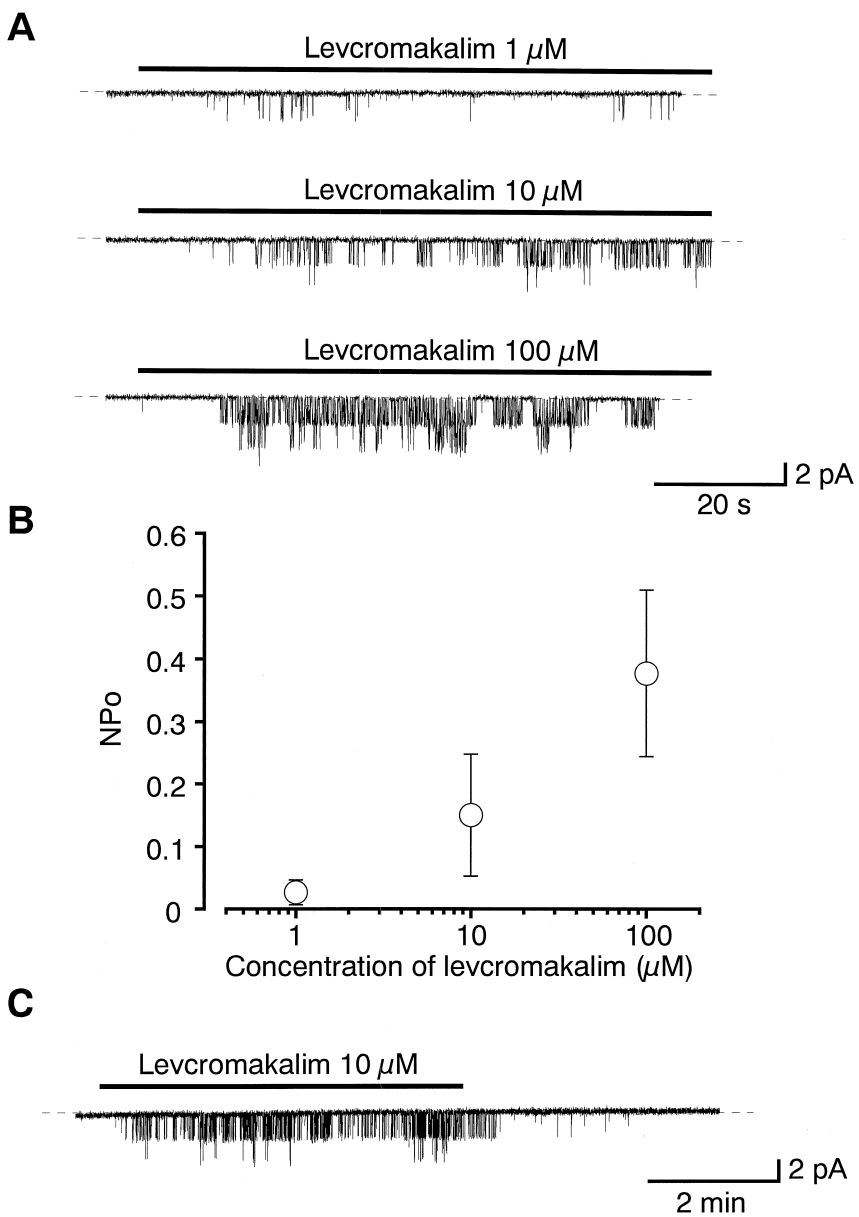

Fig. 4. Concentration-dependent activation of K_{GS} by levcromakalim. Cell-attached configuration (symmetrical 140 mM K^+ conditions) at a holding membrane potential of −50 mV. The dashed line indicates the current when the channel is not open. (A) Examples of the currents. Levcromakalim at different concentrations was rapidly applied to the same membrane patch using the concentration jump technique. (B) Concentration–response curve for mean NP_o value ± S.D. ($n = 5$). (C) Individual record showing the delay in abolition of channel activity after 10 μM levcromakalim was removed from the perfusing solution.

membrane currents. Fig. 3A shows an example of the effects of glibenclamide on the levcromakalim-induced membrane current. In an attempt to investigate the time course of the inhibitory action of glibenclamide on the levcromakalim-induced current, we plotted the current amplitude against the time after application of 5 μM glibenclamide. The inhibitory time course could be fitted with a single exponential (Fig. 3B), and the mean inhibitory time constant was 30.3 ± 3.6 s ($n = 5$).

3.4. The properties of the levcromakalim-activated K_{GS}

Under cell-attached configuration, application of various concentrations of levcromakalim by use of the concentration jump technique (from 1 to 100 μM) to the same patches induced openings of a channel with a small unitary amplitude (2.14 pA) at −50 mV (pipette and bath solutions were 140 mM K^+ containing 5 mM EGTA). The open-state probability (the NP_o value) of the levcromakalim-induced K^+ channel was concentration-dependent (Fig. 4A,B). However, onset and offset were comparatively slow, for instance, the delay for onset and offset were 15 ± 8 s ($n = 5$) and 3 min and 12 s ± 30 s ($n = 5$), respectively (Fig. 4C). The activation of the channels by levcromakalim under these cell-attached conditions was stable after the onset of the activation, and the NP_o value was independent of voltage, at least over the range from −60 to −30 mV, as shown in Fig. 5, where the voltage sensitivity of the NP_o was assessed at two concentrations of levcromakalim (10 and 100 μM).

3.5. The kinetic properties of K_{GS} in pig urethra

At concentrations higher than 50 μM, levcromakalim induced long burst-like channel opening with flickering fast opening and closing of the channel. Fig. 6 shows the current traces at −50 mV (Fig. 6A) and −90 mV (Fig. 6B). We estimated both the mean open lifetime and closed time during the burst-like channel activity according to the method of Colquhoun and Sigworth (1995), although it was not technically feasible to determine the total numbers of the activated channels in these patches. The holding membrane potential was randomly changed between −100

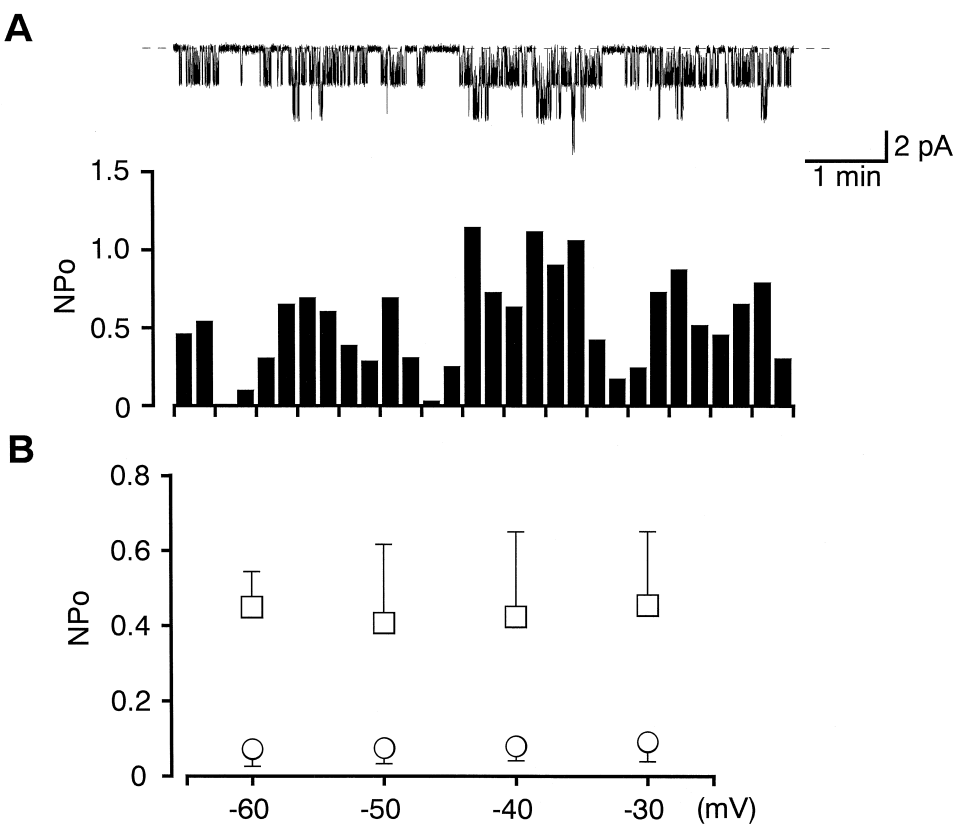

Fig. 5. Time- and voltage-independence of the levcromakalim-activated K_{GS} in cell-attached configuration (symmetrical 140 mM K^+ conditions) in the same patches. (A) A current trace (above, approximately 7.5 min duration) of channel activity induced by levcromakalim (100 μM), with the NP_o value below, calculated for every 15 s segment of the record. The dashed line indicates the current when the channel is not open. (B) The channel activity at different membrane potentials, calculated from the NP_o value (sampling rate of 80 μs for 2 min duration) in the presence of levcromakalim (open circle, 10 μM; open square, 100 μM). Each symbol is mean value of five observations with S.D.

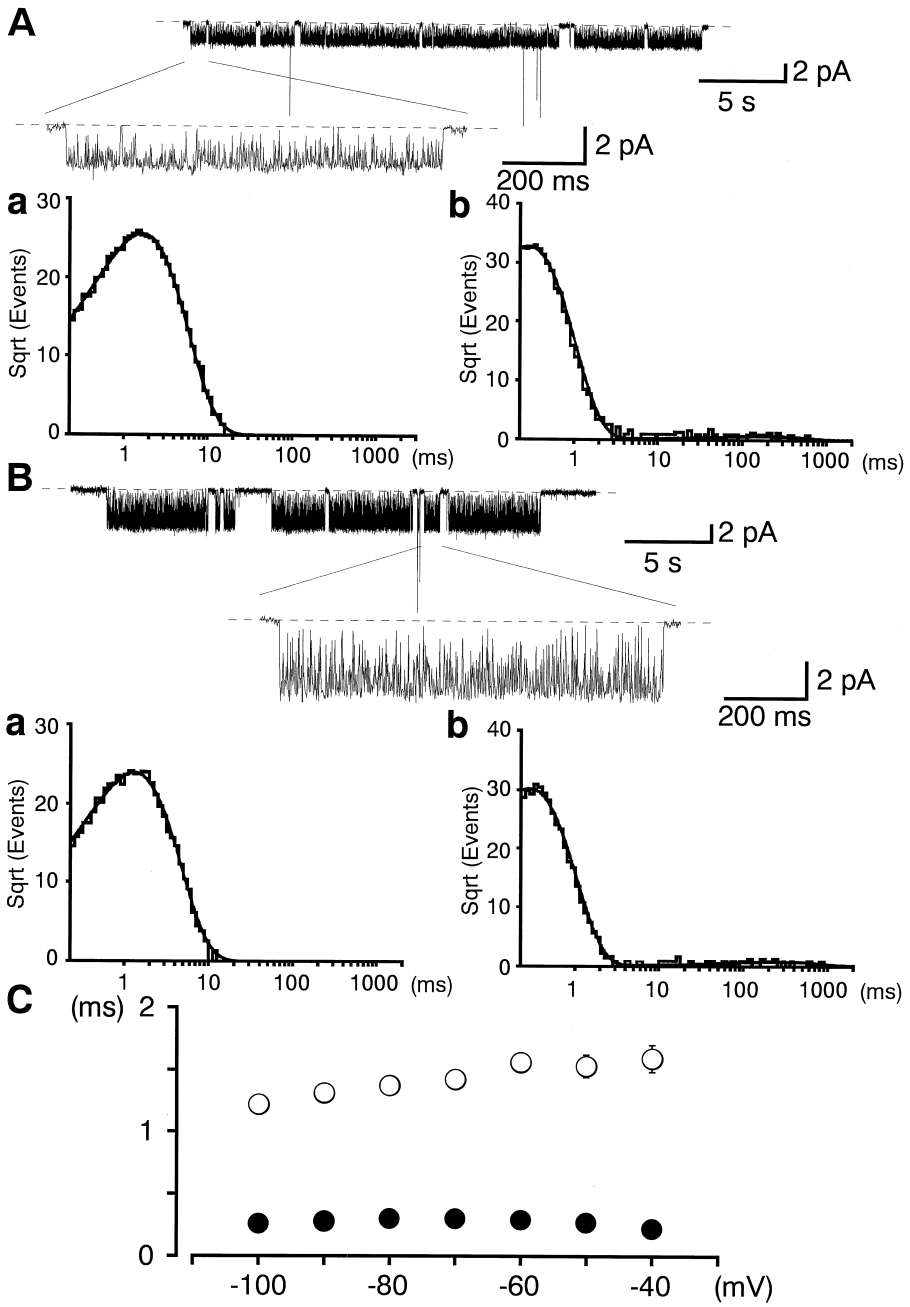

Fig. 6. Properties of long burst-like channel activities of K_{GS} induced by levcromakalim (100 μM) in cell-attached membrane patches (symmetrical 140 mM K^+ conditions). Levcromakalim induced burst-like channel activity at a holding membrane potential of −50 mV (A) or −90 mV (B). The bursting activity is shown on a slow time base above, with one burst expanded below (filtering at 2 kHz). The dashed line indicates the current base line where the channel is not open. Activation of large conductance Ca^{2+}-activated K^+ channels is occasionally observed as large downward deflections. (A and B) Time distribution graphs for the mean open lifetime and the mean closed lifetime. The abscissa shows the log of the lifetime (ms) and the ordinate shows the square root of the number of events ($n^{1/2}$). The solid curve indicates exponential fitting using the least-squares method (see Section 2). The time constants for the mean open lifetimes were 1.6 ± 0.1 ms, and 1.3 ± 0.1 ms at −50 or −90 mV, respectively ($n = 5$). The time constants for the mean closed time were 0.27 ± 0.01 ms and 0.28 ± 0.01 ms at −50 or −90 mV, respectively ($n = 5$). (C) Time constants of the open (open circle) and closed (filled circle) lifetimes of the of K_{GS} under various membrane potential levels. There is little voltage dependency between −100 and −40 mV. Mean ± S.D. ($n = 5$). Most of the S.D. bars are less than the size of the symbols.

and −40 mV after recording for at least 2 min at each membrane potential. Neither the mean open lifetime nor the mean closed time showed significant voltage-dependency (the time constants for the mean open lifetime were 1.6 ± 0.1 ms (−50 mV, $n = 5$) and 1.3 ± 0.1 ms (−90 mV, $n = 5$). The time constants for the mean closed time were 0.27 ± 0.01 ms (−50 mV, $n = 5$) and 0.28 ± 0.01 ms (−90 mV, $n = 5$), respectively, Fig. 6).

4. Discussion

4.1. Levcromakalim induces a stable and long lasting relaxation in the resting tone of pig urethra through a voltage- and time-independent activation of K_{GS}

In the present experiments, we have demonstrated that levcromakalim induces time- and voltage-independent membrane currents at membrane potentials between −120 and −30 mV with a gradual decrease of the time course of the 300-μM levcromakalim-induced current at −50 mV. This levcromakalim induces time- and voltage-independent observation was confirmed in single-channel recordings, since the NP_o value of the levcromakalim-induced K_{GS} demonstrates a voltage-independence between −60 and −30 mV. Furthermore, during burst-like channel activity, each burst consists of both fast opening and fast closing, with the appearance of fast flickering. Neither the mean open lifetime nor the mean closed time showed voltage-dependency at membrane potentials between −100 and −40 mV, and these values were quite similar to those observed in guinea pig cardiac myocytes (Trube and Hescheler, 1984). Thus, it seems plausible to assume that the levcromakalim-induced stable relaxation of the intact urethra is closely related to the voltage- and time-independent activation of K_{GS}. Since the resting membrane potential in most of intact smooth muscle lies between approximately −60 to −30 mV (vascular smooth muscle cells, Kuriyama et al., 1995; urinary tract cells, Andersson, 1993), this levcromakalim-induced voltage-independent activation of KGS would readily cause a significant membrane hyperpolarization followed by relaxation of the tissue. Although we have demonstrated the different time course between the 300-μM levcromakalim-induced current at −50 mV and the 10-μM levcromakalim-induced stable relaxation, we are not certain whether or not the decay of the levcromakalim-induced current may be due to higher concentrations of levcromakalim or different experimental conditions. On removal of levcromakalim, the amplitude of the levcromakalim-induced outward current was sustained for a short while (3 min and 38 s ± 45 s, $n = 5$) and gradually returned to the original level. Similarly, the openings of the levcromakalim-induced K_{GS} were also observed for more than 2 min after removal of levcromakalim from the bathing solution even by use of the concentration jump technique. These observations indicate that the long-lasting relaxing effect of levcromakalim is due to the long-lasting openings of K_{GS} after removal of levcromakalim.

We have further been able to demonstrate that the time course of the inhibitory effects of glibenclamide on the levcromakalim-induced membrane current at −50 mV could be fitted by a single exponential. It is reasonable to assume that glibenclamide would bind to a single site of the sulphonylurea receptor of K_{GS}, selectively inhibiting the single function of the sulphonylurea receptor of K_{GS} in pig urethra.

4.2. Pharmacological characterization of the levcromakalim-induced sustained outward membrane currents in pig urethra and clinical implications of K_{ATP} openers in urological therapy

Tolbutamide, one of the well-known sulphonylureas, did not suppress the 100-μM levcromakalim-induced outward current even at a concentration of 1 mM, thereby indicating that there is a large difference in inhibitory potency between tolbutamide and glibenclamide. Similar difference has been widely observed in other tissues (Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993). Tetraethylammonium$^+$ (≥ 1 mM), a non-selective K^+ channel blocker (Hille, 1992), partially blocked the levcromakalim-induced outward current, at a concentration close to that which causes a 50% block of K_{ATP} channels in skeletal muscle ($K_d = 6$–7 mM; Davies et al., 1989). These results suggest that the pharmacological properties of the levcromakalim-induced outward current in pig urethra may be similar to those of other tissues with respect to various types of K^+ channel blockers (Davies et al., 1989; Katnik and Adams, 1995). In the present experiments, we have been able to record that external application of the multivalent cation, La^{3+} caused a partial and irreversible inhibitory effect on the levcromakalim-induced membrane currents. It is well-known that Ba^{2+} inhibits K_{ATP} in mouse pancreatic β-cells due to its binding to a site at the external mouth of K_{ATP} (Takano and Ashcroft, 1996). Since the relative atomic mass of La^{3+} is very similar to that of Ba^{2+} (Ba^{2+}, 137.3 vs. La^{3+}, 138.9; Dawson et al., 1984), La^{3+} may inhibit the levcromakalim-activated K^+ channel in a similar manner to that of Ba^{2+}.

In urinary tract smooth muscle cells, K^+ channels play an important role in regulating the membrane potential and cellular excitability (reviewed by Andersson (1993)). K_{ATP} openers which induce urethral relaxation may have a potent clinical benefit for the treatment of the functional bladder outflow obstruction (such as a detrusor/sphincter dyssynergia, drug-induced retention of urine, post-operation urinary retention etc.). Thus, K_{ATP} openers may be useful pharmacological tool for urological therapy, especially when K_{ATP} openers are locally applied in internal urethra. The further clarification of the functional characteristics of K_{GS} may yet yield useful clinical information for the treatment of functional bladder outlet obstruction in the urological field.

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