Comparative Studies on the Relaxing Action of Several Adenosine 5'-Triphosphate-Sensitive K+ Channel Openers in Pig Urethra

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Abstract

The relaxing effects of the adenosine 5'-triphosphate (ATP)-sensitive K+ channel openers (KA, openers; diazoxide, minoxidil, pinacidil, (±)-cromakalim, (+)-cromakalim and (−)-cromakalim) were investigated on the resting tone of pig proximal urethra. In addition, patch-clamp techniques were utilized for recording cromakalim-induced ionic currents in cells dispersed from the same urethral region. The (−)-cromakalim-induced relaxation of urethral muscle strips was stable, reversible and reproducible. The rank order of potency regarding of KA, openers in lowering the resting urethral tone was (−)-cromakalim > pinacidil > diazoxide > minoxidil. KATP opener-induced urethral relaxation was suppressed by subsequent application of glibenclamide (1 μM). (+)-Cromakalim (≤10 μM) did not relax the urethra nor antagonize the (−)-cromakalim-induced urethral relaxation. However, at higher concentrations, (+)-cromakalim (≥30 μM) caused a small but significant urethral relaxation. In accordance with these observations, the relaxation induced by 5 μM (−)-cromakalim was identical to that induced by 10 μM (±)-cromakalim, as expected from a theoretical half potency for (±)-cromakalim. In whole-cell recording, (−)-cromakalim and (+)-cromakalim (100 μM) activated a glibenclamide-sensitive outward current which was due to the activation of the glibenclamide-sensitive 43 pS K+ channel (Kgs-43 pS). The potency of (+)-cromakalim to activate Kgs-43 pS was much weaker than that of (−)-cromakalim. These results indicate that the ability of KATP openers to relax pig urethral smooth muscle can be accounted for by activation of glibenclamide-sensitive K+ channels.

Key Words: enantiomer of cromakalim, KATP opener, Kgs-43 pS K+ channel, resting urethral tone

Introduction

The discovery of (±)-cromakalim introduced a novel pharmacological tool for the study of the mechanisms involved in relaxation of smooth muscle and has led to the development and synthesis of a number of cromakalim derivatives (reviewed by Evans et al., 1992). A wide variety of K+ channels has been reported as selective targets for (±)-cromakalim (or (−)-

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cromakalim) in smooth muscle cells (reviewed by Kuriyama et al., 1995). It has become apparent that the effects of (±)-cromakalim are stereospecific, the greatest activity being confined to the (−)-enantiomer (BRL 38227 or levocromakalim; Hof et al., 1988; Edwards et al., 1991; Evans et al., 1992). Thus, the purified (−)-cromakalim has been the major form utilized in recent investigations on the mechanisms involved in smooth muscle relaxation (Edwards et al., 1991; Teramoto and Brading, 1996, 1997). However, it still remains to be elucidated whether or not there is any direct interaction between (+)- and (−)-cromakalim in their ability to relax smooth muscle or activate K+ channels.

The potency of ATP-sensitive K+ channel openers (KATP openers) to relax intact smooth muscle preparations shows a wide variation depending on the types of smooth muscle, suggesting that the different potency orders may be due to species or tissue differences (Edwards et al., 1991; Zhou et al., 1995). In addition, all of these experiments were carried out in the presence of agonists in order to develop the muscle tone, which may interfere possible roles of KATP openers to relax intact preparations.

We have recently reported the presence of a glibenclamide-sensitive 43 pS K+ channel (Kgs-43 pS) in pig urethra which is activated not only by (−)-cromakalim but also by nicorandil (Teramoto and Brading, 1996, 1997, 1998; Teramoto et al., 1997a, b, 1999). In the present experiments, since urethral strips develop resting tone, we have been able to establish the rank potency order of examples from several classes of KATP, compared to that of (−)-cromakalim (which demonstrated the most potent relaxing effects on the resting urethral tone), without using agonist to activate the tissue. Furthermore, we have investigated the interaction between (+)- and (−)-enantiomers of cromakalim by use of tension and patch-clamp techniques and found that (+)-cromakalim at high concentrations activates Kgs-43 pS, which is the target K+ channel for (−)-cromakalim.

**Methods**

Fresh female pig urethra was collected from a local abattoir and transported to the laboratory in a cold modified Krebs solution (4–6°C, for composition, see below).

**Tension measurement and data analysis**

For isometric tension recording, fine strips were prepared as described previously (Teramoto and Brading, 1996, 1997). An initial tension equivalent to 1 g weight was applied to each strip, which was then allowed to equilibrate for approximately 2.5–3 h until the basal urethral tone became stable (37°C). To prevent both noradrenaline outflow from sympathetic nerve terminals and β-adrenoceptor stimulation, 3 μM guanethidine and 0.3 μM propranolol were present throughout the experiments. Data were recorded on a Macintosh computer, through "MacLab 3.5.6" (ADInstruments Pty Ltd, Australia). The value of tension was expressed as g/mg of tissue.

**Solutions and drugs**

For tension measurement, modified Krebs solution was used (mM): 137 Na+, 5.9 K+, 1.2
K\textsubscript{ATP} opener-induced urethral relaxation

Mg\textsuperscript{2+}, 2.5 Ca\textsuperscript{2+}, 133.7 Cl\textsuperscript{-}, 15.4 HCO\textsubscript{3}\textsuperscript{-}, 1.2 H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-} and 11.5 glucose which was bubbled with 97% O\textsubscript{2} and 3% CO\textsubscript{2}. In whole-cell configuration, the following solutions were used: physiological salt solution (PSS) containing (mM): Na\textsuperscript{+} 140, K\textsuperscript{+} 5, Mg\textsuperscript{2+} 1.2, Ca\textsuperscript{2+} 2, glucose 5, Cl\textsuperscript{-} 151.4, HEPES 10, titrated to pH 7.35-7.40 with Tris base; pipette solution containing (mM): K\textsuperscript{+} 140, Cl\textsuperscript{-} 140, 5 EGTA, 10 HEPES/Tris (pH 7.35-7.40). For single-channel recording, the pipette and bath solution was a high potassium solution (mM): K\textsuperscript{+} 140, Cl\textsuperscript{-} 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35-7.40) producing symmetrical 140 mM K\textsuperscript{+} conditions. Cells were allowed to settle in the small experimental chamber (80 \textmu l in volume, the bath perfusion rate, 2 ml/min). All chemicals were purchased from Sigma. Both (−)- and (+)-cromakalim were kindly provided by SmithKline Beecham Pharmaceuticals (Harlow, UK). The maximum final concentration of DMSO in the bath solution was 0.05% for tension measurements and 0.3% for patch-clamp experiments. These concentrations were shown not to affect either the urethral resting tone or K\textsuperscript{+} channels in pig urethral smooth muscle.

Cell preparation and recording procedure

An identical cell dispersion method was employed as described previously (Teramoto and Brading, 1997, 1998; Teramoto et al., 1997a, b, 1999). The experimental system used was essentially the same as that described previously (21–23°C, Teramoto and Brading, 1996, 1997, 1998; Teramoto et al. 1997b, 1999). The perforated patch technique with nystatin was performed as in our previous papers (Teramoto and Brading, 1996, 1997; Teramoto et al., 1997b, 1999).

Data analysis

The current data were low-pass filtered at 500 Hz by an 8 pole Bessel filter and analysed on a Macintosh computer using ‘MacLab 3.5.6’. For single-channel recording, the stored data were low-pass filtered at 2 kHz (-3 dB) and sampled into the computer with an interval of 80 \mu s using the PAT program (kindly provided by Dr. J. Dempster, University of Strathclyde). Conditional probability density function was fitted to the open lifetime distribution by the method of maximum likelihood (the Gaussian distribution). However, events briefer than 200 \mu s were not included in the evaluation and no correction was made for missed events. NP\textsubscript{0} values (number of channels x open state probability) were calculated for every 5 s segment of the channel recording. Data points were fitted using a least-squares fitting (Teramoto and Brading, 1997, 1998; Teramoto et al., 1997a, b, 1999).

When the peak amplitude of the 10 \mu M (−)-cromakalim-induced relaxation was normalized as 1.0, the relative amplitude of several types of K\textsubscript{ATP} opener-induced relaxation was calculated at each concentration. The curves were drawn by fitting the equation using the least-squares method,

\[
\text{Relative value of } K_{\text{ATP}} \text{ opener-induced relaxation} = \frac{1}{1+(K/D)n_{H}}
\]

where K, D and n\textsubscript{H} are EC\textsubscript{50} value, concentration of K\textsubscript{ATP} openers (\mu M) and Hill coefficient. Statistical analyses were performed with an analysis of variance (ANOVA) test (two-factor with replication). Changes were considered significant at \( p < 0.01 \), and data are expressed as mean±standard deviation (S.D.).
Results

Reproducibility of the (-)-cromakalim-induced relaxing effects on the resting tone of pig proximal urethra

When a resting tension of 1 g was applied to a circular smooth muscle strip prepared from the pig proximal urethra, spontaneous tone gradually developed. After it had reached a peak value, the tone usually decreased somewhat and eventually became stable even in the absence of any agonists (Fig. 1A). Application of 3 μM (-)-cromakalim caused a stable and reversible relaxation in the urethral resting tone, and 30 min later, reapplication of 3 μM (-)-cromakalim induced a similar relaxation, the peak amplitude of which was not significantly different from that of the first application (1.01±0.02, n=12, Fig. 1B). The time courses of the first and second relaxations were almost identical when superimposed (Fig. 1C). Similar results were obtained with lower and higher concentrations of (-)-cromakalim (300 nM, 1 μM, 5 μM and 10 μM, data not shown), thereby indicating that the relaxation of smooth muscle strips produced by (-)-cromakalim is strictly reproducible.

The effects of $K_{ATP}$ openers on the resting urethral tone

Cumulative application of $K_{ATP}$ openers produced a concentration-dependent relaxation of the resting urethral tone. Fig. 2 illustrates the responses to (-)-cromakalim, pinacidil, diazoxide and minoxidil, and shows the respective concentration-response curves, expressed relative...
Fig. 2. The relaxing effects of K<sub>ATP</sub> openers on the resting tone of pig urethra. The dashed line indicates the mean resting urethral tone. A, The effects of cumulative addition of Aa, (-)-cromakalim (30 nM-10 μM); Ab, pinacidil (1-10 μM) and after recovery, 10 μM (-)-cromakalim; Ac, diazoxide (10-300 μM) and after recovery, 10 μM (-)-cromakalim; Ad, minoxidil (100 μM-10 mM) and after recovery, 10 μM (-)-cromakalim. B, Relationships between the relative value of K<sub>ATP</sub> opener-induced urethral relaxation and the concentration of K<sub>ATP</sub> openers. The peak amplitude of the 10 μM (-)-cromakalim-induced relaxation was normalized as 1.0 (see Methods). The following values were calculated for the curve fitting by use of the least-squares method: K=304 nM, n<sub>H</sub>=2.08 ((-)-cromakalim, n=18, open circle); K=1.63 μM, n<sub>H</sub>=2.07 (pinacidil, n=12, filled circle); K=70.5 μM, n<sub>H</sub>=1.97 (diazoxide, n=12, filled square); K=991 μM, n<sub>H</sub>=2.00 (minoxidil, n=12, filled triangle). The open triangle indicates the relative value of (+)-cromakalim-induced relaxation. Each symbol indicates mean with S.D. shown by vertical lines. Most of the S.D. bars are less than the size of the symbols.

to the maximum relaxation to 10 μM (-)-cromakalim. The slope and maximum value of relaxation in each curve was identical, and the urethral tone in each case recovered to the control level after washing out the drug. The rank order of potency of the EC<sub>50</sub> values for
relaxation was (−)-cromakalim > pinacidil > diazoxide > minoxidil. The relaxations produced by these K\textsubscript{ATP} openers were suppressed by additional application of 1 \( \mu \)M glibenclamide (data not shown).

Comparison of the relaxing potencies of (±)- and (−)-cromakalim

In order to study further K\textsubscript{ATP} opener-induced urethral relaxation, cromakalim, the most potent K\textsubscript{ATP} opener in the present experiments, was utilized. Fig. 3Aa shows that application of 10 \( \mu \)M (±)-cromakalim or 10 \( \mu \)M (−)-cromakalim (8 min duration) caused urethral relaxations of nearly equal amplitude since cromakalim (≥5 \( \mu \)M) induced the maximum urethral relaxation (see Fig 2B). However, the time course of the relaxation induced by the two drugs

![Diagram](image-url)
was recognizably different, the (−)-cromakalim-induced relaxation (filled circle) reaching the maximum relaxed level much quicker than that induced by (+)-cromakalim (open circle) (Fig. 3Ab, the superimposed traces). On removal of each compound, the effects of (−)-cromakalim were longer lasting than those of (+)-cromakalim. In contrast, when 5 μM (−)-cromakalim and 10 μM (+)-cromakalim were applied for the same duration (8 min), the relaxations and time courses for each drug were almost identical (Fig. 3B, n = 6). These results suggest that the (+)-enantiomer may be inactive as either an agonist or antagonist to induce muscle relaxation.

The interaction between (−)-cromakalim and (+)-cromakalim

To investigate further the interaction between enantiomers of cromakalim, we utilized the two purified enantiomers, (+)-cromakalim and (−)-cromakalim. Whereas application of 10 μM (−)-cromakalim caused a reproducible relaxation, 10 μM (+)-cromakalim on the same strip did not induce any detectable change in the muscle tone (Fig. 4A, n = 6). As shown in Fig. 4Ba, the response to 5 μM (−)-cromakalim was not affected by the presence of 5 μM (+)-cromakalim, and the time course of the two relaxations was identical (Fig. 4Bb). Exactly the same observations were made in five other strips.

We then studied the interaction of lower concentrations of the (−)-enantiomer with higher concentrations of the (+)-enantiomer. As shown in Fig. 4C, increased concentration of (+)-cromakalim (30 μM) caused a small but significant relaxation. (−)-Cromakalim (300 nM) caused the urethral relaxation, and when 300 nM (−)-cromakalim was applied in addition to 30 μM (+)-cromakalim, an additional relaxation was observed. The sum of the two individual relaxations was not statistically different from the size of the relaxation in the presence of both drugs. Thus, it seems that the (+)-enantiomer is not acting as a competitive antagonist to the (−)-enantiomer, but has a weak agonistic action.

The interaction between (+)- and (−)-cromakalim on the glibenclamide-sensitive currents

When 100 μM (+)-cromakalim was applied in a conventional whole-cell configuration, a small but significant outward membrane current was evoked at −50 mV (Fig. 5A). Additional application of 100 μM (−)-cromakalim further enhanced the glibenclamide-sensitive outward current. Furthermore, after the amplitude of the 50 μM (−)-cromakalim–induced membrane current had reached a peak in a nystatin-perforated patch, additional application of 100 μM (+)-cromakalim repeatedly produced a small but significant increase in the amplitude of the membrane current (Fig. 5B).

To study further the interaction between (+)– and (−)-cromakalim, we recorded unitary currents. As shown in Fig. 6A, application of 100 μM (+)-cromakalim induced the opening of a 2.14 pA K+ channel at −50 mV in cell-attached configuration (symmetrical 140 mM K+ conditions). On removal of 100 μM (+)-cromakalim, the channel activation gradually decreased and then disappeared. Approximately 7 min later, the same amplitude K+ channel was activated more potently by application of 100 μM (−)-cromakalim (Fig. 6B). When the NP0 value for the 100 μM (−)-cromakalim–induced channel activation was normalized as 1.0, the
Fig. 4. The interaction of the two purified enantiomers of cromakalim on the urethral tone. The dashed line indicates the mean resting urethral tone. **A**, The effects of both 10 μM (−)-cromakalim and 10 μM (−)-cromakalim on the urethral tone. **Ba**, The comparison of the time course of the 5 μM (−)-cromakalim-induced relaxation in the presence and absence of 5 μM (−)-cromakalim. **Bb**, When both original relaxing traces in **Ba**, were superimposed, these traces overlapped completely with each other. The hatched bar indicates the application of both drugs (8 min duration). **C**, Effects of 300 nM (−)-cromakalim on the resting tone in the absence and presence of 30 μM (−)-cromakalim.

The relative NP₀ value of the 100 μM (+)-cromakalim-induced channel was 0.14±0.05 (n=5). Fig. 6C shows the effects of 100 μM (+)-cromakalim on 2.14 pA K⁺ channel which was activated in a cell-attached patch by pretreatment with 100 μM (−)-cromakalim at −50 mV. Additional application of 100 μM (+)-cromakalim further enhanced the channel activity in a repeatable manner, demonstrating an increment of the NP₀ value. Similar results were obtained in four other patches.

**Discussion**

The rank order of potency of K<sub>ATP</sub> openers in urethral smooth muscle

To date, the comparative studies on potency of K<sub>ATP</sub> openers has been examined by study
K<sub>ATP</sub> opener-induced urethral relaxation

Fig. 5. Effects of (+)-cromakalim on the membrane currents at -50 mV in whole-cell configuration. Bath solution was PSS and pipette solution was 140 mM KCl containing 5 mM EGTA. The dashed line indicates the zero-current level. A, Effects of 100 μM (+)-cromakalim and subsequent application of 100 μM (-)-cromakalim. Application of 5 μM glibenclamide suppressed the membrane current to the control level. B, Effects of 100 μM (+)-cromakalim on the 50 μM (-)-cromakalim-induced membrane current; nystatin-perforated patch.

of their relaxant effects on either excess [K+]<sub>o</sub>- or acetylcholine-induced contractions in vascular and urinary bladder smooth muscles (Edwards et al., 1991; Zhou et al., 1995). However, it is well documented that excess [K+]<sub>o</sub> activates voltage-dependent mechanisms (such as Ca<sup>2+</sup> entry through voltage-dependent Ca<sup>2+</sup> channels), Ca<sup>2+</sup>-activated mechanisms due to Ca<sup>2+</sup> entry (Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release mechanisms, Ca<sup>2+</sup>-activated K<sup>+</sup> channels, Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels etc.) and acetylcholine also activates stimulatory mechanisms (such as non-selective cation channels, muscarinic receptor-modulated pathway etc.) (reviewed by Kuriyama et al., 1995). Therefore, it is somewhat difficult to estimate the effects of K<sub>ATP</sub> openers on the glibenclamide-sensitive mechanisms alone in the presence of either excess [K+]<sub>o</sub> or agonists. Moreover, the comparative studies have not been performed at normal membrane potential and under unstimulated conditions. In the present experiments, in the absence of excess [K+]<sub>o</sub> or agonists, we have been able to evaluate the rank order of potency of K<sub>ATP</sub> openers on fresh intact tissue, utilizing a well-known K<sub>ATP</sub> opener from each of the different groups, classified on chemical structure; cromakalim (benzopyran), pinacidil (pyridine), diazoxide (benzothiadiazine) and minoxidil (pyrimidine). The rank order of potency was found to be (-)-cromakalim > pinacidil > diazoxide > minoxidil, showing a similar order to that found in other smooth muscles (rat portal vein, rat urinary bladder, Edwards et al., 1991), although the absolute EC<sub>50</sub> values of each K<sub>ATP</sub> openers differ from those obtained in rat portal vein or rat urinary bladder. Since all of the K<sub>ATP</sub> opener-induced relaxations of the resting urethral tone
Effects of the biologically-active (−)-enantiomer of cromakalim

It is known that (±)-cromakalim is a racemic mixture of two enantiomers, that the effects of (±)-cromakalim are stereoselective and that the (−)-enantiomer of cromakalim possesses the biological activity (Hof et al., 1988; Evans et al., 1992). In the present experiments, the time course and amplitude of urethral relaxation evoked by the racemic mixture could be exactly mimicked by half the concentration of (−)-cromakalim. We have also demonstrated

were suppressed by subsequent application of 1 μM glibenclamide, these results suggest that all of the $K_{ATP}$ opener-induced relaxations may be due to the activation of the glibenclamide-sensitive mechanisms in pig urethra.
that the size and time course of the 5 μM (−)-cromakalim–induced relaxation was identical in the absence and presence of equal concentrations of (−)-cromakalim, thereby indicating that 5 μM (+)-cromakalim has no effect on the muscle tone. Actually, application of (+)-cromakalim (10 μM) caused no detectable urethral relaxation. Thus, it seems that low concentrations of (+)-cromakalim (≤10 μM) has no direct relaxing effect and do not interact competitively with (−)-cromakalim. On the other hand, application of higher concentrations of (+)-cromakalim (≥30 μM) caused a small but significant relaxation, although the effects of (+)-cromakalim (30 μM) far less than that seen with 300 nM (−)-cromakalim on the same strips. The effects of (+)-cromakalim on the urethral tone are thus at least 100 times less than that of (−)-cromakalim. This potency difference between the two has been reported in other smooth muscles (Hof et al., 1988; Edwards et al., 1991). Although these reports suggest that the rudimentary activity of (+)-cromakalim may be due to contamination of (−)-cromakalim, in the present experiments, higher concentrations of (+)-cromakalim caused an additional activating effect on the (−)-cromakalim–induced muscle relaxation and membrane currents in whole-cell or single-channel recordings without showing any competitive effects. In the present experiments, Hill coefficients (n_H) calculated from the concentration–response curves for all the well-known K_ATP openers tested indicated values of approximately 2, which is close to the value observed in rat portal vein (n_H = 1.5, Hof et al., 1988). These results suggest that K_ATP openers, even though classified into different groups by chemical structures, commonly seem to bind to at least two active binding sites as full agonists in pig urethra.

The clinical implication of the effects of K_ATP openers in urological treatment

As (±)-cromakalim and pinacidil have been demonstrated to abolish the unstable contractions associated with bladder outflow obstruction in in vivo experiments and are known to cause a significant relaxation in isolated human bladder, so K_ATP openers appear as potential therapeutic tools of instability in urology ((±)-cromakalim, Foster et al., 1989; pinacidil, Malmgren et al., 1989; Hedlund et al., 1991). Our present results show that K_ATP openers are capable of producing a reduction in urethral smooth muscle tone at similar concentrations to those which evoke relaxation in the urinary bladder. These observations may support the idea that K_ATP opener–induced urethral relaxation would be an undesirable side effect for treatment of urge incontinence (Teramoto and Brading, 1996; Teramoto et al. 1997a, b). Nevertheless, the ability to produce urethral relaxation may be of value in the treatment of bladder outflow obstruction, which, if untreated, can lead to irritative urinary tract symptoms, and bladder dysfunction (Turner-Warwick et al., 1973).

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References


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