Effect of $K_{\text{ATP}}$ channel openers on myogenic and neurogenic responses in goat urinary bladder

C Vijayakumar, K Kathirvel, K K Sardar & S C Parija*
Department of Pharmacology and Toxicology, Faculty of Veterinary Sciences and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar 751 003, India

Received 2 January 2006; revised 20 September 2006

Isolated goat detrusor muscle exhibited spontaneous contractility with an irregular amplitude and frequency. The spontaneity of detrusor muscle exhibited a mean amplitude as 11.99 ± 0.83 mm and frequency as 1.37 ± 0.16/min. $K_{\text{ATP}}$-channel openers namely, cromakalim or pinacidil (10$^{-7}$-10$^{-4}$ M) added cumulatively, elicited a concentration-related inhibition of both amplitude and rate of spontaneous contractions. The mean IC$_{50}$ values for both amplitude and frequency for cromakalim were 3.3 × 10$^{-6}$ M and 2.9 × 10$^{-6}$ M, respectively; and for pinacidil were 2.0 × 10$^{-5}$ M and 1.5 × 10$^{-5}$ M, respectively. Glibenclamide, a $K_{\text{ATP}}$-channel blocker inhibited the cromakalim-induced concentration-related relaxation of spontaneous contractions with a significant increase in its mean IC$_{50}$. ACh-induced concentration-related contractile response was inhibited in the presence of either cromakalim (10$^{-4}$ M) or pinacidil (10$^{-4}$ M). The mean EC$_{50}$ value of ACh, in the presence of cromakalim (2.5 × 10$^{-3}$ M) was significantly increased as compared to the control (1.2 × 10$^{-6}$ M). In the presence of glibenclamide (10$^{-5}$ M) the inhibitory effect of cromakalim was significantly reduced with consequent decrease in the EC$_{50}$ value (1.9 × 10$^{-5}$ M). Application of EFS (30 V and 5 ms) on goat urinary bladder strips at 1, 2, 5, 10, 20 and 30 Hz elicited frequency-related contractile responses. Both cromakalim and pinacidil caused a rightward shift in the frequency-related contractile response curve with significant increase in the mean EF$_{25}$ and EF$_{50}$ values, respectively. In the presence of glibenclamide (10$^{-5}$ M), the frequency-related inhibitory response curve was shifted to left with significant (P <0.001) increase in the mean EF$_{25}$, EF$_{50}$ and EF$_{75}$. The present results suggest that in the goat detrusor muscle, agonist and EFS-induced contractile responses were more potently inhibited by cromakalim than pinacidil with activation of glibenclamide sensitive $K_{\text{ATP}}$ channels.

Keywords: Electrical field stimulation, Goat urinary bladder, $K_{\text{ATP}}$ channels openers,

K$^+$ channel openers, cromakalim and pinacidil, have been shown to be effective in reducing the level of spontaneous contractile activity in the urinary bladder following outlet obstruction in rats$^1$, guinea pigs$^2$, humans and pigs$^3$. Similarly, the mechanical and electrical activity of guinea pig$^4$ was completely abolished by cromakalim at 10$^{-5}$ M. On the other hand, pinacidil is a substantially more potent inhibitor of the amplitude of the hyper-reflexia than the frequency$^5$. Pinacidil was more effective than cromakalim in guinea pig and cromakalim was more effective than pinacidil in rabbit detrusor muscle on carbachol-induced concentration-related contractile responses and transmural electrical-field stimulated responses$^6$. Pinacidil effectively depresses the contractures induced by low (less than 40 mM) concentration of K$^+$ and by electrical stimulation. Pinacidil was a non-competitive or mixed inhibitor of both methoxamine and bethanechol stimulation, competitive inhibitor of KCl stimulation and it was substantially more effective at inhibiting field stimulation at 2 Hz, as compared to 32 Hz and equally effective against the phasic and tonic components induced by EFS$^5$. The effectiveness of cromakalim in relaxing the detrusor muscle is about an order of magnitude higher in rat and guinea pig than in humans$^6$. The effect of cromakalim, pinacidil and lemakalim was found to be similar on $^{86}$Rb release, and P1075 was more potent in resting, as well as 25 mM KCl depolarized detrusor strips suggesting that, these agents are effective potassium channel openers (PCOs) in guinea pig detrusor muscle$^7$. Cromakalim is the dualistic antagonist against carbachol$^9$ and partially inhibited the contractile response to acetylcholine in rat urinary bladder$^10$. In the human urinary bladder, glibenclamid was able to diminish the relaxant effect of cromakalim and to prevent the

*Correspondent author
Ph: +91 6742430264/9437356387
Fax: +91 674402970
email: scp4691@yahoo.co.in
cromakalim-induced hyper-polarization. Glibenclamide inhibited the voltage-dependent cardiac potassium currents at concentration above 10 μM in human ventricular myocytes. Cromakalim was capable of inhibiting the smooth muscle contraction by other mechanism additional to that of potassium channel opening effect in detrusor muscle. Likewise, it inhibits the cholinergic and NANC neuroeffector transmission in guinea pig trachea, guinea pig airways and rat detrusor muscle. Pinacidil is also having activities other than K⁺ channel opening in various tissues and might be interacting with yet another kind of potassium channel in guinea pig urinary bladder. It has been well established that the neural and peripheral control of micturition varies between human, rat, guinea-pigs, rabbits, pigs, etc. Similarly, responsiveness to different ion channel ligands also differs from one species of animal to other. In ruminants, informations are almost lacking in understanding the role of these ion channels in normal urinary bladder. The present study has been carried out on goat urinary bladder with the objective to establish urinary bladder as a prototype smooth muscle model for ruminants and to assess the effects of cromakalim and pinacidil on agonist and EFS-induced contractile responses on this tissue.

Materials and Methods

Black Bengal goats (2.0-2.5 years old) of either sex weighing between 12.5 to 15.0 kg were employed. Drug solutions—Acetylcholine chloride (Sigma), Atropine (Sigma), Cromakalim (Smith Kline and Beecham), Diltiazem (Sigma), EGTA (Sigma), Glibencamide (Hoechst), Pinacidil (Leo) were used.

The whole urinary bladder from goat of was collected immediately after slaughtering and flushed with chilled Tyrode’s solution (4°C) and brought to the laboratory. Each urinary bladder was weighed, dissected longitudinally on the midline through ventral surface. Two transverse strips with an approximate size of 8 mm were obtained from the mid-region of the urinary bladder and tissue was cut at mid-dorsal section, thus creating four strips of similar length. The mucous membrane was removed from each strip and further employed for isotonic contraction studies as there was 100-fold increase in mean EC₅₀ of ACh in denuded tissue than non denuded one. The denuded detrusor muscle, approximately of 2.5 cm × 8 mm was suspended in a thermostatically controlled (37.0 ± 0.5 °C) organ bath containing 20 ml of Tyrode solution (CaCl₂.2H₂O, 1.9; glucose, 5.5; KCl, 5.9; MgSO₄.7H₂O, 0.5; NaCl, 138; NaHCO₃, 6; NaH₂PO₄.2H₂O, 0.5; pH, 7.4), bubbled with O₂ (pH, 7.4) under a 2 g passive tension and equilibrated for a period of 90 min. The tissue was washed every 15 min interval and isotonic contractions were recorded in the kymograph. After the equilibration period of 90 min, the effect of cromakalim and pinacidil was examined on spontaneous contractions and contractions induced by low KCl and ACh on the detrusor muscle. Similarly, to assess the effect of KATP channel modulators (cromakalim, pinacidil and glibenclamide) on neurogenic response, the detrusor muscle strip was suspended in between two parallel electrodes and electrically stimulated (30 V, 5 ms) with a stimulator (Physiograph stimulator, Biodevices, India) at different frequencies (1, 2, 5, 10, 20 and 30 Hz). After taking control EFS-induced response, the tissues were exposed to KATP channel modulators for 5 min before taking subsequent response.

Statistical analysis—Results are given as mean ± S.E. Student’s t-test was employed to test for significance at the level of P<0.05. Inhibitory concentration (IC₅₀) or Effective concentrations (EC₅₀) values were calculated by regression analysis.

Results

Characteristics of spontaneous contractility in detrusor muscle strips obtained from the goat urinary bladder (GUB)—The goat detrusor muscle exhibited spontaneous contractility that was irregular in amplitude and frequency. A regional variation in the contractility was noted where strips taken away from the body portion exhibited an increased contractility in the strips towards the neck region and spontaneous activity was minimal in strips towards the apex region. In the present study detrusor strips from mid-region of the body of urinary bladder were taken for contraction studies.

Effect of cromakalim and pinacidil on the amplitude and rate of spontaneous contractions and blockade by glibenclamide—The spontaneity of detrusor muscle exhibited a mean amplitude and frequency, 11.99 ± 0.83 mm (n=10) and 1.37 ± 0.16/min (n=10), respectively. Cromakalim or pinacidil (10⁻⁷-10⁻⁴ M) added cumulatively, elicited a concentration-related inhibition of both amplitude and rate of spontaneous contractions. The complete inhibition of these responses was observed at 10⁻⁴ M for both these agents. The mean inhibitory
concentration-related contractile response curve of both cromakalim and pinacidil was shifted to right (Figs 1A, B, 2A, B and 3). The mean IC50 value for both amplitude and frequency for cromakalim was $3.3 \times 10^{-6}$ $M$ and $2.9 \times 10^{-6}$ $M$, respectively; and for pinacidil was $2.0 \times 10^{-5}$ $M$ and $1.5 \times 10^{-5}$ $M$, respectively. On comparison the mean IC50 of cromakalim was significantly ($P<0.001$) lower than the pinacidil.

Pre-incubation of the tissues with glibenclamide ($10^{-4}$ $M$), a $K_{ATP}$ channel blocker, the inhibitory effect of cromakalim on spontaneous contractile response was partially reversed with significant ($P<0.001$) increase in IC50 for both amplitude and frequency (Fig.1). But in the presence of glibenclamide, IC50 of pinacidil for both amplitude and frequency was not significantly altered as compared to control (Fig. 2).

**Effect of glibenclamide on the inhibitory effect of cromakalim and pinacidil on ACh-induced concentration-related contractile response**—ACh ($10^{-9}$–$10^{-4}$ $M$), added cumulatively elicited a concentration-related contractile response in the goat detrusor muscle strips. The threshold concentration and concentration for maximal response ($E_{max}$) were $10^{-8}$ $M$ and $10^{-4}$ $M$, respectively. In the presence of atropine ($10^{-6}$ $M$), ACh mediated responses were almost abolished. There was a rightward shift of the ACh-induced concentration-related contractile response curve in the presence of either cromakalim ($10^{-4}$ $M$) or pinacidil ($10^{-4}$ $M$) (Fig. 4A and B). The
mean EC_{50} of ACh in presence of cromakalim (2.5 \times 10^{-3} M) or pinacidil (3.9 \times 10^{-6} M) were significantly (P < 0.001) increased as compared to the ACh control (1.2 \times 10^{-6} M). In presence of glibenclamide (10^{-4} M) the inhibitory effect of cromakalim and was significantly (P <0.001) reduced with consequent decrease in EC_{50} but potentiated the effect of pinacidil on the ACh response.

**Effect of EFS on goat detrusor muscle strips and sensitivity to atropine**—Application of EFS (30 V and 5 ms) on goat urinary bladder strips at 1, 2, 5, 10, 20 and 30 Hz caused frequency-related contractile responses (Fig. 5A and B). With relation to magnitude of contractile response, a delayed response was observed at 1 and 2 Hz with time to peak 28.0 ± 3.3 and 21.0 ± 0.8 sec, respectively and, quick response was observed at 5 and 10 Hz with time to peak 25.0 ± 2.9 and 28.0 ± 4.4 sec, respectively. In the presence of atropine (10^{-6} M), EFS-induced contractile response was marked inhibited.

**Effect of cromakalim and pinacidil on the EFS-mediated contractile response and reversal by glibenclamide**—Frequency-related contractile response curve was shifted to right in the presence of cromakalim with significant (P < 0.001) increase in the mean EF_{25} value (20.12 ± 2.96 Hz), as compared to control (1.03 ± 0.65 Hz). The comparison of EF_{25} was made as the mean EF_{Bmax} (41.0 ± 5.7%; n=6) was lower than 50% response. The inhibitory effect of cromakalim (10^{-4} M) on EFS-induced contractile response was reversed in the presence of glibenclamide (10^{-5} M) (n=6).
VIJAYKUMAR et al.: EFFECT K<sub>ATP</sub> CHANNEL OPNERS ON CONTRACTILE ACTIVITY

189

glibenclamide (10<sup>-4</sup>M), resulting in a leftward shift of the frequency-related contractile response curve (Fig. 5A). Similarly, in the presence of pinacidil (10<sup>-4</sup> M), the EFS-induced contractile response was shifted to right with significant (P < 0.001) increase in mean EF<sub>50</sub> value (17.26 ± 1.88 Hz), as compared to control (9.11 ± 0.45 Hz). In the presence of glibenclamide (10<sup>-4</sup> M) and pinacidil (10<sup>-4</sup> M), the frequency-related inhibitory response curve was shifted to left with significant (P < 0.001) increase in EF<sub>50</sub> (Fig. 5B).

Discussion

Both cromakalim and pinacidil caused a concentration-related inhibition of the spontaneous contractions of the goat detrusor strips with a maximal inhibition occurring at 100 μM. Similarly, at equi-active concentration, cromakalim exhibited about 40 and 50-fold more potency than pinacidil in depressing the amplitude and frequency, respectively. Our present finding is well in agreement with earlier findings on isolated guinea pig<sup>20,21</sup> and human detrusor muscles<sup>19,22</sup>. A high concentration of cromakalim (10<sup>-6</sup>–10<sup>-5</sup> M), abolished the spikes, as well as frequency and there was concentration-dependent hyper-polarisation of the cell membrane<sup>4,23</sup>. Spontaneous contractile activity was abolished in the present study which is in accordance with similar effects demonstrated with pinacidil on detrusor muscle<sup>24</sup>. Cromakalim opened a potassium channel having the property similar to ATP-dependent K<sup>+</sup> channels in vascular smooth muscle<sup>25</sup>. Supporting such a view, the relaxant effects of several potassium channel openers in the rat detrusor were antagonized by glibenclamide<sup>26</sup>. Grant and Zuzack<sup>27</sup> also demonstrated that cromakalim opens ATP-sensitive potassium channels in the guinea pig detrusor. Studies on isolated human detrusor muscle and on bladder tissue from several animal species have shown that K<sub>ATP</sub> channel openers reduce not only spontaneous contractions<sup>28</sup>, but also contractions induced by electrical stimulation, carbachol, and low, but not high external K+ concentrations<sup>29</sup>. Based on these findings, it is conceivable that (i) spontaneous contractions of goat detrusor smooth muscles is also sensitive to both the K<sub>ATP</sub> channel openers pinacidil and cromakalim and membrane hyperpolarization may be one of the major mechanisms of relaxation by these PCOs (ii) these PCOs exhibited a rank order of potency, cromakalim>pinacidil.

Glibenclamide, a sulfonylurea compound, has been reported to block ATP-sensitive K<sup>+</sup> channels in pancreatic β-cells, as well as other vascular and non vascular smooth muscles<sup>28,30-35</sup>. As observed in guinea pig<sup>18,25</sup>, rat<sup>26</sup> and human bladder<sup>7</sup>, the effect of cromakalim and pinacidil on detrusor muscle seems to be mediated by glibenclamide-sensitive potassium channels as glibenclamide was able to diminish the relaxant effect of both PCOs. In the goat bladder, the results obtained with cromakalim are in accordance with its well established K<sub>ATP</sub> channel activation proposed. But the result of the present study contains discrepancies with respect to pinacidil and glibenclamide inhibition. Therefore, this strongly suggested that cromakalim and pinacidil in goat bladder activate the types of K<sup>+</sup> channels that share same pharmacological properties with an ATP-
regulated potassium channel as identified in pancreatic β-cells. Glibenclamide-insensitive component of the relaxant effect of these PCOs may be due to a mechanism other than K\(^+\) channel opening as suggested in human bladder\(^7,19,26\), guinea pig bladder\(^{18,24,44}\), rabbit bladder, dog coronary arteries\(^{16,36}\) and rabbit portal vein\(^{17}\).

In muscarinic receptor stimulation studies, both cromakalim and pinacidil caused a clear cut rightward shift of the ACh-induced concentration-response curve elicited on normal goat detrusor muscle and further depressed the maximal response by 50 and 20\%, respectively. This observation is in accordance with the preliminary findings of Foster and Brading\(^{21}\) investigating the effect of cromakalim in normal pig bladder and pinacidil in human bladder\(^{19}\). The present results showed that cromakalim was more potent than pinacidil in goat detrusor muscle in inhibiting ACh-mediated responses, which is similar in rabbit but opposite in guinea pig urinary bladder with respect to carbachol-mediated response\(^6\). Malmgren \textit{et al.}\(^1\) found that pinacidil was more effective in reducing the micturition pressure than cromakalim in rat bladder. In the presence of glibenclamide, cromakalim-induced rightward shift of concentration-response curve to ACh was significantly reversed with consequent reduction of EC\(_{50}\) by 132-fold. In contrast, glibenclamide caused a further shift of the contractile response curve to ACh in the presence of pinacidil though ACh-induced concentration related contractile response curve did not change significantly. In goat urinary bladder, the potentiation of relaxing effect of pinacidil by glibenclamide cannot be interpreted with the available information. A significant relaxant effect has been observed on ACh-induced contractile response by higher concentration of cromakalim and pinacidil. The inhibition of agonist-induced contraction by the PCOs under study has been believed to be due to opening of potassium channels and subsequent hyperpolarisation of cells of rat\(^1\) and guinea pig urinary bladder\(^4,37\).

It was shown in the present study that the frequency-related contractile response induced by electrical field stimulation at 1–30 Hz in the isolated detrusor muscle was inhibited by atropine at all frequencies with a maximal inhibition (80\%) occurring at 20 Hz. Thus, an atropine resistant component ranging from 18-22\% over 1-30 Hz was observed. It has been known that there is an atropine-resistant component in contraction induced by parasympathetic or transmural nerve stimulation in the bladder of several species\(^{38,41}\) and stimulation at low frequencies showing greater resistance to atropine. Hence the cholinergic component of contraction appears markedly as the stimulation frequencies are increased and contractile responses to low frequencies which might be mediated by primarily ATP. In most mammalian species, part of the bladder contraction induced by electrical stimulation of nerves is resistant to atropine\(^{29}\). The proportion of ATP-mediated response to the total contraction varies with species and the frequency of stimulation. Thus, in bladder strips from rats and guinea pigs, atropine had little effect on the response to single nerve stimuli, but at 20 Hz, 75\% of the response was resistant to atropine. In bladders from rabbits, mice, and pigs, 60, 70, and 25\%, respectively, was resistant to atropine\(^{42,43}\). Based on the above findings, the contractile response induced by EFS in isolated goat detrusor smooth muscle obtained here consists of cholinergic and ATP (about 20\%) component.

In goat detrusor smooth muscle strips, we observed that the maximum response to electrical field stimulation was depressed and that there was a rightward shift in the frequency-response curve in the presence of cromakalim and pinacidil. Cromakalim exhibited about 2.5-fold more inhibition than pinacidil at EF\(_{25}\). This result was similar to that of rabbit bladder and opposite to guinea pig detrusor muscle\(^6\). In the presence of glibenclamide, the relaxant effect of cromakalim and pinacidil on EFS-induced contractile response was diminished partially and completely, respectively.

In this tissue it is well conceivable that this relaxant effect of cromakalim and pinacidil is consistent for spontaneous contractions and contractions induced by ACh and EFS, but a significant variability was observed with blockade by glibenclamide while examining the sensitivity of K\(_{ATP}\) channels to this PCO blocker. The partial sensitivity to glibenclamide could be due to denudation of detrusor that results in impairment of either NO-c-GMP-PKG pathway contributing activation of K\(_{ATP}\) channels as observed in guinea-pig urinary bladder myocyties\(^{44}\), or metabolic stress\(^{45}\) and decreased intracellular ATP\(^{46}\). Similarly, other electrophysiological studies employing patch-clamp techniques revealed that intracellular acidosis in follicular cells\(^{47}\) and alteration of cytosolic factors in pig urethral tissues\(^{47}\) resulted in
different glibenclamide-sensitivity of ATP-sensitive K⁺ currents.

Molecular basis of K<sub>ATP</sub> channels in different tissues indicates that there is functional diversity which results from cell-specific expression of different subunit proteins. Cloning of K<sub>ATP</sub> channels has resulted four pore-forming, inwardly rectifying channel subunits (Kir6.x) and four modulatory sulphonylurea receptor subunits (SUR.x) that are members of the ATP-binding cassette (ABC) super-family of proteins. To date, two Kir6 isoforms, Kir6.1 and Kir6.2, and two SUR isoforms, SUR1 and SUR2, have been identified. It is well accepted that different combinations of Kir6.x and SUR.x isoforms/variants yield tissue-specific K<sub>ATP</sub> channel subtypes with different features and distinct functional properties. For instance, Kir6.1–SUR2B in murine colon, Kir6.1–Kir6.2–SUR2B in guinea-pig stomach, Kir6.2–SUR1 in pancreatic β cell, Kir6.1 and Kir6.2–SUR1 and SUR2B in pig urethra and Kir6.2–SUR2A cardiac cells further support that various transcripts do exist in different cell types which are responsible for functional variation of K<sub>ATP</sub> channels. RT-PCR studies suggest that the Kir6.1–SUR2B channel is likely to be the predominant isoform of native K<sub>ATP</sub> channel in some smooth muscles and the molecular properties of native K<sub>ATP</sub> channels are due to expression of more than one type of K<sub>ATP</sub> channel in smooth muscle under study. These results suggest that multiple types of native K<sub>ATP</sub> channels exist in different species and types of smooth muscle and that mixed populations of Kir6.x and SURs subunits form hybrid K<sub>ATP</sub> channels which form the basis of a consistent variability in regulation of K<sub>ATP</sub> channels by sulphonylurea receptor ligands. Thus, the variability in glibenclamide sensitivity observed in goat detrusor could further be analyzed employing electrophysiological and molecular biological techniques.

References

6. Rizk D E, Arafat K, Ki-Sharkawy T Y, Comparison of the inhibitory effect of cromakalim and pinacidil (PCOs), with those oxybutynin on stimulated guinea pig and rabbit detrusor muscle strips, Arch Gynecol Obstet, 265 (2001) 145.


