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Calcium-dependent and calcium-mimicking pathways regulate lead-induced myometrial contraction in water buffaloes

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ABSTRACT

Lead produced dose-dependent contractile effect on buffalo myometrium and it was more marked on tonicity and amplitude while almost negligible on phasic contractions and frequency. In the presence of Ca^{2+}-free Ringer Locke solution (RLS) and nifedipine, lead-induced uterotonic effect was significantly attenuated with decrease in E_{max} thus, suggesting major role of extracellular Ca^{2+} in uterotonic effect of lead and involvement of L-type Ca^{2+}-channels in mediating this response. Direct excitatory effect of cyclopiazonic acid (CPA) on myometrium indicates possible Ca^{2+}-influx via store operated calcium channels (SOCC). Lead failed to evoke any excitatory effect in the concurrent presence of nifedipine (1µM) and CPA (10µM), thus intracellular calcium seems to have negligible role in lead-induced myometrial contraction. In Ca^{2+}-free high K^+ (80 mM) depolarizing solution, lead-induced excitatory effect was attenuated by GF 109203X; which suggests permeation of lead through L-type Ca^{2+} channels to replace Ca^{2+} which directly activates protein kinase C (PKC) to produce excitatory effect. Thus lead seems to interact with calcium in buffalo myometrium in a dynamic fashion and exerts calcium-depending and calcium-mimicking effects; and therefore, lead is likely to have adverse effect on reproductive functions in buffaloes.

Key words: Buffalo, Lead, Myometrium, PKC, VDCC

Ruminants are most susceptible to lead toxicity due to their feeding behavior and ability to convert lead particles to soluble lead acetate. Higher lead was documented in maternal blood and placenta of aborting cattle and buffaloes and also soil and vegetation from different parts of India (Gowda *et al*. 2003). Lead reportedly induces infertility, miscarriage, premature membrane rupture, pre-eclampsia, pregnancy hypertension and premature delivery (Flora *et al*. 2011). Lead increases amplitude of spontaneous contraction with reduction in frequency induced by oxytocin and $PGF_{2\alpha}$ in bovine myometrium (Yildirim and Macun 2013), and produces inhibitory effect on rat myometrium (Nakade *et al*. 2016). Lead evokes diverse actions (excitatory or inhibitory) on different tissues by modulating membrane receptors, downstream signaling molecules, calcium signaling or neurotransmitter release (Hore *et al.* 2013, Nakade *et al.* 2016).

Spontaneous myometrial rhythmicity and uterotonics-induced myometrial contraction are regulated by calcium, and lead alters the dynamics of calcium either by interfering with the activity or metabolism of calcium or replacing it in physiological processes (Gupta and Fahim 2007). Lead exhibits extracellular calcium-independent vaso-reactive action in rat (Valencia *et al.* 2001) while blocks Ca^{2+} entry through VDCC and/or stimulates α-adrenoceptors-adenylyl cyclase-cAMP pathways in rat myometrium (Nakade *et al.* 2016). Calcium homeostasis mechanisms in myometrial smooth muscle differ from that in vascular beds, as myometrium exerts spontaneous rhythmicity with phasic and tonic contractions which too differ between cyclic and pregnant animals. Effect of lead on endocrine regulation is well delineated in females (Qureshi and Sharma 2012) but limited studies have been undertaken to evaluate its impact on cellular signaling especially calcium regulating pathways in uterine smooth muscle. In view of the continuous exposure of buffaloes to lead and its ability to alter calcium homeostasis in smooth muscles to produce adverse effects on reproduction, present study was undertaken to unravel the effect of lead on buffalo myometrium and its underlying mechanistic pathways, especially calcium signalling cascade.

MATERIALS AND METHODS

Chemicals: Dilutions of the required concentration were

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made in the freshly prepared Ringer-Locke solution (RLS) on the day of use. The solvents used had no marked effect on response of tissue at the concentration(s) used. Stock solution of lead acetate (100 mM) was prepared in distilled water.

Tissue source: Diestrous stage uteri along with ovaries were collected from the local abattoir immediately after slaughter from adult cyclic nondescript buffaloes and transported to laboratory in chilled (4.0 ± 0.5°C) Ringer-Locke solution (RLS) having pH of 7.4. Experimental protocol was approved by the Institutional Animals Ethics Committee as per the provision of CPCSEA of Government of India.

Tissue preparation and tension measurements: Tissue preparation and tension measurements were done as per Sharma *et al.* (2014). Effect of lead on tonic and phasic contractions and alterations in amplitude and frequency of myometrial spontaneity was recorded in isolated myometrial strips of buffaloes up to a maximum concentration of 0.1mM (10^{-4}M) starting from 0.1nM (10^{-10}M). Further, to unravel the mechanistic pathways of lead-induced alterations in myometrial activity, with particular reference to calcium regulatory pathways, effect of lead was studied in normal RLS, Ca^{2+}-free RLS, nifedipine (1µM), cyclopiazonic acid (CPA; 10µM), nifedipine (1µM) + CPA (10µM), GF 109203X (5µM), Ca^{2+}-free high K^+ RLS and Ca^{2+}-free high K^+ RLS + GF 109203X (5µM).

Data recording and statistical analysis: Mean integral tension (MIT) for 8 min along with E_{max} and EC_{50} values were calculated as per Sharma *et al.* (2014). Isometric developed tension (IDT) values were obtained by measuring the mean amplitude of all the contractions recorded over a period of time and frequency of contraction was determined as the mean number of contractile cycles observed during the same period (Chaud *et al.* 1997). Amplitude of the tonic contractions was measured from the baseline while phasic contractions were measured by subtracting the average cyclic maxima from the mean integral tension of contractions (Kawamata *et al.* 2007). Results are expressed as mean ± SEM. Multiple mean values were analyzed using two-way ANOVA followed by Bonferroni post hoc test to compare between different treatments.

RESULTS AND DISCUSSION

Lead (0.1nM - 0.1mM, at 1 log dose unit) produced concentration-dependent excitatory effect on buffalo myometrium as shown in Fig. 1A and effect was significantly (P<0.05) higher on tonic contractions while almost negligible on phasic contractions (Fig. 1B). It also produced dose-dependent increase in amplitude of contractions but effect on frequency of myometrial

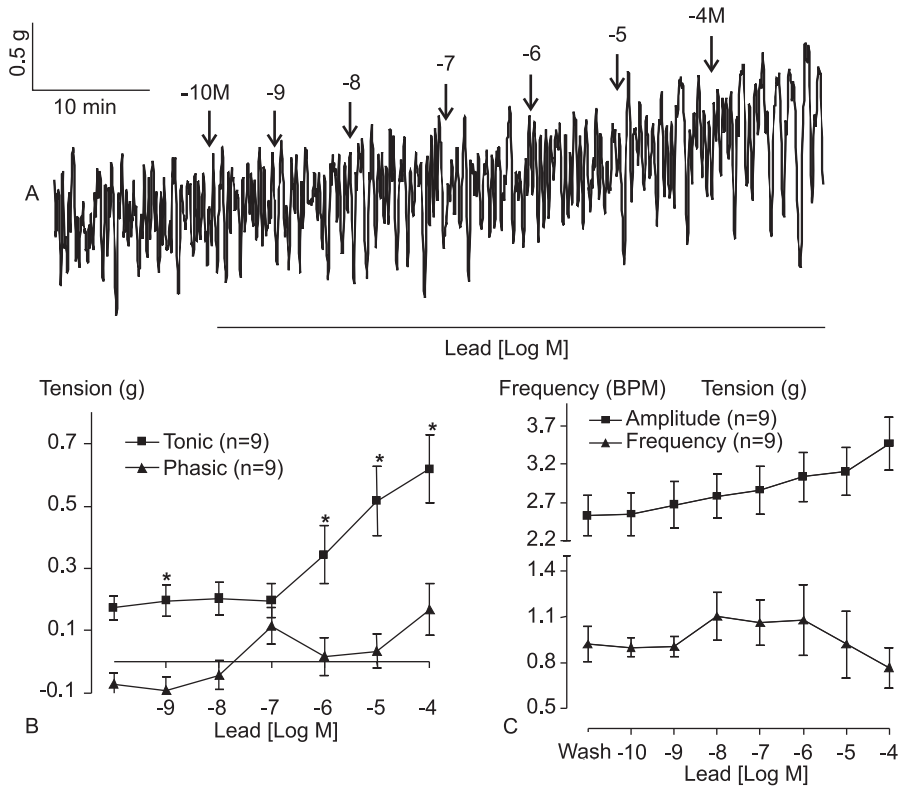

Fig.1 (A–C). Representative physiograph recording showing effect of cumulative concentrations of lead (0.1nM – 0.1mM) on spontaneity of myometrial strips (A), effect on tonic and phasic contractions (B; n=9) and basal amplitude and frequency of contractions (C; n=9) on myometrium of non-pregnant buffalos. Data were analyzed by two-way ANOVA followed by Bonferoni post hoc tests. *P<0.05 vs phasic.

Table 1. Comparative potency (pD_2) and efficacy (E_{max}) of lead on isolated myometrial strips of buffaloes in the absence and presence of different modulators of calcium signalling pathways

Treatment	pD_2	E_{max} (g)
Lead (n=9)	6.41 ± 0.21	0.69 ± 0.08
Ca^{2+}-free RLS + Lead (n=6)	7.7 ± 0.76	0.13 ± 0.04*
Nifedipine (1µM) + Lead (N=6)	8.12 ± 0.70	0.21 ± 0.05*
GF 109203X (5µM) + Lead (n=6)	6.33 ± 0.63	0.31 ± 0.11*
Ca^{2+}-free high K^+-depolarizing solution + Lead (n=6)	6.66 ± 0.41	0.45 ± 0.09
Ca^{2+} free high K^+ depolarizing + GF109203X (5µM) + Lead (n=6)	4.74 ± 1.01	0.04 ± 0.02*

Data expressed as Mean ± SEM, n denotes the number of animals, *P<0.05 vs lead alone.

contraction was inconsistent (Fig. 1C). The minimum threshold concentration of lead for eliciting apparent contractile effect was 0.1 nM and maximal effect was observed at 0.1 mM as shown in the representative physiograph recording (Fig. 1A). Fig. 2 illustrates the cumulative dose-response curve of lead on buffalo myometrium. Maximum myometrial contraction (E_{max}) and potency (pD_2) of lead are shown in Table 1.

Effect of lead was studied separately in Ca^{2+}-free RLS and in the presence of nifedipine, a L-type Ca^{2+} channel blocker, in normal RLS to explore the possible role of extracellular calcium in lead-induced myometrial contraction in buffaloes. After pre-incubation of myometrial strips either in Ca^{2+}-free RLS or with nifedipine (1µM) for 30 min, myometrial rhythmicity was completely abolished. Following complete removal of Ca^{2+} from RLS (Ca^{2+}-free RLS) or blockade of Ca^{2+} gating through L-type calcium channels, the dose response curve of lead was significantly

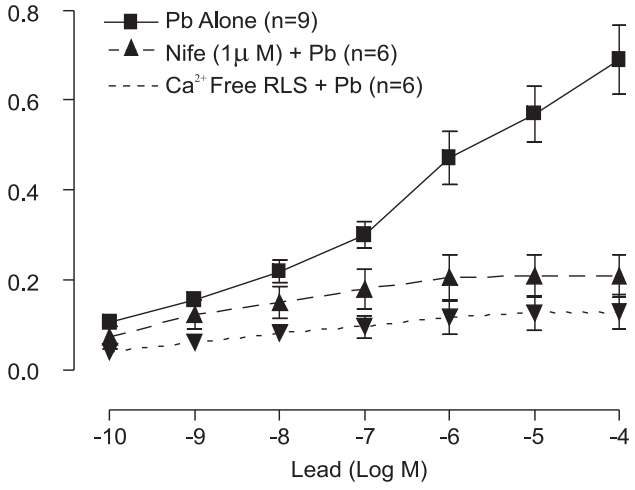

Fig.2. Cumulative concentration response curves of lead in normal RLS, in Ca^{2+}-free RLS and in the presence of nifedipine (1 µM) on isolated myometrial strips of non-pregnant buffaloes. Vertical bars represent SEM. Data were analyzed by two-way ANOVA followed by Bonferoni post hoc tests. *P<0.05 vs lead alone.

(P<0.05) shifted towards right (Fig. 2) with significant decrease in maximal efficacy as evident from the values of E_{max} and pD_2 of lead in Ca^{2+}-free RLS and in the presence of nifedipine (Table 1).

Lead evoked concentration-dependent contractile effect on buffalo myometrium contrary to the inhibitory effect on rat myometrium (Nakade *et al.* 2016). Compared to rat aortic rings (Valencia *et al.* 2001), relatively much lower concentration of lead (0.1 mM Vs 3.1 mM) was required to induce maximal contractile effect on buffalo myometrium; thus suggesting higher sensitivity of buffalo myometrium to lead. Possibility of such differences due to involvement of different signaling pathways in different smooth muscle and/ or between different species cannot be ruled out.

Calcium is the primary signal for activation of smooth muscle contractile proteins and it plays an important role in generating uterine myogenic spontaneity (Tribe 2001) or uterotonic effect of spasmogens (Sharma *et al.* 2014). Significant attenuation of lead-induced myometrial contraction in buffalo myometrium in Ca^{2+}-free RLS in the present study suggests dependence of uterotonic effect of lead on extracellular Ca^{2+} and L-type Ca^{2+} channels seem to play major role in initiation of contractile effect and this observation is in conformity with the report of Sharma *et al.* (2014) in histamine-induced myometrial contractions in buffaloes. Extracellular calcium-dependent contractile effect of lead has also been reported in rabbit mesenteric artery (Watts *et al.* 1995) and canine tracheal smooth muscles (Sopi *et al.* 2009). But on the contrary, vaso-reactive action of lead in rat aortic rings is known to be extracellular calcium-independent (Valencia *et al.* 2001).

Myometrial strips were pre-incubated with cyclopiazonic acid (CPA 10µM) alone, a selective sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) blocker, and 10µM CPA + 1 µM nifedipine before recording the myometrial response to 10 µM of lead. In this protocol, Ca^{2+} entry through VDCC was inhibited by nifedipine and thereafter, two successive doses of histamine (10 µM each) were used to release the intracellular Ca^{2+} store from SR while simultaneously blocking the reuptake of Ca^{2+} back to SR using CPA. Lead failed to evoke any contraction in myometrial strips in the presence of intracellular calcium as shown in Fig. 3A. In a separate experimental protocol, myometrial tissues were mounted in Ca^{2+}-free RLS and effect of lead was studied in the presence of CPA (10 µM). As shown in Fig. 3B, compared to the effect in normal RLS, lead failed to evoke any appreciable contractile effect. Lead also failed to produce any contractile effect following pre-incubation of myometrium with nifedipine and CPA (Fig. 3C) even in the absence of spasmogen-induced stimulation as shown in Fig. 3A.

Excitatory effect of lead on buffalo myometrium after blockade of sarcoplasmic reticulum Ca^{2+} pump by CPA indicates increased availability of intracellular Ca^{2+} in buffalo myocytes due to elevation in Ca^{2+} influx via store operated calcium channels (SOCC) and this finding is in conformity with rat uterine myocytes (Shmigol *et al.* 1999).

After blocking the entry of extracellular Ca^{2+} through L-type Ca^{2+} channels and allowing the release of Ca^{2+} from SR while simultaneously blocking SERCA by CPA to prevent the reuptake of Ca^{2+} back into SR, lead failed to evoke any contractile effect on buffalo myometrium. This observation evidently suggests that cytosolic Ca^{2+} derived from intracellular sources plays negligible role in mediating lead-induced contractile effect in buffalo myometrium and our findings are in agreement with the observations of Valencia *et al.* (2001) who have also reported intracellular Ca^{2+}-independent contractile effect of lead in rat aorta.

To investigate the involvement of protein kinase C (PKC) pathway in uterotonic action of lead, myometrial strips were pre-incubated with GF 109203X (5 µM), a selective protein kinase C inhibitor, for 45 min before recording myometrial response to lead. As shown in Fig. 4, lead-induced myometrial contraction was significantly ($P<0.05$) attenuated in the presence of PKC inhibitor but without any change in potency. As evident from the E_{max} and pD2 values of lead in the presence of GF 109203X (Table 1).

To assess whether lead produces uterotonic action directly by replacing Ca^{2+}, cumulative dose response of lead was recorded in Ca^{2+}-free high K^+ (80 mM) depolarizing solution. To overcome the influence of calcium in this protocol, myometrial strips were initially exposed to Ca^{2+}-free Ringer-Locke solution containing 3 mM EGTA and thereafter, two successive washings were given with this solution at 4-5 min interval and thereafter, tissues were incubated with Ca^{2+}-free high K^+-depolarizing solution without EGTA (to open the L-type Ca^{2+} channels) before recording the dose response of lead as shown in Fig. 5A. The E_{max} and potency values of lead are presented in Table 1.

To elucidate whether contractile action of lead on buffalo myometrium is PKC- dependent or not, effect of lead was studied in Ca^{2+}-free high K^+ depolarizing solution in the presence of GF109203X (5µM). GF109203X significantly ($p<0.05$) attenuated the lead-induced myometrial contraction when compared to that of lead alone in Ca^{2+}-free high K^+-depolarizing solution or lead alone in normal RLS as depicted in Fig. 5B, C, respectively and the respective E_{max} and pD_2 values of lead are presented in Table 1.

Protein kinase C (PKC) is an integral component of the signal transduction in calcium messenger system. Calcium activates PKC in the range of 0.1-10 µM (Watts *et al.* 1995) similarly lead also activates PKC at lower concentrations by interacting with Ca^{2+} activated site in C_2 domain (Sun *et al.* 1999). At micro-molar concentration, lead is reported to increase PKC *beta* and decrease PKC *alpha* translocation from cytosolic to particulate fraction in H4-IIE-C3 hepatoma cell (Tonner and Heiman 1997). In the present study, GF109203X, a PKC inhibitor, significantly inhibited lead-induced myometrial contraction; thus suggesting lead-

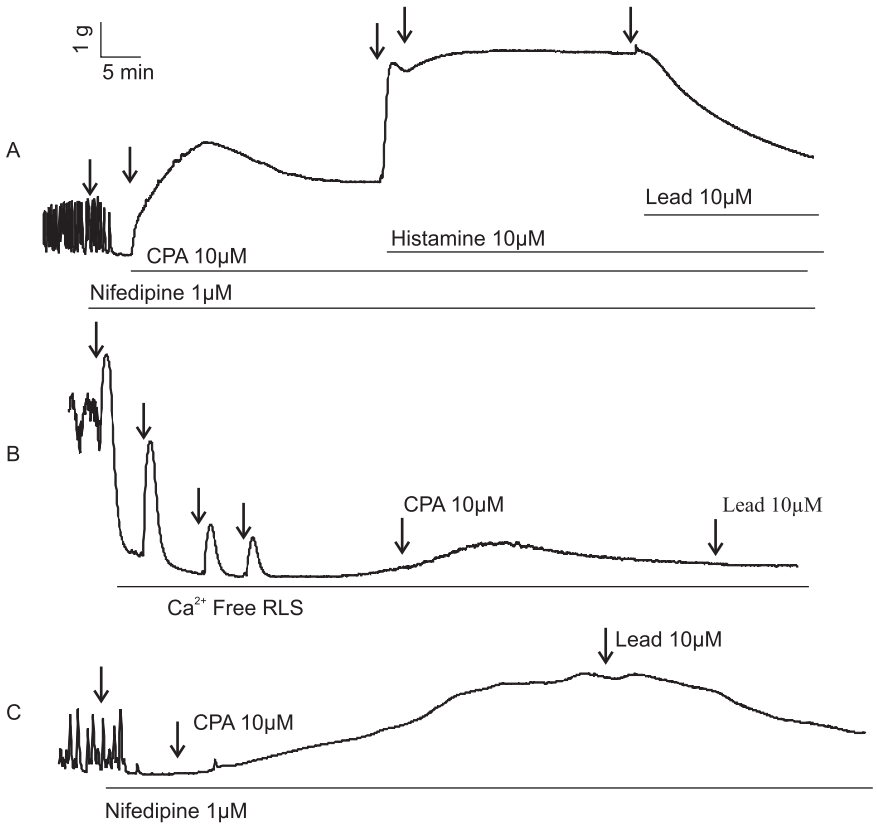

Fig.3. Representative physiograph recordings showing effect of lead (10 µM) in the presence of nifedipine (1 µM), CPA (10 µM) and two successive doses of histamine (10 µM each) in normal RLS (A), in the presence of CPA (10 µM) in Ca^{2+}-free RLS (B) and in the presence of nifedipine (1 µM) and CPA (10 µM) in normal RLS (C) on isolated myometrial strips of non-pregnant buffaloes.

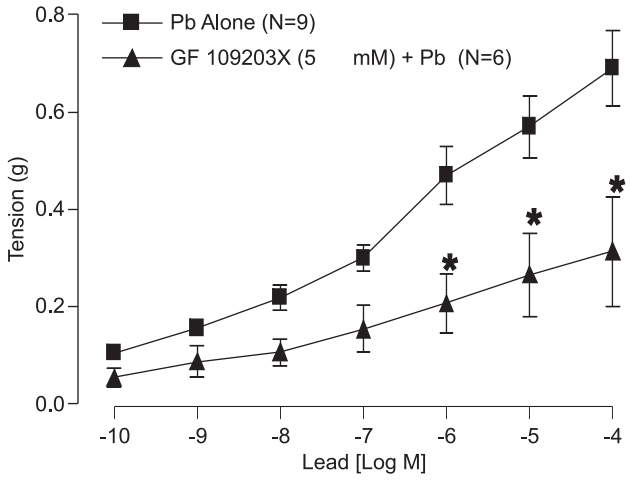

Fig.4. Cumulative concentration response curves of lead in the absence and presence of GF109203X (5 µM) on isolated myometrial strips of non-pregnant buffaloes. Vertical bars represent SEM. Data were analyzed by two-way ANOVA followed by Bonferroni post hoc tests. *P<0.05 Vs lead alone.

in Ca^{2+}-free high K^+ (80 mM) solution and found that lead produced myometrial contraction even in the absence of extracellular calcium. In this protocol, when membrane L-type Ca^{2+} channels were opened by the depolarizing solution, lead permeated into the cell through Ca^{2+} channels and evoked its contractile effect. Entry of lead through L-type Ca^{2+}-channels have been reported in different tissues (Marchetti 2013).

Lead-induced myometrial contraction was attenuated by GF109203X even in the absence of calcium in Ca^{2+}-free high K^+-depolarising solution, thus suggesting direct activation of protein kinase C by lead in the absence of extracellular Ca^{2+}. Our observation on buffalo myometrium is in agreement with PKC-dependent calcium mimicking response of lead in vascular smooth muscle (Watts *et al.* 1995). Therefore, similar consequences of direct activation of PKC by lead at low concentrations over a longer period of time in buffalo myometrium and consequent deleterious effects on reproduction in buffaloes cannot be ruled out.

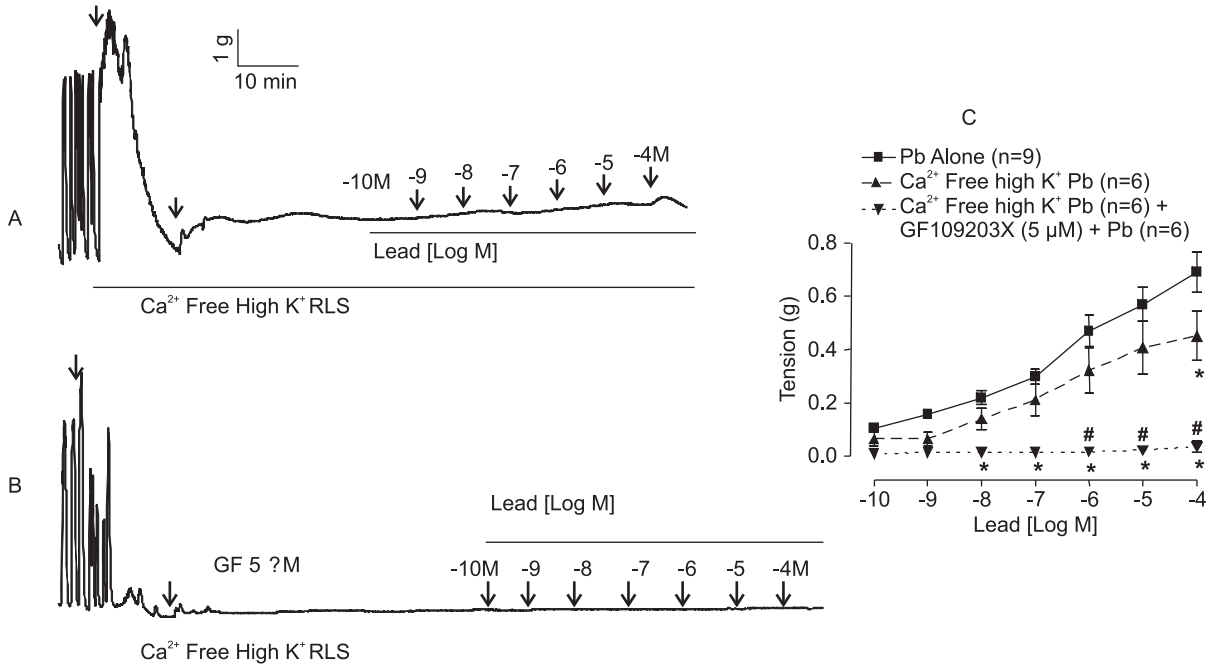

Fig.5. Effect of cumulative concentrations of lead (0.1nM – 0.1mM) in the absence (A) and presence of GF109203X (5 µM) in Ca^{2+}-free high K^+ (80 mM) solution (B) while figure C depicts the cumulative concentration response curves of lead alone in normal RLS and in the absence and presence of GF109203X (5 µM) in Ca^{2+}-free high K^+ depolarizing solution. Vertical bars represent SEM. Data were analyzed by two-way ANOVA followed by Bonferoni post hoc tests. *p <0.05 Vs lead alone in normal RLS, #p<0.05 Vs lead in Ca^{2+}-free high K^+-depolarizing solution.

induced possible activation of PKC and the uterotonic effect is similar to lead-induced vasoconstriction (Watts *et al.* 1995). But no such information is available on myometrium of any species including buffaloes.

In the absence of required concentration of calcium for activation of PKC, lead is reported to activate PKC directly (Schanne *et al.* 1997) to exert a calcium-mimicking response. To establish the Ca^{2+}-replacing and Ca^{2+}-mimicking action of lead, we investigated the effect of lead

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