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POTASSIUM CHANNELS OPENER PINACIDIL HAVE MULTIPLE EFFECTS ON KCl-ELICITED CONTRACTIONS OF ISOLATED NON-PREGNANT RAT UTERUS

NOVAKOVIĆ RADMILA*, MILOVANOVIĆ S**, ĆUPIĆ V*** and GOJKOVIĆ-BUKARICA LJILJANA*

*University of Belgrade, Faculty of Medicine, Serbia
**University of East Sarajevo, Faculty of Medicine, Foca, Federation BH
***University of Belgrade, Faculty of Veterinary Medicine, Serbia

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The effects of K^+ channel opener, pinacidil on contractions provoked by contraction-stimulating KCl were investigated on isolated uterus of non-pregnant rats in oestrus. Pinacidil produced a more potent inhibition of 20 mM KCl–elicited contractions (pD_2 = 6.57 μM) than of 40 or 80 mM KCl–elicited contractions (pD_2 = 5.11 and 5.19 μM, respectively). Glibenclamide, a selective blocker of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels, antagonized the pinacidil-induced inhibition of contractions elicited by 20 mM KCl in a competitive manner. However, the pinacidil-induced inhibition of contractions provoked by 40 and 80 mM KCl glibenclamide was unable to prevent them. Pinacidil ability to completely relax the non-pregnant uterus pre-contracted with K^+-rich solution suggests that K^+ channel-independent mechanism(s) also plays a part in its relaxant effect.

Key words: high K^+ solution, K_{ATP} channels, pinacidil, rat uterus

INTRODUCTION

Contractions of smooth muscles are regulated by the intracellular Ca^{2+} level, and the sensitivity to Ca^{2+} of the contractile elements in response to changes in the environment surrounding the cell. Uterine contractile activity is determined by the increase in intracellular free Ca^{2+} concentration in the myometrial cells. Potassium channels (K^+ channels) activation has an inhibitory effect on uterine contractile activity through hyperpolarization by changing the membrane potential away from the threshold required to generate an action potential of myometrial cells.

The numerous physiological mechanisms that control uterus contractility by involving the modulation of ion currents have led to the elaboration and investigation of various therapeutic methods.

Pinacidil is an antihypertensive agent, which can relax various smooth muscles (Davies *et al*., 1996; Gojkovic and Kazic, 1999; Gojkovic-Bukarica *et al*., 2010), including the animal (Piper *et al*., 1990; Mandi *et al*., 2005; Novakovic *et al*., 2007) and human uterus (Morrison *et al*., 1993, Khan *et al*., 1998). The mechanism

of action has not yet been fully established, but has been named that relaxation responses are associated with the opening of K^+ channels and characterized by its ability to cause cell hyperpolarization by primarily increasing potassium ion permeability (Bray *et al*., 1987).

The aim of this study was to determine the relaxant properties of pinacidil on contractions provoked with contraction-stimulant KCl on the isolated uterus of non-pregnant rats and their sensitivity to glibenklamide, a potent blocker of adenosine 5'-triphosphate – sensitive potassium channels (K_{ATP}).

MATERIALS AND METHODS

General Methods or Tissue Preparation

Experiments were carried out on virgin female Wistar rats weighing 200 – 250 g. This investigation conforms to the principles outlined in the "Good Laboratory Practice" and was approved by the Medical Ethics Committee of the Military Medical Academy. Rats were pretreated 24h before the experiment with 17β-oestradiol benzoate (100 μg/kg, i.p.) according to the method of Hughes and Hollingsworth (1995). Uterine horns were cut into longitudinal segments approximately 1 cm long and mounted in a 10 mL organ chamber containing PSS. The temperature in the organ bath was maintained at 30°C and the solution was continuously aerated with 95% O_2 and 5% CO_2 (pH~7.4). Strips were equilibrated at passive tension of 1 g for 1h. Isometric tension was measured with isometric force transducer "K 30, Hugo Sachs" (Freiburg, Germany) and recorded on a 2-channel recorder "R60, Rikadenki" (Tokyo, Japan). The mechanical responses were measured as integrated tension by the method of Granger *et al.* (1985).

Experimental procedure

After equilibration, uterus strips were stimulated with KCl (20, 40, 80 mM) to induce contraction and allowed a 60 min period to assess the control contractile performance. On each strip only one KCl concentration was tested. KCl remained in contact with the preparation until the plateau of contraction was reached and after reaching the plateau the tissue was washed. After further 5 min the process was repeated and the cumulative concentration-response curve to pinacidil was obtained by adding increasing logarithmic molar concentrations (10 nM - 0.1 mM). Subsequent concentrations were added to the organ bath after the previous concentration had produced its equilibrium response or after 10 min if no response was obtained. Relaxation produced by each concentration of pinacidil was measured and expressed as a percentage of the maximum possible relaxation (i.e., relaxation back to the baseline tension). Experiments followed the multiple curve design. The strips were washed and allowed to return to control contractions elicited by KCl.

In separate experiments, after twitch responses became consistent, glibenclamide was added into the bathing solution, at least 20 min before exposure to pinacidil. Pinacidil was reintroduced into the bath and the concentration-effect values were obtained by the same procedure as before.

Vehicle- and time-matched control experiments were done.

Drugs and solutions

The following drugs were used: pinacidil monohydrate (Leo Pharmaceuticals) and glibenclamide, KCl (Sigma Chemical Co., St. Louis, MO, USA). Stock solution of pinacidil was dissolved in dilute acid solution (0.1 N HCl) to make a stock solution of 100 μM with a further dilution in PSS. Glibenclamide was dissolved in polyethylene glycol. KCl was dissolved in distilled water. Where KCl was used as the spasmogen the stated concentration excludes KCl present in PSS. PSS had the following composition (in mM): NaCl 137, KCl 5.36, $CaCl_2 \cdot 2H_2O$ 0.41, $MgCl_2 \cdot 6H_2O$ 0.19, Na_2HPO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.04. All drugs were added directly to the bath in a volume of 100 μL and the concentrations given are the calculated final concentrations in the bath solution.

Analysis of data

EC_{50} value is defined as the concentration of pinacidil required to produce 50% of the maximum response of KCl-elicited contractions, and it was determined for each curve by using a non-linear least square fitting procedure of the individual experimental data, and presented as pD_2 (pD_2=-log EC_{50}). The results are expressed as the mean ± standard error of the mean (S.E.M.); *n* refers to the number of trials. Statistical difference between means was determined by one-way ANOVA and Student's *t*-test, a value of $p<0.05$ was considered statistically significant. All calculations were done by using the computer program Graph Pad Prism (Graph Pad Software Inc., San Diego, USA).

RESULT

Application of KCl (20, 40, 80 mM) caused a rapid, phasic contraction followed by a prolonged sustained plateau (tonic component) (Fig. 1A-C).

Pinacidil (10 nM – 0.1 mM) significantly induced a concentration-dependent relaxation ($p<0.05$) of the spasm evoked by 20 mM KCl with pD_2 value of 6.57± 0.2 μM (maximal response 100 ± 0 %, n = 7). Glibenclamide (1 - 10 μM) produced a significant rightward shift (pD_2 value of 5.68 ± 0.3 in the presence of 10 μM glibenclamide, $p<0.01$, n=5) of the concentration-response curve to pinacidil in a concentration-dependent manner, without suppression of the maximal response ($p>0.05$) (Fig. 2A).

Pinacidil (10 nM – 0.1 mM) inhibited KCl (40, 80 mM) induced contractions in a concentration-dependent manner with pD_2 values of 5.11 ± 0.2 μM and 5.19 ± 0.2 μM respectively (maximal inhibition of 100 ± 0 % and 97.20 ± 2.3 %, n = 5, 6). The administration of glibenclamide (1 - 10 μM) did not produce a significant shift to the right ($p>0.05$) of the concentration-response curve for pinacidil (pD_2 values: 5.40 ± 0.2 μM in the presence 10 μM glibenclamide on 40 mM KCl-elicited contractions, and 5.26 ± 0.5 μM on 80 mM KCl-elicited contractions, respectively, $p>0.05$, n = 7, 8) (Fig. 2B, 2C).

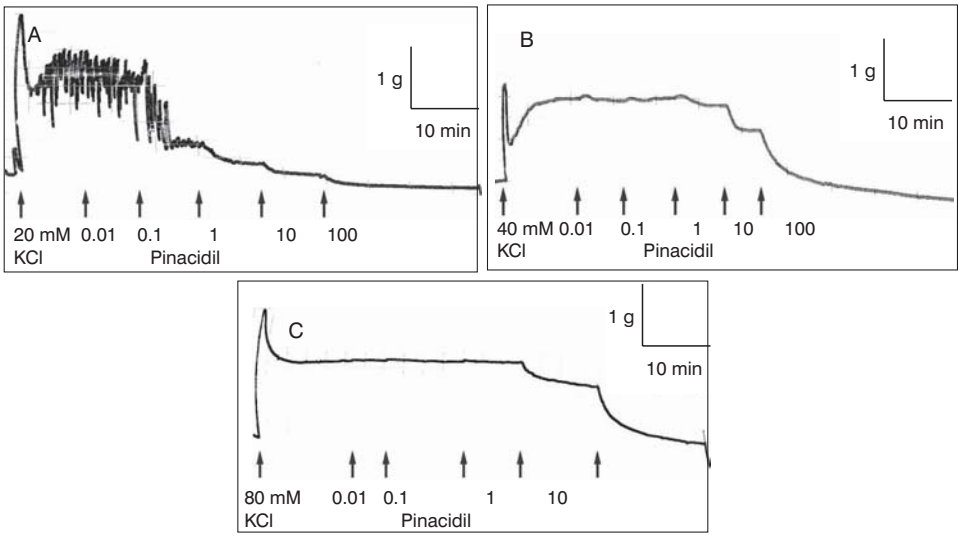

Figure 1. Original recordings show the typical effects of pinacidil on contractile activity of the non-pregnant rat myometrium: (A) contractions provoked by 20 mMKCl; (B) contractions provoked by 40 mM KCl; (C) contractions provoked by 80 mM KCl. The concentration of pinacidil is expressed as μM

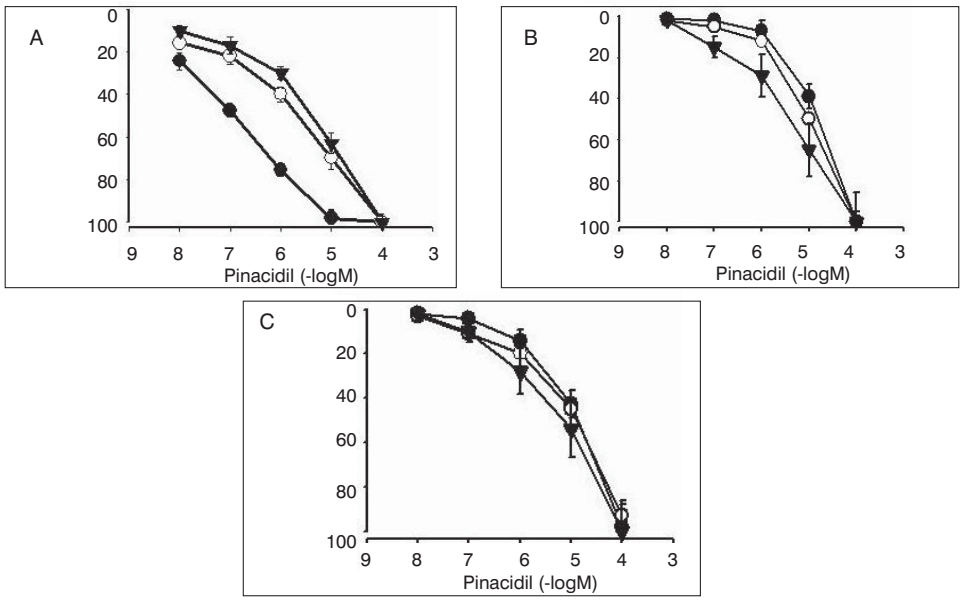

Figure 2. Effect of pinacidil on tension development in the isolated uterus of the non-pregnant rat treated with KCl 20 mM (A), 40 mM (B), 80 mM (C). Effects are shown in the absence (●) and in presence of glibenclamide (1 μM, ○; 10 μM ▼). Responses are expressed as a percentage of the maximum possible relaxation. The points are the means and the vertical lines show the s.e.means (n = 7-10)

Glibenclamide (10 μM) had no effect on the resting tone of the preparations or on contractions elicited by KCl (percent of contractions were: 105.0 ± 8.8% in the absence and 104.6 ± 10.5% in the presence of glibenclamide, $p>0.05$, $n = 4$, data not shown).

DISCUSSION

Pinacidil and other potassium channel openers open K^+ channels, hyperpolarize the membrane, inhibit Ca^{2+} influx, decrease cytosolic Ca^{2+} level and inhibit contractions (Gojkovic-Bukarica *et al*., 2010; Itoh *et al*., 1995; Novakovic *et al*., 2007). In opposite, all spasmogenic response to KCl can be explained by Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Edvards *et al*., 1986). In the present study we partly confiremed former reports.

Pinacidil inhibits 20 mM KCl-elicited contractions of smooth muscles of the rat uterus in a concentration-dependent manner with a potency $pD_2 = 6.57$. This value is higher then obtained for the canine mesenteric artery (5.88; Masuzawa *et al*., 1990) and is similar to those reported for the intestinal smooth muscle (6.19; Davies *et al*., 1996) and guinea-pig pulmonary artery (6.12; Eltze, 1989). Addition of 20 mM KCl to the extracellular compartment, giving rise to an extracellular potassium concentration that would result in increasing the membrane potential (–59 to –46 mV) (Morrison *et al.,* 1993). Accordingly, it may be concluded that 20 mM KCl induced membrane depolarisation increased $[Ca^{2+}]_i$ (Trujillo *et al.,* 2000) whereas membrane hyperpolarization induced by pinacidil decreased the $[Ca^{2+}]_i$ available for contraction (Wray *et al*., 2007).

Glibenclamide is a well documented and potent blocker of the K_{ATP} channel and a large number of pharmacological studies have involved the use of glibenclamide to antagonize the relaxant effects of K^+ channel openers (Jovanovic *et al*., 1994). In the present study glibenclamide was found to produce a significant rightward shift in a concentration-dependent manner, with no suppression of the maximum of the concentration-response curves for pinacidil. The obtained affinity of glibenclamide indicates that pinacidil has an action involving glibenclamide-sensitive, K_{ATP} channel in rat uterus.

It has been shown that the equilibrium potential for potassium (– 31 mV) could be obtained by addition of ≥ 40 mM KCl (Morrison *et al.,* 1993). Unexpectedly, pinacidil reduces uterine spasm elicited by 40 and 80 mM KCl concentrations with low potency (5.11, 5.19) suggesting a mechanism of action other than K^+ channel opening. Similar data is published for rat uterus (Piper *et al*., 1990), canine mesenteric artery (Masuzawa *et al*., 1990), rabbit aorta (Cook, 1989), guinea-pig trachea (Nielsen-Kudsk, 1988) and for the human internal mammary artery (Gojkovic *et al*., 1997). In contrast, it has been shown that in the human non-pregnant (Kostrzewska *et al*., 1996) and pregnant myometrium (Morrison *et al*., 1993) pinacidil did not inhibit contractions provoked by high K^+ (>40 mM). The reason for this might be the lower concentrations of pinacidil (0.01 - 10 μM) used in this study. The fact that glibenclamide (10 μM) was unable to prevent the inhibition of KCl contractions (>40 mM) induced by 100 μM of

pinacidil indicated the presence of an additional K_{ATP} channel - independent mechanism(s) of pinacidil action. Previously it has been showed on vascular smooth muscle that relaxant response to pinacidil have multiple sites of action: indirecly by reducing neurotransmitter release (Quast, 1993), by interaction with intracellular Ca^{2+} stores (Erne and Hermsmeyer, 1991; Greenwood and Weston, 1993), by inhibition of the receptor - mediated GTP binding protein - coupled Ca^{2+} sensitization (Anabuki *et al*., 1990), by inhibition of inositol-1,4,5-triphosphate (IP_3) syntheses (Itho *et al*., 1990). Interestingly, Trujillo *et al*. (2000) showed in the rat uterus, that high K^+ solutions in addition to their well known effect on Ca^{2+} influx, activate other cellular processes like increased total IP_3 acumulation. This is in agreement with the present results and suggest that pinacidil has dual effects on rat uterine smooth muscle contractions: to decrease intracellular Ca^{2+} by activating K^+channels and perhaps decrease IP_3 syntheses. Thus, the relaxation of uterine smooth muscle related to reduction of intracellular Ca^{2+} produced by pinacidil is due to hyperpolarization of the plasma membrane resulting in not only the closure of voltage-dependent Ca^{2+} channels, but also in the inhibition of production of IP_3 and Ca^{2+} release from intracellular stores.

The present data show that pinacidil exhibits potent relaxant properties in the rat non - pregnant uterus in oestrus and confirm the possibility that this agent may possess therapeutic potential in the treatment of motility disorders. Further, the results on the basis of glibenclamide affinity, are consistent with the existence of a glibenclamide – sensitive K_{ATP} channel in the rat uterus. The observations that pinacidil has additional, K^+ channel-independent mechanism(s) of action on contractions elicited by high K^+ solutions, need further evaluation.

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Address for correspondence:
Ljiljana Gojkovic-Bukarica, M.D., PhD, Associate Professor of Pharmacology
Department of Clinical Pharmacology, Pharmacology and Toxicology
School of Medicine, University of Belgrade
11129 Belgrade, Serbia
E-mail: bukarica@rcub.bg.ac.rs

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EFEKTI PINACIDILA - OTVARAČA KALIJUMOVIH KANALA NA KONTRAKCIJE IZOLOVANOG NEGRAVIDNOG UTERUSA PACOVA IZAZVANE KALIJUM HLORIDOM

NOVAKOVIĆ RADMILA, MILOVANOVIĆ S, ĆUPIĆ V i GOJKOVIĆ-BUKARICA LJILJANA

SADRŽAJ

U ovom radu su prikazani efekti pinacidila, koji ima osobinu da otvara kalijumove kanale, na kontrakcije izazvane kalijum hloridom na modelu izolovanog negravidnog uterusa ženki pacova tokom estrusa. Pinacidil dovodi do snažnije inhibicije kontrakcija izazvanih sa 20mM KCl ($pD_2 = 6.57\ \mu M$) u poređenju sa kontrakcijama izazvanim sa 40 ili 80 mM KCl ($pD_2 = 5.11$ i $5.19\ \mu M$). Poznato je da je glibenclamid selektivni blokator adenozin-3-fosfat senzitivnih K^+ (K_{ATP}) kanala antagonizuje pinacidilom indukovanu kompetitivnu inhibiciju kontrakcija izazvanih pomoću 20 mM KCl-a. Međutim, pinacidilom indukovana inhibicija kontrakcija, izazvanih sa 40 i 80 mM KCl-a nije se mogla prevenirati glibenclamidom. Sposobnost pinacidila da dovede do potpune relaksacije negravidnog uterusa ženki pacova pre kontrakcije izazvane rastvorom kalijuma ukazuje na to da u relaksaciji učestvuje i mehanizam nezavisan od kalijumovih kanala.