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The Effect of Potassium Channel Opener Pinacidil on the Non-Pregnant Rat Uterus

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Abstract: The effects of the K^+ channel opener, pinacidil on the spontaneous rhythmic contractions and contractions provoked by electrical field stimulation (50 Hz) or by oxytocin were investigated in the isolated uterus of the non-pregnant rat in oestrus. Pinacidil produced more potent inhibition of oxytocin-elicited contractions than of spontaneous rhythmic contractions or electrical field stimulation-induced contractions. Glibenclamide, a selective blocker of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels, antagonized the pinacidil-induced inhibition of contractions elicited by oxytocin in a competitive manner. However, the pinacidil-induced inhibition of electrical field stimulation-elicited contractions and spontaneous rhythmic contractions was antagonized non-competitively by glibenclamide. In the uterine strips pre-contracted with 80 mM K^+, the pinacidil-induced maximal relaxation was not affected. The present data show that pinacidil exhibits potent relaxant properties in the rat non-pregnant uterus in oestrus and therefore should be taken into account as a possible agent for treatment of dysmenorrhoea. Based on glibenclamide affinity, it appears that the inhibitory response to pinacidil involves K_{ATP} channels. We need further investigations to explain why the interaction between glibenclamide and pinacidil in this experimental model depends on the nature of contractions. The ability of pinacidil to completely relax the rat non-pregnant uterus pre-contracted with K^+-rich solution suggests that K^+ channel-independent mechanism(s) also play a part in its relaxant effect.

It is generally accepted that primary dysmenorrhoea during menstruation is a consequence of excessive uterine activity. Some observations suggest that dysmenorrhoea is a repercussion of decreased uterine blood flow caused by the hypercontractility of the uterus muscle and local contractions of the uterine vessels [1]. The mechanism that triggers uterine spasm is currently unclear, although different factors or events seem to be involved. The control of uterine contractility is modulated by various endogenous stimuli, including neurotransmitters, and polypeptide hormones, particularly oxytocin. In addition, spontaneous depolarization of the membrane of uterine smooth muscle cells and activity of pacemaker cells leads to contractions and maintenance of spontaneous contractile activity that may be involved in the uterine spasm [2]. The ideal agent for prevention and treatment of uterine spasm has not yet been found. Cyclooxygenase inhibitors are the mainstay of treatment but they are not sufficiently efficacious in all patients [3].

Pinacidil is a member of the K^+ channel opener group that is a structurally heterogeneous group of compounds that relax smooth muscle by activation of K^+ channels in the plasmalemma. Previously we have shown that pinacidil induces relaxation of the isolated human uterine artery by activation of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels in the vascular smooth muscle [4]. As well, it has been suggested that pinacidil inhibited smooth muscle contractions of the human pregnant uterus probably by activation of plasmalemmal K_{ATP} channels [5]. However, in the same experimental model, Khan et al. [6] demonstrated that pinacidil activated the large conductance Ca^{2+}-dependent K^+ channels. The effect of pinacidil on the non-pregnant uterus is still not well defined.

Taking into consideration that the search for drugs capable of modifying uterine contractility is warranted, the present study was designed to examine the inhibitory effects of pinacidil on the contractility of the non-pregnant rat uterus, and to define the contribution of K_{ATP} channels in this pinacidil action.

Materials and Methods

Experiments were carried out on virgin female Wistar rats weighing 200–250 g. The study was conducted in accordance with the ethical standards in Directive 86/609/EEC, 'European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes', 1986, and with the 'Guiding Principles in the Use of Animals in Toxicology', adopted by the Society of Toxicology in 1989. The rats were pre-treated 24 hr before the experiment with 17β-oestradiol benzoate (100 μg/kg, subcutaneously) according to the method of Hughes and Hollingsworth [7]. Uterine horns were cut into longitudinal segments of approximately 1 cm length. Strips were mounted for isometric recording under 1 g tension in physiological salt solution (PSS) maintained at 37°C, gassed with 95% O_2 and 5% CO_2 and equilibrated for 1 hr. Isometric tension was measured with isometric force transducer 'K30, Hugo Sachs' (Freiburg, Germany) and recorded on a 2-channel recorder 'R60, Rikadenki' (Tokyo, Japan).

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Experimental procedures.

Spontaneous rhythmic contractions and contractions elicited by oxytocin or KCl. After equilibration, some preparations (32%, 24/75 preparations) had spontaneous rhythmic contractions. When the contractions reached a steady state (for nearly 60 min.), a cumulative concentration–response curve to pinacidil was obtained by adding increasing logarithmic molar concentrations (10 nM–0.1 mM). A subsequent concentration was added to the organ bath after 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 a multiple curve design.

In a separate series of experiments, uterus strips were stimulated with oxytocin or KCl to induce contractions and allowed for 30 min. period to assess control contractile performance, and then pinacidil was added to the bath cumulatively.

In order to test the involvement of K_{ATP} channels in a mechanism of action of pinacidil, a highly selective potassium channel blocker, glibenclamide was used according to the following protocol: (i) concentration–response curve to pinacidil, followed by three washes, addition of glibenclamide, and 20 min. equilibration period and (ii) concentration–response curve to pinacidil in the presence of glibenclamide.

Contractions of the rat uterus evoked by electrical field stimulation. After equilibration, isometric contractions were elicited by EFS (50 Hz, with square wave pulses of 0.3 msec. duration and supramaximal voltage) delivered through two parallel platinum wire electrodes from a 'Grass S44' electronic stimulator. The preparations were allowed to stabilize for at least 30 min. until twitch responses became consistent, before addition of drugs. Concentration–response curve was constructed by addition of pinacidil to the bathing solution directly in a cumulative way, taking the amplitude of response measured immediately before the addition of a drug as a control (100%). Higher concentrations of pinacidil were added only when the previous concentration has produced its equilibrium response. In separate experiments, after twitch responses became consistent, glibenclamide was added into the bathing solution, at least 20 min. before exposure to pinacidil.

In order to confirm that electrical field stimulation-induced contraction is mediated by neurotransmitter release from cholinergic nerve endings, tetrodotoxin (1 μM) or atropine (1 μM) was added into the bath, 20 min. before applying electrical field stimulation. Vehicle- and time-matched control experiments were done.

In experiments where contraction was evoked by oxytocin or electrical field stimulation, the strips that exhibited spontaneous rhythmic contractions were eliminated from this study. The reason for eliminating these preparations from further study was overlapping of their contractions and contractions induced by oxytocin or electrical field stimulation that make necessary measurements impossible.

Drugs and solutions. The following drugs were used: pinacidil monohydrate (Leo Pharmaceuticals, Copenhagen, Denmark); glibenclamide, oxytocin, tetrodotoxin, atropine and 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 further dilution in PSS. Glibenclamide was dissolved in polyethylene glycol. Oxytocin was dissolved in distilled water. The PSS had the following composition (in mM): NaCl 137, KCl 5.36, $CaCl_2$ $2H_2O$ 0.41, $MgCl_2$ $6H_2O$ 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.

Treatment of data and statistics. EC_{50} value is defined as the concentration of pinacidil required to produce 50% of the maximum response of elicited and spontaneous 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 means ± standard error of the mean (S.E.M.); n refers to the number of experiments. Statistical difference between means was determined by one-way ANOVA and Student's t-test, a P-value of <0.05 was considered statistically significant. The calculation of pA_2 values for glibenclamide was determined from a Schild plot [8] where $\log_{10}$ (DR-1) was plotted against the $\log_{10}$ molar concentration of glibenclamide. DR is the dose ratio for each concentration of the antagonist. The slope of the plotted regression line was calculated and used to assess the nature of antagonism. The significance of the Schild plot linearity was tested by ANOVA. The closeness of the slope to unity was tested by Student's t-test and was not considered different from unity if $P > 0.05$. The frequency of spontaneous rhythmic contractions and oxytocin-induced contractions was calculated as a number of cycles in a 10-min. period of time. All calculations were done by using the computer program Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA).

Results

Effects of pinacidil on the spontaneous rhythmic contractions. Longitudinal muscle strips of the rat uterus exhibited spontaneous rhythmic contractions of constant amplitude 1.29 ± 0.08 g and frequency 8.10 ± 1.90 mHz (fig. 1A). Pinacidil (10 nM–0.1 mM) inhibited spontaneous rhythmic contractions in a concentration-dependent manner with a pD_2 value of 5.96 ± 0.2 μM (maximal response 98.4 ± 0.8%, n = 14) (table 1). Glibenclamide (3–10 μM) produced a significant rightwards shift of the concentration–response curve to

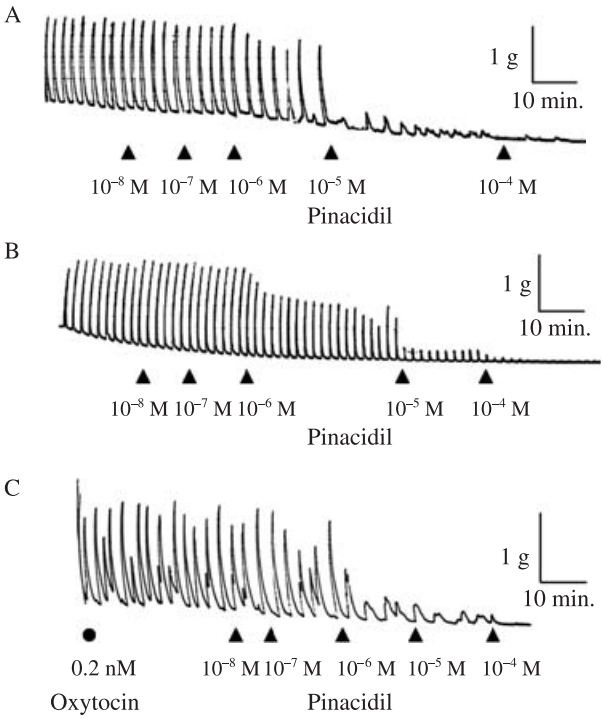

Fig. 1. Original recordings show the effects of pinacidil (closed triangle, 10 nM–0.1 mM) on contractile activity of the non-pregnant rat myometrium: (A) spontaneous rhythmic contractions, (B) contractions provoked by electrical field stimulation (50 Hz, square wave pulses of 0.3 msec. duration and supramaximal voltage) and (C) contractions provoked by oxytocin (closed circle, 0.2 nM).

Table 1.

The effect of pinacidil on spontaneous rhythmic contractions (SRC), contractions evoked by electrical field stimulation (EFS) and oxytocin-induced contractions of the isolated rat uterus in the absence and presence of glibenclamide (Schild analysis).

		Schild analysis for interaction with glibenclamide		
Myometrial contractions	pD_2 (mean ± S.E.M.)	*r*	slope	pA_2
SRC	5.96 ± 0.20^{a}	0.88***	$1.93 \pm 0.17^{\dagger}$	$5.59 \pm 0.22^{1,2}$
Induced by EFS	6.02 ± 0.20^{b}	0.97**	$0.58 \pm 0.15^{\dagger}$	5.92 ± 0.26^{1}
Induced by oxytocin	$6.52 \pm 0.08^{a,b}$	0.99*	0.94 ± 0.08	5.99 ± 0.09^{2}

$^{a,b}P < 0.01$, *P > 0.05, **P < 0.05, ***P < 0.01, $^{\dagger}P < 0.01$, $^{1,2}P < 0.05$; *r* and mean slope was compared to 1.

pinacidil ($P < 0.05$) in a concentration-dependent manner, without suppression of the maximal response ($P > 0.05$) (fig. 2A). The data from the experiments with glibenclamide were analysed as described by Arunlakshana and Schild [8] (fig. 2B, table 1). The experiments with glibenclamide did not yield a straight line ($r = 0.88$, $P > 0.05$). The mean slope was significantly different from unity (1.93 ± 0.17; $P < 0.01$) and the pA_2 value was 5.59 ± 0.22.

The effects of pinacidil on the contractions elicited by electrical field stimulation.

Preliminary experiments revealed that tetrodotoxin (1 μM, n = 4) or atropine (1 μM, n = 4) abolished (100%, both) the contractile responses to electrical field stimulation, indicating that these responses were mediated via stimulation of cholinergic nerves.

The amplitude of electrical field stimulation-elicited contractions was 1.43 ± 0.07 g (fig. 1B). Pinacidil (10 nM–0.1 mM) inhibited electrically induced contractions in a concentration-dependent manner with a pD_2 value of 6.02 ± 0.2 (maximal inhibition of $94.3 \pm 2.3\%$, n = 17). Glibenclamide (1–30 μM) was found to produce a significant rightward shift in a concentration-dependent manner ($P < 0.01$), without suppression of the maximum of the concentration–response curve for pinacidil (fig. 2C). When subjected to Schild analysis (table 1), the experiments with glibenclamide did not yield a straight line ($r = 0.97$; $P < 0.05$). The mean slope was significantly different from unity (0.58 ± 0.15; $P < 0.01$) and the pA_2 value was 5.92 ± 0.26 (fig. 2D).

The effects of pinacidil on the contractions elicited by oxytocin.

Application of oxytocin (0.2 nM) to the uterus strips produced regular phasic contractions of constant amplitude 1.50 ± 0.04 g and frequency 6.68 ± 0.85 mHz as shown in fig. 1C. Pinacidil (10 nM–0.1 mM) inhibited oxytocin-induced contractions in a concentration-dependent manner with pD_2 value of 6.52 ± 0.1 μM (maximal responses: $100 \pm 0\%$, n = 15) (table 1). The effects of glibenclamide (1–30 μM) on oxytocin-elicited contractions were concentration-dependent and induced a significant rightwards shift ($P < 0.01$) of the concentration-effect curve for pinacidil (fig. 2E) without suppression of the maximal pinacidil-induced responses ($P > 0.05$). When subjected to Schild analysis, the experiments with glibenclamide yielded a straight line ($r = 0.99$, $P > 0.05$) with a mean slope not significantly different from unity (0.94 ± 0.08; $P > 0.05$) and the following pA_2 value was thus obtained, 5.99 ± 0.09 (fig. 2F, table 1).

Glibenclamide in the applied concentrations did not alter the resting tone of the uterus and did not modify the spontaneous rhythmic contractions or contractions evoked by electrical field stimulation or oxytocin (n = 4 all, data not shown). The effect of pinacidil on electrical field stimulation- and oxytocin-induced contractions was partly reversible (after 30 min. of washing the contractions were <50% of control contractions).

The effects of pinacidil on the contractions elicited by KCl.

Pinacidil (10 nM–0.1 mM) caused potent inhibition of the contractions evoked by 80 mM of KCl, with pD_2 value of 5.19 ± 0.1 μM (maximal response $97 \pm 2\%$, n = 5) ($P < 0.05$) (fig. 3). This effect of pinacidil was insensitive to glibenclamide (30 μM) with pD_2 value of 5.26 ± 0.3 μM (maximal response $98 \pm 3\%$, n = 5) ($P > 0.05$).

Discussion

The pharmacology of pinacidil has been studied on a wide variety of smooth muscle preparations, including cardiac vascular, tracheal, bladder and neuronal preparations [4,9–11]. However, only few studies have shown that pinacidil induces relaxation of rat [12], goat [13] and human uterus [1,5,6]. Here, we compared the relaxant effect of pinacidil on the spontaneous rhythmic contractions, neurogenic contractions and contractions evoked by oxytocin in a non-pregnant rat uterus.

Pinacidil inhibited the oxytocin-induced contractions of uterus strips more potently than the spontaneous rhythmic contractions or electrical field stimulation-evoked contractions. The order of potency (pD_2) for pinacidil was: oxytocin (6.52) > electrical field stimulation (6.02) > spontaneous rhythmic contractions (5.96). Interestingly, the sensitivity of electrical field stimulation-induced contractions to pinacidil was comparable with the sensitivity of spontaneous rhythmic contractions to this drug. The complete inhibition of oxytocin-induced contractions was achieved with 10 μM of pinacidil compared to 10 times higer value obtained on the spontaneous rhythmic contractions or electrical field stimulation-evoked contractions. The potency of pinacidil obtained here was in

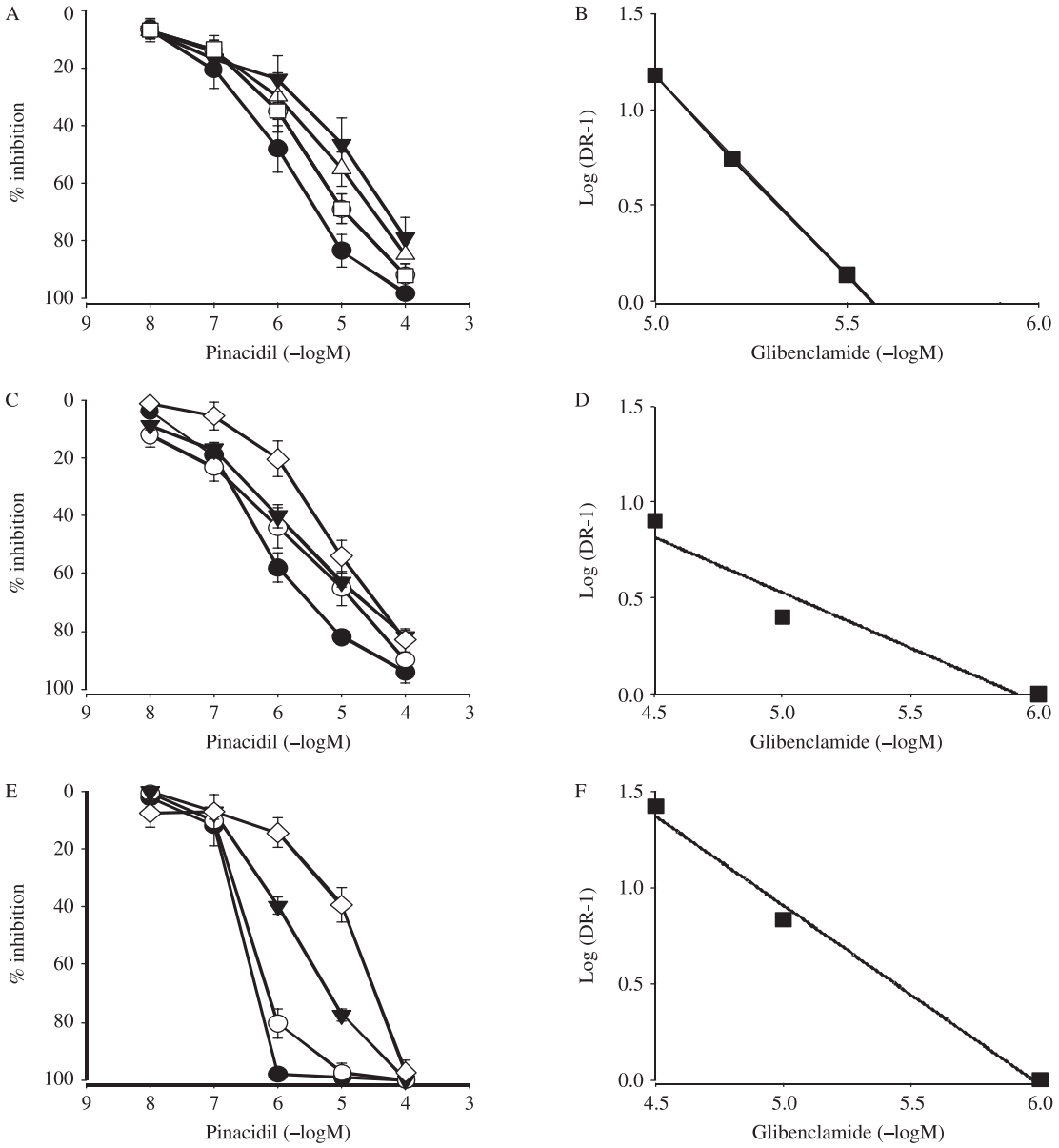

Fig. 2. Antagonism of the inhibitory effect of pinacidil by glibenclamide on tension development in the isolated uterus of the non-pregnant rat. Concentration–response curves for pinacidil on the spontaneous rhythmic contractions (SRC) (A), on the contractions provoked by electrical field stimulation (EFS) (C) and contractions evoked by oxytocin (E) in the absence (closed circle) and presence of glibenclamide (1 μM, open circle; 3 μM, open square; 6 μM, open triangle; 10 μM, closed triangle and 30 μM, open diamond). Schild plot of log (dose ratio-1) against –log (glibenclamide) for pinacidil-glibenclamid (closed square) antagonism on SRC (B), EFS- (D) and oxytocin-provoked contractions (F). The intercept on the abscissa scale gives the apparent pA_2 value; y = –1.93x + 11.10, $r = 0.88$ (B); y = –0.58x + 3.41, $r = 0.97$; (D) y = –0.94x + 5.6, $r = 0.99$ (F). The amplitude of contractions just before addition of pinacidil was taken as 100%. The points are the means and the vertical lines show the S.E.M. (n = 14–17).

good agreement with its potency for spontaneous rhythmic contractions and oxytocin-elicited contractions in a non-pregnant human uterus [5] and pregnant goat myometrium [13]. The pD_2 value for pinacidil obtained on spontaneous rhythmic contractions (5.96) in our study is lower than the pD_2 obtained on the non-pregnant (7.80) and pregnant (6.46) human myometrium [1,5]. The potency (pD_2) of pinacidil (6.52) against oxytocin-induced contractions is higher than that found for pregnant human myometrium (6.13, 6.40) [5,6]. These differences indicate species selectivity for pinacidil. In addition, in the same experimental model, potency of levromakalim (6.56) to inhibit the oxytocin-induced contractions was comparable with the potency of pinacidil found here [7].

Among the K^+ channel openers, the mechanism of action of pinacidil is not as well defined as for levcromakalim or nicorandil. Numerous investigators have suggested that the effect of pinacidil on the smooth muscle is mediated by K_{ATP}

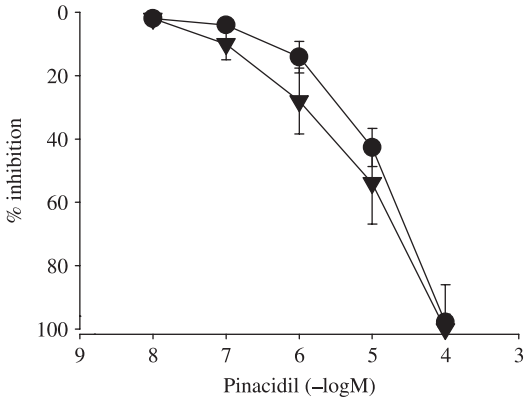

Fig. 3. Effect of pinacidil on tension development to 80 mM KCl in the isolated uterus of non-pregnant rats. Effects are shown in the absence (closed circle) and in the presence of glibenclamide (30 μM, closed triangle). Responses are expressed as a percentage of the maximum possible relaxation. The points are the means and the vertical lines show the S.E.M. (n = 5).

channels [9]. Glibenclamide is known to be one of the most selective blockers of K_{ATP} channels [14]. In the present study, glibenclamide reversed the inhibition induced by pinacidil on spontaneous rhythmic contractions and elicited contractions, in a concentration-dependent manner with potency comparable to those described in a various smooth muscles [10,11,15], including animal [12,13] and human uterus [1,5,6]. The obtained pA_2 value of 5.59 is consistent to those reported for K_{ATP} channels on the human internal mammary artery (5.46) [16]. The slope of the Schild plot for glibenclamide against pinacidil was different from unity indicating that the antagonism was not competitive. Accordingly, we confirmed that K_{ATP} channels are involved in the pinacidil effect on the spontaneous rhythmic contractions of rat uterus. However, the non-competitive nature of antagonism between glibenclamide and pinacidil suggested different binding sites or additional mechanism of pinacidil action [17]. Indeed, it hase been shown that big Ca^{2+}-activated K^+ (BK_{Ca}) channels have been implicated in the maintenance of the resting membrane potential, and possibly in the generation of spontaneous activity in smooth muscles [18]. Khan et al. have previously suggested that the relaxation of human pregnant uterus evoked by pinacidil may be at least in part due to activation of BK_{Ca} channels [19]. Further investigations have to be performed in order to define the role of those channels in the pinacidil action.

The contractions of the longitudinal muscle of the rat uterus in response to electrical field stimulation recorded in our experiments are neurogenic in nature, as this effect was abolished by tetrodotoxin. This result agrees with data of Houdeau et al. [20]. We have also found that atropine inhibited the rat uterus electrical field stimulation-induced contractions, suggesting that this response was mediated by acetylcholine release from cholinergic nerve ending that are dominant in the rat and human uterus [20,21]. The recent studies have shown that pelvic plexus neurones (i.e. the source for uterine parasympathetic nerve fibres) mainly project to the caudal region of the rat uterine horns, suggesting that cholinergic motor effects are predominant in the genital tract [22,23]. The obtained affinity ($pA_2 = 5.92$) of glibenclamide indicates that pinacidil induced inhibition of electrical field stimulation-elicited contractions probably by activation of K_{ATP} channels. Our observation are comparabile with data obtained on the electrical field stimulation-evoked contractions in the rat intestinal smooth muscle where pinacidil-induced inhibition was antagonized by glibenclamide [15]. The result that glibenclamide antagonized the pinacidil action on neurogenic contractions in a non-competitive manner is in accordance with the conception that pinacidil, at high concentrations (10 and 100 μM), have more than one binding site [24,25]. According to this, it seems that the pre-junctional action in addition to post-junctional consequence may be included in the inhibitory effect of pinacidil on the neurogenic contractions. Indeed, it has been reported that in airway smooth muscle preparations [26], in the rat urinary bladder [27], in the rat vas deferens [28] and in the rabbit portal vein [11], pinacidil inhibits release of neurotransmitters. In contrast, in the guinea-pig and rabbit mesenteric arteries, pinacidil and cromakalim had no effect on presynaptic neurotransmitter release [29,30]. However, these observations need further evaluation.

The slope of the Schild plot for glibenclamide against pinacidil on oxytocin-elicited contractions was not different from unity indicating that this antagonism was competitive. Our data agree with the observations of Morrison et al. [5] and Khan et al. [6] that revealed a competitive antagonism on the oxytocin-stimulated contractions in the human uterus. The pA_2 value for glibenclamide was similar to those found for K_{ATP} channels in the smooth muscle of human and rat uterus [5,12], and comparable to those obtained in other smooth muscle experimental models [4,10,11].

The concentration response curves for pinacidil obtained on spontaneous rhythmic contractions or contractions evoked by electrical field stimulation and oxytocin appear to show that there is significant relaxation evoked by high concentrations of pinacidil (>1 μM) that is residual to glibenclamide. To characterize this residual relaxation, experiments with high potassium were performed. The ability of high concentrations of pinacidil (10 and 100 μM) to reduce uterine contractions elicited by 80 mM of KCl suggested that pinacidil has an additional mechanism of action different from K^+ channel activation. The fact that glibenclamide (30 μM) was unable to prevent this effect of pinacidil further confirmed that K_{ATP} channels are not involved. Similar results were noted for the rat uterus [12], human internal mammary artery [16] and canine mesenteric artery [31]. In contrast, it has been shown that in the human pregnant [5] and non-pregnant myometrium [1], 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, and a species selectivity of pinacidil. Here, we have shown that the inhibitory effect of high concentration of pinacidil (100 μM) is preserved in any conditions and until

now, no one has offered an appropriate explanation for the mechanism of pinacidil action used in concentrations higher than 100 μM. Itoh et al. have shown that pinacidil directly inhibits contractile elements in rabbit mesenteric artery [25]. These results, including the results presented here, suggest that pinacidil has potassium channel-dependent, as well as potassium channel-independent mechanisms of action.

Thus, the present data show that pinacidil exhibits potent relaxant properties in the rat non-pregnant uterus in oestrus and therefore should be considered a possible agent for treatment of dysmenorrhoea. On the basis of glibenclamide affinity, it appears that the relaxant response to pinacidil involves smooth muscle K_{ATP} channels. However, we need further investigations to explain why the interaction between glibenclamide and pinacidil in this experimental model depends on the nature of contractions. Its ability to completely relax the rat non-pregnant uterus pre-contracted with K^+-rich solution suggests that K^+ channel-independent mechanism(s) also play a part in its relaxant effect.

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