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Drotaverine interacts with the L-type Ca^{2+} channel in pregnant rat uterine membranes

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Abstract

The effect of the isoquinoline derivative, drotaverine on the specific binding of [^{3}H]nitrendipine and [^{3}H]diltiazem to pregnant rat uterine membranes was examined. Drotaverine inhibited the specific [^{3}H]nitrendipine and [^{3}H]diltiazem bindings with IC_{50} values of 5.6 and 2.6 μM, respectively. Saturation studies showed that diltiazem caused a significant increase in the maximum binding density without changing the K_D of [^{3}H]nitrendipine while drotaverine increased both the K_D and the B_{max} of [^{3}H]nitrendipine. The dissociation kinetics of both [^{3}H]nitrendipine and [^{3}H]diltiazem were accelerated by drotaverine. These results suggest that drotaverine has a negative allosteric interaction with the binding sites for 1,4-dihydropyridines and 1,5-benzothiazepines on the L-type Ca^{2+} channel in pregnant rat uterine membranes, which may have implications as to the potential usefulness of this drug in aiding child delivery.

Keywords: Ca^{2+} channel, L-type; [^{3}H]Nitrendipine; [^{3}H]Diltiazem; Uterus, pregnant rat; Drotaverine

1. Introduction

Control of the smooth muscle in the uterus (myometrium) is of vital importance during pregnancy and parturition. Uterine muscle contractions can be stimulated by many endogenous and exogenous substances through various mechanisms (for review, see Wray, 1993). Uterine activation occurs late in pregnancy, which prepares it to perform the contractions typical of labor (Olson et al., 1995).

Increased intracellular calcium concentration is essential for triggering contraction in uterine smooth muscle cells (Inoue and Sperlakis, 1991; Taggart et al., 1996). A pivotal mechanism for increasing intracellular calcium concentration is through uterine voltage-dependent L-type Ca^{2+} channels (Young et al., 1993; Sanborn, 2000). Emerging evidence indicates the existence of gestational changes among the myriads of mechanisms recruited near term to coordinate Ca^{2+} signalling and force production needed for the development of effective uterine contractions, which lead to progressive dilatation of the uterine cervix and delivery of the fetus (Taggart et al., 1996). Involvement of L-type Ca^{2+} channels in regulation of these processes is most probable.

The L-type Ca^{2+} channels are multimeric protein complexes that are expressed in many different cell types including striated (skeletal and cardiac) and smooth muscle cells (Davila, 1999; Triggle, 1999). The α1 subunit is the principal subunit of the L-type Ca^{2+} channel and contains the voltage-gated channel pore. Six distinct genes have been identified encoding homologous α1 subunits. The "cardiac" (α1C-a) and the "smooth muscle" (α1C-b) proteins are splice products of the class-C Ca^{2+} channel α1 gene (Welling et al., 1997; Triggle, 1999; Davila, 1999). These α1C subunits are the major targets for all known Ca^{2+} channel blockers, such as dihydropyridines (e.g. nitrendipine, nifedipine, isradipine), benzothiazepines (e.g. diltiazem and clentiazem) and the phenylalkylamines (e.g. verapamil, desmethoxyverapamil). These L-type-selective Ca^{2+} channel antagonists bind to separate binding sites, which are allosterically linked by negative or positive heterotropic interactions (Catterall and Striessnig, 1992; Spedding and Paoletti, 1992; Hockerman et al., 1997; Varadi et al., 1999; Triggle, 1999). These types of allosteric interaction have been shown to be species, temperature, tissue, cation and ligand dependent. The α1C-a and α1C-b

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isoforms have different sensitivities to dihydropyridines displaying no clearcut differences in their electrophysiological properties (Davila, 1999).

L-type Ca^{2+} channel is present in both the non-pregnant and pregnant uterus but is up-regulated near term (Inoue and Sperlakis, 1991; Yoshino et al., 1997; Okabe et al., 1999). Ca^{2+} channel antagonists have been shown to inhibit uterine smooth muscle contractility both in vitro and in vivo; moreover, Ca^{2+} channel blockers seem to have the greatest effect on myometrial relaxation (Higby and Suiter, 1999).

More recently, an endogenous inhibitor of uterine contractions acting at the dihydropyridine binding sites of the uterine L-type Ca^{2+} channels was described to be released in greater amounts from fetal membranes before term than at term (Carroll et al., 2001). This observation further emphasizes the potential role of uterine L-type Ca^{2+} channel blockade in maintaining uterine quiescence and in regulating the transition into labor.

The isoquinoline derivative, drotaverine is a potent spasmolytic agent which has been on the market for more than 35 years. Drotaverine was shown to inhibit human cAMP-specific phosphodiesterase type 4 enzyme with an IC_{50} value of 3.3 μM (Blaskó, 1998; Kapui, 1998). More recently, a controlled clinical trial has been performed which has shown some important effects for drotaverine on some parameters of physiological deliveries, i.e. drotaverine significantly shortened the time course of dilatation stage and significantly lowered the incidence of cervical rupture (Demeter et al., 1999).

The purpose of the present study was to verify whether drotaverine has Ca^{2+} channel blocking properties in pregnant uterine smooth muscle which may contribute to its spasmolytic effect elicited by inhibition of phosphodiesterase type 4 enzyme. Preliminary experiments showed that drotaverine could effect the binding of [^{3}H]nitrendipine.

2. Materials and methods

2.1. Materials

[^{3}H]Nitrendipine (87 Ci/mmol) and D-*cis*-[^{3}H]diltiazem (84 Ci/mmol) were purchased from New England Nuclear. Nitrendipine, D-*cis*-diltiazem hydrochloride were obtained from Sigma. Drotaverine hydrochloride was supplied by Chinoin (Tó utca 1-5, Budapest, Hungary, H-1045).

2.2. Animals

Primigravid Sprague–Dawley rats at days 18–20 of the 21-day gestation period were used. At the time of the acute experiment, the duration of gestation was confirmed by comparing the fetal size with known standards. Tissues from implantation sites were not used. Rats were anaesthetized with ether and killed by decapitation. The abdomen was opened and both uterine horns were removed and separated from fat deposits and mesenteric attachments.

2.3. Membrane preparation

Pregnant rat uterine membranes were prepared as previously described (Batra and Popper, 1989) with minor modifications. The myometrium was homogenized in 10 vol of sucrose–HEPES buffer (0.25 M sucrose, 10 mM HEPES–NaOH, pH 7.2) and centrifuged at 12 000 × *g* for 15 min at 4 °C. The supernatant was pelleted by means of two consecutive centrifugation cycles (40 000 × *g*, 1 h, 4 °C) with intermittant resuspension of the pellet in fresh buffer. The microsomal pellet obtained was suspended in 50 mM Tris–HCl buffer (pH 7.2 at 25 °C) and used immediately for binding assay. The protein concentration of the resultant membrane preparation was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.4. Radioligand binding

For [^{3}H]nitrendipine competition studies, aliquots of the membrane preparation (300–500 μg protein) were incubated with 0.3 nM [^{3}H]nitrendipine in 50 mM Tris–HCl buffer (pH 7.2) in a final volume of 1 ml for 90 min at 25 °C under reduced room lighting. Nonspecific binding was defined by 1 μM unlabeled nitrendipine. For [^{3}H]diltiazem competition studies, aliquots of the membrane preparation (1 mg protein) were incubated with 10 nM [^{3}H]diltiazem in a final volume of 0.5 ml of 50 mM Tris–HCl buffer (pH 7.2) containing 0.1% bovine serum albumin for 90 min at 25 °C under reduced room lighting. Nonspecific binding was determined by 10 μM unlabeled diltiazem. After incubation, the samples were filtered through Whatman GF/B ([^{3}H]nitrendipine binding) or GF/C pretreated with 0.3% polyethylenimine ([^{3}H]diltiazem binding; Chatelain et al., 1993) and washed three times with 5 ml of ice-cold buffer using a cell harvester. The radioactivity remained on the filter was counted in 3 ml of OptiPhase HiSafe (Wallac) scintillation cocktail at an efficiency of approximately 50%.

Dissociation kinetics were measured by preincubating [^{3}H]nitrendipine (0.5 nM) or [^{3}H]diltiazem (5 nM) with membrane protein for 90 min at 25 °C. Then an excess of unlabeled nitrendipine (1 μM) or unlabeled diltiazem (10 μM) was added with or without (control) other drugs. At defined time, the samples in triplicate were filtered.

2.5. Data analysis

The IC_{50} values and Hill slopes for competition binding data were obtained using a nonlinear method (SigmaPlot for Windows Version 4.00). The equilibrium dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were estimated by linear regression analysis after Scatchard transformation of the radioligand saturation binding data.

The dissociation rate constant (k_{-1}) was determined from linear regression analysis of log B_t/B_e vs. time, where B_t and B_e were the specific binding at t time and at equilibrium, respectively. The dissociation half-life ($t_{1/2}$) is equal to log $0.5/k_{-1}$.

All experiments were performed in triplicate and data are expressed as mean ± S.E.M. of three to five independent experiments. Statistical analysis was performed by using a one-sample, two-tailed t-test and one-way analysis of variance, followed by Dunnett's test. All the calculations were performed by SAS® 6.12 (SAS® Institute, Cary, NC). Values of $P<0.05$ were considered statistically significant.

3. Results

Fig. 1 shows the effects of drotaverine, nitrendipine and diltiazem on the binding of [^{3}H]nitrendipine (Fig. 1A) and [^{3}H]diltiazem (Fig. 1B) to pregnant rat uterine membranes.

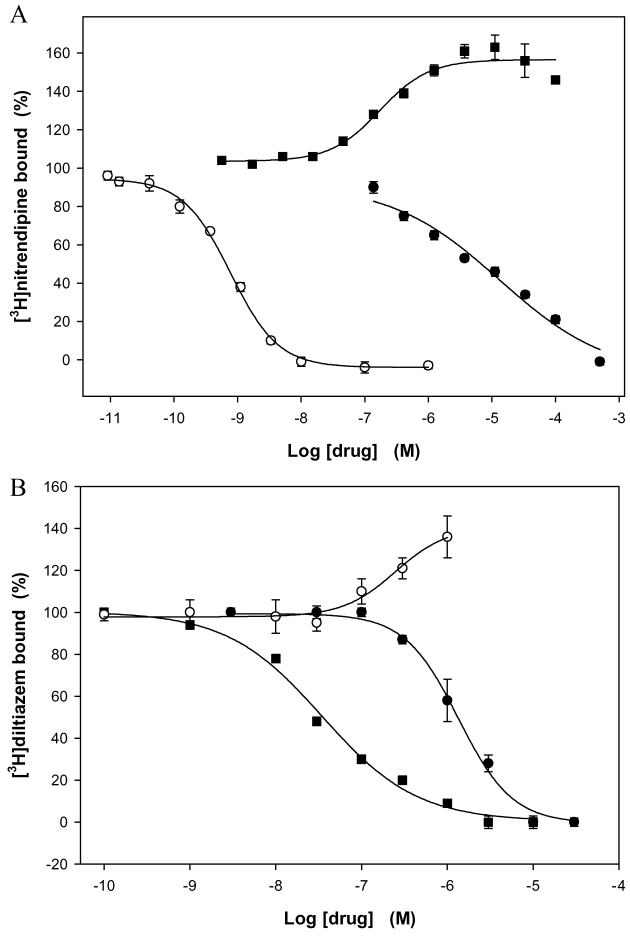

Fig. 1. Effects of increasing concentrations of unlabeled nitrendipine (○), drotaverine (●) and diltiazem (■) on the specific [^{3}H]nitrendipine (0.3 nM) binding (A) and [^{3}H]diltiazem (10 nM) binding (B) to pregnant rat uterine membranes. Data are means ± S.E.M. of five (A) and three (B) independent experiments performed in triplicate.

Nitrendipine competitively inhibited [^{3}H]nitrendipine with an IC_{50} of 0.40 ± 0.07 nM (K_i = 0.25 ± 0.04 nM, n = 5). The value of Hill slope for nitrendipine was close to unity suggesting the presence of a homogenous population of nitrendipine binding sites in the pregnant rat uterine membranes. Specific binding of [^{3}H]nitrendipine was completely displaced by drotaverine in a concentration-dependent fashion. Drotaverine inhibited [^{3}H]nitrendipine binding with an IC_{50} value of 5.6 ± 1.11 μM (n = 5). The value of Hill slope for drotaverine at displacing [^{3}H]nitrendipine binding was significantly ($P<0.001$) less than unity (0.49 ± 0.03) suggesting a more complex interaction than a simple competition for a single common site between [^{3}H]nitrendipine and drotaverine. Diltiazem markedly enhanced the binding of [^{3}H]nitrendipine to pregnant rat uterine membranes at 25 °C, with a maximal stimulation of 156 ± 5%. The value of half-maximal enhancement concentration (ED_{50}) was equal to 134 ± 17 nM (n = 5).

The specific binding of [^{3}H]diltiazem at a concentration of 10 nM was completely inhibited both by diltiazem (IC_{50} = 35 ± 6.6 nM, n = 3) and drotaverine (IC_{50} = 2.6 ± 0.77 μM, n = 3). The values of Hill slopes for both diltiazem and drotaverine were not significantly different from unity. Nitrendipine increased the specific binding of [^{3}H]diltiazem in a concentration-dependent manner up to 136%.

We performed Scatchard analysis to determine [^{3}H]nitrendipine saturation isotherms with and without 5 μM drotaverine, a concentration causing approximately 50% inhibition of [^{3}H]nitrendipine binding to pregnant rat uterine membranes (Fig. 2, solid circles). Drotaverine resulted in a remarkable reduction in the apparent affinity of [^{3}H]nitrendipine (K_D = 1.55 ± 0.28 nM, n = 5, $P<0.001$), as compared to control treatment, and a pronounced increase of B_{max} value (B_{max} = 70.6 ± 10.8 fmol/mg protein, n = 5, $P<0.01$). These results also suggest that drotaverine interacts with [^{3}H]nitrendipine binding sites in a noncompetitive and allosteric manner. Diltiazem, the well-known allosteric enhancer of [^{3}H]nitrendipine binding, at a concentration of 5 μM significantly increased the B_{max} value (B_{max} = 68.2 ± 6.5 fmol/mg protein, n = 5, $P<0.01$) while the apparent K_D value of [^{3}H]nitrendipine binding was not affected (Fig. 2, solid squares).

We did not succeed in performing saturation analysis of [^{3}H]diltiazem binding to pregnant rat uterine membranes because of the high level of nonspecific binding (>60% of the total binding when radioligand concentration was increased above 40 nM). However, total and nonspecific bindings of [^{3}H]diltiazem were increased in a concentration-dependent manner, indicating that the radioligand is valid for labeling of the relevant binding site in pregnant rat uterine membranes.

The value of Hill slope for drotaverine at displacing [^{3}H]diltiazem approximated unity which might be indicative of a competitive interaction for drotaverine with the [^{3}H]diltiazem binding sites.

According to Ehlert (1988), the inhibition of radioligand binding to specific binding sites by an allosteric modulator is likely to be observed as apparent competitive inhibition when the magnitude of negative heterotropic cooperativity is large. Therefore, we examined the effects of drotaverine on the dissociation kinetics of [^{3}H]nitrendipine and [^{3}H]diltiazem. Effects on modification of the dissociation rate of a radioligand by an unlabeled drug have been widely used to indicate whether a compound is acting at the same site as the radioligand (unchanged dissociation rate) or at a different, allosterically linked site (faster or slower dissociation rate) (Spedding and Paoletti, 1992).

The effects of drotaverine on the dissociation kinetics of [^{3}H]nitrendipine and [^{3}H]diltiazem at 25 °C were assessed to further clarify the mechanism of the drotaverine interaction with these two binding sites on the L-type Ca^{2+} channel (Table 1). The dissociation rates of [^{3}H]nitrendipine and [^{3}H]diltiazem were best fitted as monoexponential processes. Drotaverine, at a concentration of 30 μM, significantly increased the dissociation rate constant of both radioligands, while the dissociation rate constant of [^{3}H]nitrendipine and that of [^{3}H]diltiazem was allosterically decreased by diltiazem and nitrendipine, respectively.

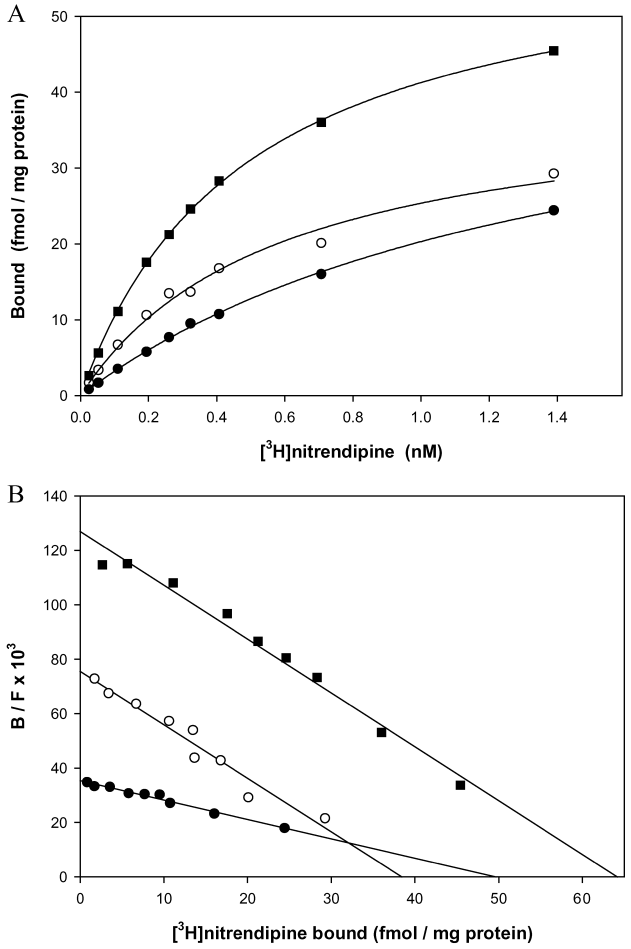

Fig. 2. (A) Effect of drotaverine and diltiazem on the equilibrium saturation binding of [^{3}H]nitrendipine to pregnant rat uterine membranes. The typical results of the equilibrium saturation binding of [^{3}H]nitrendipine are shown. Equilibrium binding was measured in the absence (○) and in the presence of 5 μM drotaverine (●), or in the presence of 5 μM diltiazem (■). Nonspecific binding was determined in the presence of 1 μM nitrendipine. (B) Scatchard plot of the data is shown in panel (A). K_D (nM) and B_{max} (fmol/mg protein) values are summarized in the text.

Table 1
Effects of drotaverine, diltiazem and nitrendipine on dissociation rate constant (k_{-1}) and on dissociation half-life ($t_{1/2}$) of [^{3}H]nitrendipine and [^{3}H]diltiazem from pregnant rat uterine membranes

	k_{-1} (min^{-1})	$t_{1/2}$ (min)	Linearity
[^{3}H]nitrendipine			
Control (nitrendipine 1 μM)	0.026 ± 0.006	11.81 ± 0.014	− 0.99 ± 0.01
+ drotaverine 30 μM	0.055 ± 0.005 *	5.55 ± 0.57 * *	− 0.97 ± 0.03
+ diltiazem 10 μM	0.013 ± 0.002 * *	22.84 ± 3.14 *	− 0.97 ± 0.01
[^{3}H]diltiazem			
Control (diltiazem 10 μM)	0.013 ± 0.002	22.88 ± 2.55	− 0.99 ± 0.003
+ drotaverine 30 μM	0.062 ± 0.006 *	4.90 ± 0.49 * *	− 0.98 ± 0.005
+ nitrendipine 1 μM	0.005 ± 0.001 * *	65.14 ± 7.44 *	− 0.97 ± 0.002

Values are mean ± S.E.M. from three independent experiments performed in triplicate. Significance of difference from individual control data analysed by one-way ANOVA.
* $P < 0.001$.
** $P < 0.05$.

4. Discussion

In the present study, drotaverine was found to interact both with 1,4-dihydropyridine (nitrendipine) and 1,5-benzothiazepine (diltiazem) binding to the L-type Ca^{2+} channels of pregnant rat uterine membranes with IC_{50} values of 5.6 and 2.6 μM, respectively.

Scatchard analysis of [^{3}H]nitrendipine binding to pregnant rat uterine membranes indicated a single population of homologous binding sites with a K_D value of 0.47 nM and a B_{max} value of 33.5 fmol/mg protein. Similar high affinity binding for [^{3}H]nitrendipine was previously reported in uterine membranes (Grover et al., 1984; Yousif et al., 1985; Batra and Popper, 1989).

The results from the competition and saturation studies suggested that both drotaverine and diltiazem interact with [^{3}H]nitrendipine binding sites in an allosteric manner. The value of Hill slope for drotaverine at displacing [^{3}H]nitrendipine was significantly less than unity, moreover, drotaverine affected both the K_D and B_{max} values for [^{3}H]nitrendipine binding.

Diltiazem increased the binding of [^{3}H]nitrendipine, which was achieved by increasing the number of binding

sites available without affecting the apparent affinity for [^{3}H]nitrendipine. Similarly, nitrendipine increased [^{3}H]diltiazem binding. Allosteric interactions of diltiazem binding with other binding sites on the Ca^{2+} channel was earlier observed in other systems (e.g. Schoemaker and Langer, 1985; Schoemaker et al., 1987; Chatelain et al., 1993).

The dissociation kinetics of [^{3}H]nitrendipine and [^{3}H]diltiazem were slowed down by diltiazem and nitrendipine, respectively, clearly demonstrating a mutually positive heterotropic allosteric interaction between the nitrendipine and diltiazem binding sites of the L-type Ca^{2+} channels of pregnant rat uterine membranes, which is also in line with the mutual promotion of each others binding (see above).

On the other hand, the dissociation kinetics of both [^{3}H]nitrendipine and [^{3}H]diltiazem were accelerated by drotaverine. These results suggested that drotaverine interacted with a binding site other than that for 1,4-dihydropyridines (nitrendipine) and 1,5-benzothiazepines (diltiazem) and the binding site for drotaverine was linked to these two principal binding sites in a negative allosteric manner.

Verapamil, at a concentration of 10 μM, was reported to inhibit both the contractions and the increase of the intracellular Ca^{2+} concentration in isolated porcine myometrial strips (Kitazawa et al., 2000) indicating a specific interaction for the phenylalkylamine-type verapamil with the uterine L-type Ca^{2+} channels.

Further studies are needed to clarify whether the site for drotaverine action on the L-type Ca^{2+} channels of pregnant rat uterine membranes is either identical to that for phenylalkylamines or whether drotaverine interacts with a site different from the three described binding sites.

Earlier in vitro studies revealed a significant relaxing effect of drotaverine on pregnant rat uterine horn preparation precontracted with oxytocin (0.001 U/ml) with an IC_{50} value of 5.1 μM (Tanev et al., 1982).

Our present finding concerning a specific interaction of drotaverine with the uterine L-type Ca^{2+} channels may be indicative for a Ca^{2+} channel blocking potency of drotaverine. Further electrophysiological studies or measurement of the intracellular Ca^{2+} concentration would be needed to verify a direct inhibition by drotaverine of the Ca^{2+} influx via the uterine L-type Ca^{2+} channels.

More recent data suggest that capacitative (or store-operated) Ca^{2+} entry in pregnant human myometrium may be sufficient to elicit a contraction even in the presence of an L-type Ca^{2+} channel blocker (Tribe, 2001). In addition to it, although a change in intracellular Ca^{2+} concentration is the most important mechanism for the control of contractile activity in myometrium, several other mechanisms are able to modulate or perhaps even initiate tension generation, or to inhibit it, producing relaxation (Bolton et al., 1999; Sanborn, 2000; Tribe, 2001). Thus, in order to demonstrate unequivocally the role of drotaverine interaction with the L-type Ca^{2+} channels in the spasmolytic effect observed in pregnant rat uterine horn preparation, further studies in the same uterus, on the effect of drotaverine on radioligand binding, intracellular Ca^{2+} concentration, and contractility will be required.

Relaxation between contractions during normal labor seems to be a much welcomed respite. A blockade of Ca^{2+} influx via the uterine L-type Ca^{2+} channels may be a potential mechanism of action in suppressing uterine dysfunctions linked with an increase in uterine muscular activity and hence exerting an increase in the relaxation periods (Wray, 1993; Tribe, 2001).

Recently, a controlled open, phase IV clinical trial has been performed demonstrating prominent effects for drotaverine on some parameters of human deliveries (Demeter, 1998). Drotaverine, given at a dose of 40 mg (i.m.) in the stage of cervical dilatation phase to parturients, significantly ($P=0.005$) shortened the time course of dilatation stage and highly significantly ($P=0.001$) lowered the incidence of cervical ruptures, which led to decreased obstetrical complications. No increase in the risk of atonia was observed. Although drotaverine caused some lowering in the blood pressure, this effect may be of beneficial influence on the stress accompanying labor (Demeter et al., 1999).

It should be noted, however, that in the present study, the interaction of drotaverine with the uterine L-type Ca^{2+} channels has been examined in rat and not in human. Although there are differences between rat and human myometrium (Tribe, 2001; Wray et al., 2001), the implication of drotaverine for human childbirth could be speculated. On the basis of our present results, we assume that drotaverine, exerting a negative allosteric effect on the binding sites for 1,4-dihydropyridines and 1,5-benzothiazepines of the L-type Ca^{2+} channel in pregnant myometrium, decreases the Ca^{2+} influx through the channel, which may lead to a transient suppression of the uterine muscular hyperactivity and, as a consequence, an increase in blood and oxygen supply to both the myometrium and the fetus. The potential Ca^{2+} channel blocking potency of drotaverine may play a significant role in its effect on the improvement of the outcomes of deliveries.

In conclusion, in the present study, we have shown a specific interaction for drotaverine with the L-type Ca^{2+} channels in pregnant rat uterine membranes. In light of this observation, we hypothesize that a Ca^{2+} channel modulating potency may contribute to drotaverine's spasmolytic effect elicited by inhibition of phosphodiesterase type 4 enzyme. These dual mechanisms of action may explain the prominent efficacy of drotaverine on human deliveries, and moreover, drotaverine may provide a useful alternative for treating premature labor.

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