Estrous cycle-dependent differences in responsiveness to prostaglandins and contractile agents in sheep (*Ovis aries*) cervical smooth muscle

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We investigated the influence of the phase of the estrous cycle on mechanical responses elicited in sheep cervix by potassium chloride (KCl), acetylcholine chloride (ACh), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_1 (PGE_1). The cervix of adult ewes ($n = 48$) were classified according to the presence or absence of corpora lutea (luteal or follicular phase, respectively). Muscle strips of the circular and longitudinal layers were prepared in an organ bath and coupled to an isometric force transducer. Concentration–response curves were obtained noncumulatively. KCl and ACh produced concentration-dependent contractions in all preparations in both phases of the estrous cycle. However, maximum effect, EC_{50} and slope values of KCl and ACh were not significantly different between muscle layers, as well as between the phases of the estrous cycle. The prostanoid, $PGF_{2\alpha}$, produced a significant reduction in the amplitude of spontaneous contractions for all preparations. The depressant effect of $PGF_{2\alpha}$ on spontaneous contractions of circular smooth muscle was significantly greater during the follicular than the luteal phase, whilst the depressant effect of $PGF_{2\alpha}$ on the longitudinal layer did not differ between phases of the estrous cycle. PGE_1 significantly reduced the amplitude of spontaneous contractions on circular but not on longitudinal preparations. In conclusion, we have characterized with *in vitro* preparations of circular and longitudinal muscle layers of ewes during the follicular and luteal phases of the estrous cycle, the parameters of the K- and ACh-induced contractions on cervix and the efficacy of $PGF_{2\alpha}$ and PGE_1 on inhibition spontaneous contractile activity.

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INTRODUCTION

The Brazilian sheep flock consists of 15 million animals, with approximately 58% raised in the northeastern region (IBGE, 2004). Sheep reared in this area represent an important economic source for breeders, where they are used mainly for meat and skin production. However, production efficiency of most local animals from northeastern Brazil is low, owing to management conditions but especially because of the low genetic quality of the flock. Widespread genetic improvement of sheep is dependent on the ability to use reproductive technologies, such as artificial insemination and embryo transfer.

There are major limitations in the application of reproductive technologies in sheep, due to the inability to relax or dilate the cervix. Anatomically, the cervix of sheep is a rigid, long, tightly closed structure crossed by five to seven offset spiral folds that form crypts, believed to hold ejaculated spermatozoa (More, 1984). The second fold cranial to the vagina generally presents the greatest obstacle to instrument insertion through the sheep cervix (Croy *et al.*, 1999). Relaxation of the cervix would provide the dilatation necessary to facilitate noninvasive artificial insemination. Cervical relaxation would also permit nonsurgical embryo recovery or transfer 'per vaginum' (Mylne *et al.*, 1992; McKelvey, 1999).

To overcome the sheep cervical barrier, some attempts have been made using physical (Halbert *et al.*, 1990), chemical (Barry *et al.*, 1990) or mechanical (Wulster-Radcliffe & Lewis, 2002) methods. However, these attempts produced variable or inconsistent results, perhaps due to the lack of knowledge about ewe cervical tissue responsiveness under different physiological conditions.

This work is a part of a larger project whose main objective is to promote the dilatation of sheep cervix to facilitate the use of reproductive technologies. The aim of the present study was to explore the effects of the phase of the estrous cycle on responsiveness to a depolarizing agent [potassium chloride (KCl)], acetylcholine and selected prostanoids on sheep cervical tissues. Additionally, it was possible to study the longitudinal and circular muscle response.

MATERIALS AND METHODS

Chemicals and drugs

The following chemicals and drugs were used in this experiment: acetylcholine chloride (ACh) (Sigma Chemical Co., St Louis, MO, USA), KCl (Reagen, Rio de Janeiro, Brazil), and synthetic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Coopers, São Paulo, Brazil) and prostaglandin E_1 (PGE_1) (Searle, São Paulo, Brazil).

All agents, except for PGE_1, were dissolved in distilled water and added directly to an organ bath. Prostaglandin E_1 was dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co.), and this solution was added to the organ bath. The maximum concentration of DMSO in the bath solution was 0.5%.

Animals and tissue collection

Cycling adult local ewes ($n = 48$) aged 22.0 ± 1.9 (mean ± SEM) months were obtained from local farms. The management of experimental animals was in agreement with institutional and internationally accepted welfare guidelines [American Veterinary Medical Association (AVMA), 2001]. Sheep were slaughtered by captive bolt and reproductive tracts were collected. The cervix was removed and transported to the laboratory in modified Tyrode solution (in mM: NaCl 136.0, KCl 5.0, $MgCl_2$ 0.98, $CaCl_2$ 2.0, NaH_2PO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.5, pH 7.4) at 5 °C within 2–3 h. The ewes were nonpregnant, and prior to collection of cervix, the ovaries were removed and macroscopically examined to determine the phase of the estrous cycle. The absence or presence of corpora lutea was indicative of the follicular or luteal phase, respectively. In addition to external examination, the ovaries were sectioned to examine for the presence of internal corpora lutea.

Tissue preparations

After dissecting off the connective tissues, muscle strips parallel to the direction of the circular and longitudinal muscle fibers were prepared. Two or three muscle preparations were obtained from each cervix. Each muscle strip (4–5 mm in width, 9–10 in length and 1 mm in thickness) was suspended vertically in an organ bath (5 mL) containing modified Tyrode solution, at 37 °C, bubbled continuously with air. The tissue segments were loaded with an initial tension of 1 g and allowed to equilibrate for 60 min to establish reproducible spontaneous contractility. The 1 g tension was determined to be the optimal in our previous assays with this tissue. To test for viability, preparations were stimulated to contract by adding 60 mM KCl. Before each experiment, the KCl was washed out twice at 5-min intervals and replaced with modified Tyrode solution. The segments were attached to a hook and to an isometric force transducer (Force transducer FT03; Grass Instruments, Quincy, MA, USA) connected by a transducer cable (Grass transducer cable TAV-7 REV-1; Grass Instruments) to a four-channel polygraph chart recorded (Model PM-1000; Grass Instruments). All signals from the force transducers were recorded and stored in a computerized system (WINDAQ 1.65; DATAQ Instruments, Akron, OH, USA).

Procedure and recording data

After the viability test, the cervical strips were then exposed to ascending concentrations of well-defined pharmacological agonists that act on other smooth muscles: a depolarizing agent (KCl), a muscarinic receptor agonist (ACh) and two prostanoid receptor agonists ($PGF_{2\alpha}$ and PGE_1). According to putative effects, the agents were used as contractile (ACh and KCl) and relaxant ($PGF_{2\alpha}$ and PGE_1) agents. Concentration–response curves for all agents were obtained noncumulatively, by the addition of single doses, and each tissue preparation was used only once.

After equilibration, ACh (0.01–80.0 μM), KCl (10.0–120.0 mM), $PGF_{2\alpha}$ (0.5–30.0 μM) or PGE_1 (0.1–10.0 μM) were added at 15-min intervals. For the KCl concentration–response curves 5.0 mM KCl (K^+ Tyrode concentration) was included as the baseline concentration. The tissue strips were left at each concentration of test agent until an effect reached steady-state (approximately 5 min). After this time of exposure, the strips were washed twice with modified Tyrode solution and stabilized. Concerning the prostanoid agonists, spontaneous contractile activity that occurred during the equilibration time was recorded for 5 min to obtain the amplitude of reference contraction. In the case of the PGE_1, strips mounted simultaneously were tested with DMSO, which served as negative controls for experimental segments.

Calculations and statistical analysis

The contractile amplitude was determined by measuring the peak height of 'plateau' contraction (g tension) relative to basal tonus (0 g tension). ACh and KCl responses were expressed as contraction force (g) relative to the basal tonus. On the other hand, prostanoid responses were calculated as a percentage of the reference contraction, where 100% represented the amplitude of spontaneous movements demonstrated before the addition of the first concentration of $PGF_{2\alpha}$ or PGE_1. The contractile and relaxant responses were compared between

the phases of the estrous cycle (follicular and luteal) or muscle layers (circular and longitudinal).

The data were expressed as mean ± SEM of individual values obtained from at least four different tissue preparations. Concentration-effect curves for KCl and ACh contraction-inducing agonists were calculated fitting the data to the following Hill equation:

$$E = \frac{(\alpha[A]^{nH})}{([A]_{50}^{nH} + [A]^{nH})},$$

where E is the effect measured, $[A]$ is the concentration of the agonist, $[A]_{50}$ is the midpoint location (EC_{50}), α is the maximal asymptote (maximum effect) and nH is the Hill slope. Fitting was calculated using the SigmaPlot software (Version 9.0; Systat, San Jose, CA, USA). Prostaglandin-related data were analyzed using ANOVA and *post hoc* analysis test (Dunnet's test) for statistically significant difference. A probability level of <0.05 was accepted as being significant.

RESULTS

The concentration-dependent effect induced by depolarization at different KCl concentrations in sheep cervical smooth muscle is shown in Fig. 1. This agent was able to elicit contractions significantly greater than the basal tonus ($P < 0.05$). In both phases of the estrous cycle, the onset of KCl-induced contractile responses started at concentrations between 10.0 and 20.0 mM

and peaked between 80.0 and 100.0 mM. Maximum effect, EC_{50} and slope values were not significantly different ($P > 0.05$) between muscle layers, as well as between the phases of the estrous cycle (Table 1).

The concentration–response curve for ACh is shown in Fig. 2. In both phases of the estrous cycle, the onset of ACh-induced contractile responses started at concentrations between 0.03 and 0.1 μM and peaked between 60.0 and 80.0 μM. Maximum effect, EC_{50} and slope values were not significantly different ($P > 0.05$) between muscle layers, as well as between the phases of the estrous cycle (Table 2).

The prostanoid, $PGF_{2\alpha}$, produced a significant reduction ($P < 0.05$) in the amplitude of spontaneous contractions for all preparations. $PGF_{2\alpha}$, at 3.0, 10.0 and 30 μM, depressed spontaneous contractions of circular smooth muscle significantly more during the follicular than the luteal phase (Fig. 3a). In contrast, the depressant effects of $PGF_{2\alpha}$ on longitudinal smooth muscle did not differ between phases of the estrous cycle (Fig. 3b). In particular, in longitudinal muscle, at 1.0 and 10.0 μM, $PGF_{2\alpha}$-induced increase in amplitude of spontaneous contractions, however, did not reached statistical significance when compared with control (Fig. 3b).

PGE_1 significantly ($P < 0.05$) reduced the amplitude of spontaneous contractions on circular but not on longitudinal preparations (Fig. 4). Reduction of contractions was statistically significant ($P < 0.05$ when compared with control) for all concentrations of PGE_1 tested (0.1–10.0 μM) in circular muscle in follicular phase. Reduction of contractions only reached
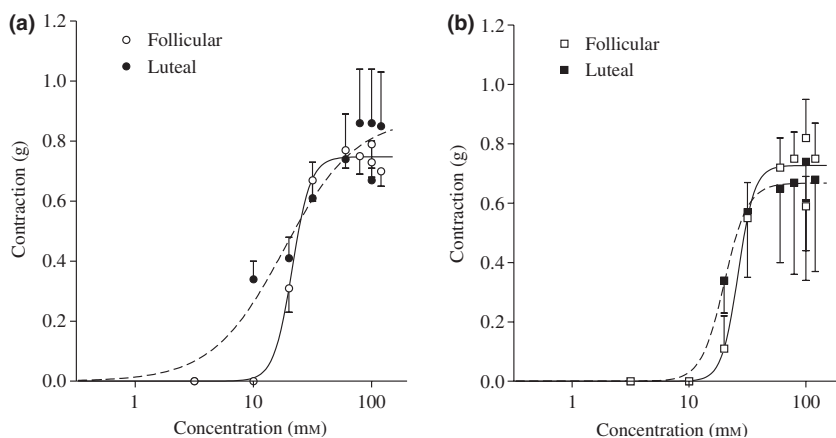

Fig. 1. Mean noncumulative concentration–response curves for KCl in circular (a) and longitudinal (b) cervical muscle from ewes in the follicular and luteal phase of the estrous cycle. Data were mean ± SEM of at least four experiments. Nonlinear regression curves are presented for muscle response in follicular (filled line) and luteal (dotted line) phases. *Significantly different in relation to the basal tonus ($P < 0.05$).

Table 1. Maximum effect, EC_{50} and slope values for KCl in cervical smooth muscle (circular and longitudinal layers) from ewes in follicular and luteal phase of the estrous cycle

Phase of the estrous cycle	Maximum effect (g) ± SEM		EC_{50} (μM) ± SEM		Slope (nH) ± SEM	
	Circular	Longitudinal	Circular	Longitudinal	Circular	Longitudinal
Follicular	0.75 ± 0.03	0.76 ± 0.05	21.15 ± 1.07	26.61 ± 2.85	6.77 ± 2.67	4.97 ± 2.16
Luteal	0.93 ± 0.25	0.68 ± 0.11	20.57 ± 11.91	24.80 ± 6.18	1.50 ± 1.63	6.25 ± 6.68

Not significant difference between phases of the estrous cycle or muscular layers were found ($P > 0.05$; $n > 4$ in all cases).

Fig. 2. Mean noncumulative concentration–response curves for ACh in circular (a) and longitudinal (b) cervical muscle from ewes in the follicular and luteal phase of the estrous cycle. Data were mean ± SEM of six experiments. Nonlinear regression curves are presented for muscle response in luteal (dotted line) and follicular (filled line) phases. *Significantly different in relation to the basal tonus ($P < 0.05$).
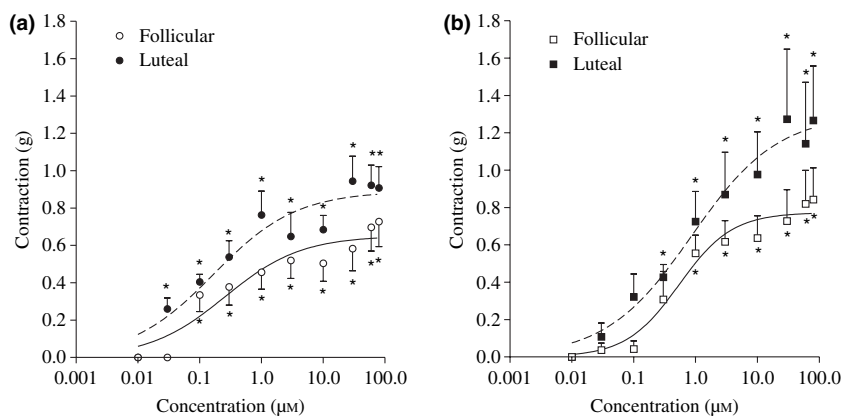
Table 2. Maximum effect, EC_{50} and slope values for ACh in cervical smooth muscle (circular and longitudinal layers) from ewes in follicular and luteal phase of the estrous cycle

Phase of the estrous cycle	Maximum effect (g) ± SEM		EC_{50} (μM) ± SEM		Slope (nH) ± SEM	
	Circular	Longitudinal	Circular	Longitudinal	Circular	Longitudinal
Follicular	0.65 ± 0.08	0.77 ± 0.08	0.26 ± 0.30	0.54 ± 0.33	0.71 ± 0.53	1.01 ± 0.58
Luteal	0.89 ± 0.09	1.29 ± 0.31	0.17 ± 0.21	0.87 ± 1.02	0.65 ± 0.43	0.62 ± 0.57

Not significant difference between phases of the estrous cycle or muscular layers were found ($P > 0.05$; $n = 6$ in all cases).

Fig. 3. Mean noncumulative concentration–response curves for $PGF_{2\alpha}$ in circular (a) and longitudinal (b) cervical muscle from ewes in the follicular and luteal phase of the estrous cycle. Data were mean ± SEM of at least five experiments. *Significantly different in relation to the spontaneous contractions ($P < 0.05$). (a,b) Significant difference ($P < 0.05$) at each $PGF_{2\alpha}$ concentration.
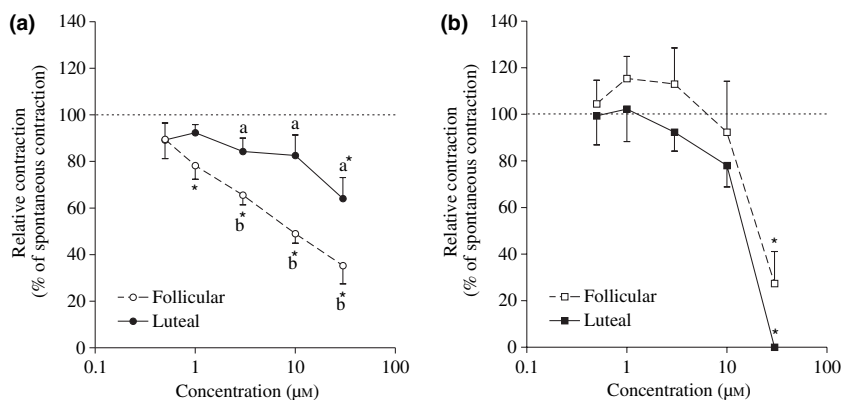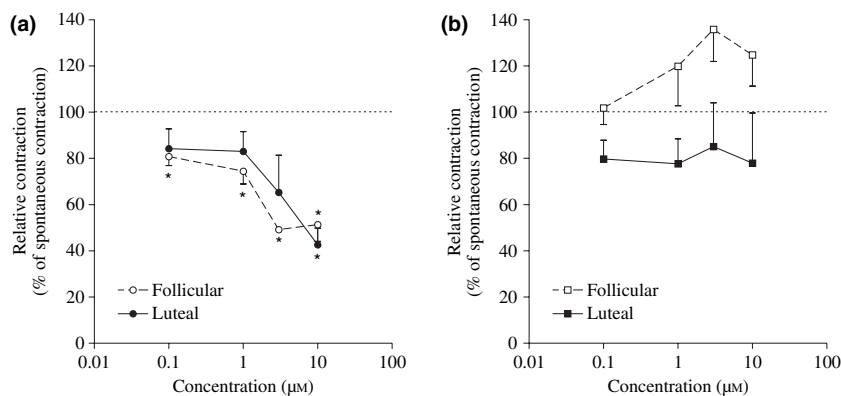

Fig. 4. Mean noncumulative concentration–response curves for PGE_1 in circular (a) and longitudinal (b) cervical muscle from ewes in the follicular and luteal phase of the estrous cycle. Data were mean ± SEM of at least four experiments. *Significantly different in relation to the spontaneous contractions ($P < 0.05$).

significance at 10 μM ($P < 0.05$ when compared with control). Depression of contractions by PGE$_1$ did not differ between phases of the estrous cycle (Fig. 4a). PGE$_1$ (0.1–10 μM) did not significantly alter the amplitudes of spontaneous movements of longitudinal muscle in both phases of the estrous cycle when compared with control (Fig. 4b).

DISCUSSION

The use of noninvasive reproductive technologies in sheep is limited due to the cervix anatomy, which restricts the passage of the instruments through the uterine lumen. Knowledge about the cervical profile under different physiological conditions (Garcia-Villar et al., 1982a, 1984) as well as when submitted to various bioactive agents (Garcia-Villar et al., 1982b; Ayad et al., 2004) is important in the development of useful drugs for reproductive biotechniques in sheep.

In the present study, we examined the influence of the phase of the estrous cycle (follicular vs. luteal) on mechanical responses elicited in sheep cervix by KCl, ACh, PGF$_{2\alpha}$ and PGE$_1$. Our aim was to characterize the effects (contractile and/or relaxant) of these agents on the tissues and, secondly, to identify differences between the phases of the estrous cycle and/or muscle layers. A conventional Tyrode solution (Tyrode, 1910), a nutrient solution with 2.7 mM K$^+$, has been used in studies of cervical and uterine muscle in rodents (Prendergast et al., 2006). However, this is species dependent. Sheep plasma K$^+$ concentrations are between 3.8 and 5.4 mM (Pugh, 2005). We thus used a modified Tyrode solution with 5.0 mM KCl, allowing our study to be used as a background for future physiological and pharmacological studies of sheep tissues. As K$^+$ threshold concentration for inducing contraction lies between 10.0 and 20.0 mM (Fig. 1), it is reasonable to assume that 5.0 mM KCl did not alter the spontaneous contraction and contractile response of agonists.

Thus, KCl and ACh caused well-defined concentration-dependent contractions in sheep cervical smooth muscle. Both exogenous and endogenous ACh produce uterine contractions through the action of muscarinic receptors (Houdeau et al., 2003). The muscarinic receptors, M$_2$ and M$_3$, produce pharmacomechanical coupling in the myometrium of several species: rabbit (Batra, 1990), guinea-pig (Boxall et al., 1998), rat (Choppin et al., 1999) and sow (Kitazawa et al., 1999). However, no studies have been performed to identify the muscarinic receptors in the sheep cervix.

On studying the effect of prostaglandins on cervical muscle, we found a relaxant effect distributed differently according to the muscle layers and phases of estrous cycle. Whilst PGF$_{2\alpha}$ depressed the cervical spontaneous motility in both phases, at a given concentration of PGF$_{2\alpha}$ caused a greater reduction of tone in circular smooth muscle in the follicular than in the luteal phase. Similar responses were observed for prostaglandins (F$_{2\alpha}$ and E$_2$) in the cornu, corpus and cervix of the nonpregnant porcine uterus (Cao et al., 2005). These authors reported a relaxant effect of PGF$_{2\alpha}$ on cervical circular muscle at concentrations similar to that used in our experiment. PGF$_{2\alpha}$ (1.0 and 3.0 μM) induced an increase in the amplitude of spontaneous motility on longitudinal muscle in follicular phase which was, however, not statistically significant. This absence of significance should be taken with caution. This is because another study (Cao et al., 2005) reported similar results, with PGF$_{2\alpha}$ increasing spontaneous motility at concentrations smaller than 10.0 μM and depressing at higher values.

PGE$_1$ reduced the tone of circular smooth muscle during both phases of the estrous cycle, but had no significant effect on longitudinal muscle. The absence of an effect of PGE$_1$ on longitudinal muscle at follicular phase here reported should be interpreted cautiously as it is reported (Cao et al., 2005) that PGE$_2$, another inhibitor of spontaneous contractions, increased motility at low concentrations.

We hypothesize that this response variability (contraction/relaxation) can often be produced by prostaglandins in the longitudinal muscles of the female genital tract. The variations in responsiveness could be due to different factors, such as animal species (Crankshaw, 2001), distribution and/or concentration of receptors (Cao et al., 2005; Schmitz et al., 2006), physiological condition (Audicana et al., 1998; Kershaw et al., 2007) and type of prostaglandin tested (Cao et al., 2005).

There are only few studies identifying the effects of the estrous cycle phases on cervical responsiveness, in contrast to the multitude of them concerning physiological and drug-induced uterine activity. This work describes the responsiveness of sheep cervical muscle to ACh, PGF$_{2\alpha}$ and PGE$_1$ with regard to the follicular or luteal phases.

In relation to the studies with ACh, we observed no significant differences between the estrous cycle phases for maximum effect amplitudes and EC$_{50}$ values produced in both cervical muscle layers. The few in vivo studies performed in sheep (Garcia-Villar et al., 1982a) and bovine (Hirsbrunner et al., 2003) cervical muscle have found no influence of estrous cycle phase in spontaneous motility. However in rat, results in myometrium indicated an increase in cholinergic control during the follicular phase (estrus), with a higher sensitivity to ACh and without changes in nerve density (Houdeau et al., 2003), compared with the luteal phase (diestrus).

We observed a greater depressive effect of PGF$_{2\alpha}$, at a given concentration, on spontaneous motility of circular muscle at follicular phase. This suggests that the circular layer from ewes at follicular phase have higher sensitivity to PGF$_{2\alpha}$. Several studies have proposed that the high levels of estradiol (Kershaw et al., 2005, 2007), oxytocin (Ellwood et al., 1980; Calder & Greer, 1990; Shemesh et al., 1997; Ayad et al., 2004) and FSH (Mizrachi & Shemesh, 1999) associated with estrus and ovulation stimulate the release of prostaglandins from cervical and uterine cells. The latter substance acts as a paracrine agent, stimulating cervical relaxation and myometrial contraction (Stys et al., 1981; Ledger et al., 1983). Furthermore, studies performed with rat nonpregnant myometrium have correlated progesterone action (alone or in association with

estrogens) with an increase in prostaglandin-mediated contraction (Engstrom, 2001; Vedernikov *et al.*, 2003).

The physiological-functional aspect could explain the exact opposite between the results presented in our study and those of the current literature with other uterus sections (cornu and corpus). Despite the anatomical proximity, they play complementary roles in the female reproductive tract. The differences in spontaneous motility (Hirsbrunner *et al.*, 2006) as well as in responsiveness to bioagents (Ledger *et al.*, 1983; Ayad *et al.*, 2004; Cao *et al.*, 2005) for the uterine regions (cornu, corpus and cervix) probably account for the functional contractility of the organ (Cavaco-Gonçalves *et al.*, 2006).

Taken together, our results led us to hypothesize that, in specific phases of the sheep estrous cycle, the cervical response to some agents can differ from that in other parts of the uterus (cornu and corpus). For example, during the follicular phase, when the uterine contractile activity is evident and mediated by cholinergic nervous stimulation, the responsiveness to ACh is increased (Houdeau *et al.*, 2003). However, in the same phase, the cervix must be dilated (circular layer in relaxation) to permit physiological process, for example, in the transport of spermatozoa after mating (Selaive-Villarroel & Kennedy, 1983; Toutain *et al.*, 1985) or artificial insemination (Cavaco-Gonçalves *et al.*, 2006). On the other hand, uterine motility must be reduced causing the quiescent status during the luteal phase (Garcia-Villar *et al.*, 1982a) and pregnancy (Garcia-Villar *et al.*, 1984; Kitazawa *et al.*, 2003). In addition, the increase in the sheep cervical sensitivity to ACh would allow its maintenance in an active contractile state (Garcia-Villar *et al.*, 1982a, 1984). Additionally, the dilatation of the cervix is made difficult by the low sensitivity to prostaglandins in inducing relaxation.

In conclusion, we have characterized with *in vitro* preparations of circular and longitudinal muscle layers of ewes during the follicular and luteal phases of the estrous cycle, the parameters of the K- and ACh-induced contractions on cervix and efficacy of $PGF_{2\alpha}$ and PGE_1 on inhibition spontaneous contractile activity. To our knowledge, this is the first *in vitro* pharmacological study characterizing sheep cervical response, taking into account the muscle layer and the phase of the estrous cycle. These studies about differences between the phases of estrous cycle for genital tract responsiveness are a prerequisite for the development of drugs acting on this organ. Further studies are necessary for a better control of cervical response to different bioagents and to facilitate the use of noninvasive reproductive technologies in sheep.

ACKNOWLEDGMENTS

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