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ROLE OF Ca^{2+} ON UTERINE FORCE STIMULATED BY A GLYCOSIDE FROM THE ROOT OF *DALBERGIA SAXATILIS*

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Abstract : Uterine muscle contraction is dependent on external Ca^{2+} and Ca^{2+} release from cytoplasmic storage sites. In this study, the mechanism of Ca^{2+} mobilization in uterine muscle cells by glycoside, *dalsaxin*i, isolated from the root of *D. Saxatilis* was investigated in the rat. Uterine muscle contractility stimulated by dalsaxin was concentration dependent (ED_{50} 0.13 mg/ml) and was significantly attenuated (85%; $P<0.01$) in Ca^{2+}–free physiological solution and in solutions containing verapamil (0.06-0.48 µmol). The small transient contraction observed in Ca^{2+}–free medium was further suppressed by caffeine (2 mmol) and completely abolished in solutions containing Lanthanum chloride [(La^{3+}), 2 mmol]. Contractions stimulated by the glycoside were unaffected by amiloride (50–83 µmol) in Ca^{2+}–free and Ca^{2+}–containing media. Dalsaxin also altered the pattern of uterine contraction stimulated by high potassium depolarization from fast-phasic to a sustained but transient plateau. It is concluded that dalsaxin causes uterine muscle contraction by mobilizing external Ca^{2+} through predominantly a voltage-dependent Ca^{2+} channel.

Key words : dalsaxin, calcium, glycoside, cAMP, depolarization, uterus, contraction, channel

INTRODUCTION

The role of intracellular calcium, $[Ca^{2+}]_i$, in the excitation-contraction coupling of a variety of muscular tissues is well established (1,2). In smooth muscle cells, elevation of $[Ca^{2+}]_i$ is due primarily to release from intracellular storage sites such as the sarcoplasmic retculum and influx from extracellular compartment. External Ca^{2+} influx is thought to involve voltage-dependent (VOC) as well as receptor-operated (ROC) Ca^{2+} channels of the plasma membrane. Voltage-activated Ca^{2+} current of the myometrium is predominantly of the L-type (3). This increase in $[Ca^{2+}]_i$ is a pre-requisite for calcium-calmodulin interaction and activation of myosin light chain kinase (MLCK) activity. Phosphorylation of the 20,000 dalton light chain of smooth muscle myosin by MLCK increases myosin ATPase activity and promotes contraction (4).

In the continued search for uterine spasmogen of plant origin with minimum of

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side effects but maximum potency, aqueous ethanolic root extract of *Dalbergia saxatilis* (Leguminosae) was screened for uterine muscle contractility. A triterpenoid glycoside (dalsaxin) was isolated from the extract as an active contractile principle. The isolated glycoside was found to be partially soluble in water but completely soluble in warm methanol. Acid hydrolysis showed that it contained fructose as the only hydrolysable sugar (unpublished observations). Uterine muscle responses to the glycoside were unaffected by varying concentrations of atropine sulphate but significantly inhibited by isoprenaline, and by atipamezole hydrochloride, an α_2-adrenoceptor antagonist. This finding suggests that dalsaxin's contractile activity is mediated via cell membrane α_2-adrenoceptor activation. In the present study, the role of Ca^{2+} in the *in vitro* uterine force generated by this active principle was investigated in the rat.

METHODS

Animals

Female Wistar rats of breeding age, weighing approximately 250 g were used for the experiments. They were supplied by the Faculty of Agriculture, University College, Dublin and housed under specific pathogen free (SPF) conditions with a 14 hr/10 hr light/dark schedule. The animals were provided standard rat chow and tap water *ad libitum*. They were primed with estradiol benzoate (Sigma, USA) at a dose of 0.1 mg/kg body weight (subcutaneously) 24 hours before the commencement of the experiments.

Preparation of uterine muscle tissue and isometric contraction studies:

The animals were killed by stunning and decapitation. The uterine horns were trimmed free of fat and transfered to Krebs solution that was continuously bubbled with 0_2 (95%), Co_2 (5%) gas mixture, maintained at 37°C (pH 7.4). The physiological solution had the following composition (mmol): NaCl (118), $CaCl_2$ (2.5), KCl (4.7), $NaHC0_3$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), and glucose (11).

Uterine strip, about 12 mm in length was cut out and suspended vertically in a 30 ml organ bath by means of ligatures attached at one end to a specimen holder and at the other to a myograph attached to an isometric force displacement transducer/amplifier. The latter was connected in parallel to a physiological recorder (MacLab/2e instrument) and an LC 475 Macintosh computer screen for displaying isometric contractions. Resting tension in the muscle strip was readjusted, just sufficient to remove the slack, and the preparation allowed to stabilize usually within 40 min of mounting.

After spontaneous contractions were regular, the role of extracellular Ca^{2+}, $[Ca^{2+}]_o$ on dalsaxin modulated uterine force was assessed by replacing $CaCl_2$ in the perfusate with 2.0 mmol EGTA (Sigma, USA). The incubation time with EGTA was 5 minutes and was maintained constant throughout the experiments for maximum chelation of extracellular Ca^{2+}. Contractile response to dalsaxin in Ca^{2+}–free medium was assumed to reflect Ca^{2+} release from

intracellular storage sites. In experiments in which the trivalent cation, lanthanum chloride (La^{3+}; Sigma, USA) was used to prevent Ca^{2+} efflux across the plasma membrane, EGTA was omitted so that the solution was nominally Ca^{2+}–free. Only 2.0 mmol La^{3+} was used where necessary.

The ability of dalsaxin to release Ca^{2+} from the sarcoplasmic reticulum via Ca^{2+}–induced, Ca^{2+}–release (CICR) mechanism was verified with caffein (Merck, Germany) to a bath concentration of between 2–10 mmol. Verapamil (0.06–30 µ mol; Knoll AG, Germany) was used as a voltage-dependent Ca^{2+} channel blocker. Excess K^+ (140 mmol) depolarization was used to further assess the contribution of VOC to dalsaxin mediated myometrial contraction.

To study the effect of other ion fluxes, amiloride (50–80 µ mol; Sigma, USA) was used as an inhibitor of Na^+/H^+ exchanger system.

In all the experiments, a minimum of 3 min. was allowed for individual tissue responses before being washed 2–3 times with Krebs solution. Except otherwise indicated, concentration of test substances given in the text are all final nutrient bath concentrations.

Analysis of Data

Differences between the means of the control and the experimental groups were compared using paired and unpaired Student's t–test as appropriate. In the legend to figures, n is the number of animals used in each experiment.

RESULTS

Utrine muscle responses to dalsaxin in a normal physiological solution containing 2.5 mmol $CaCl_2$ were depicted by forceful but transient single contraction which increased in both amplitude and frequency as the concentration of the test drug was increased. The ED_{50} was 0.13 mg/ml, with 0.04 mg/ml as the lowest active concentration (Fig. 1).

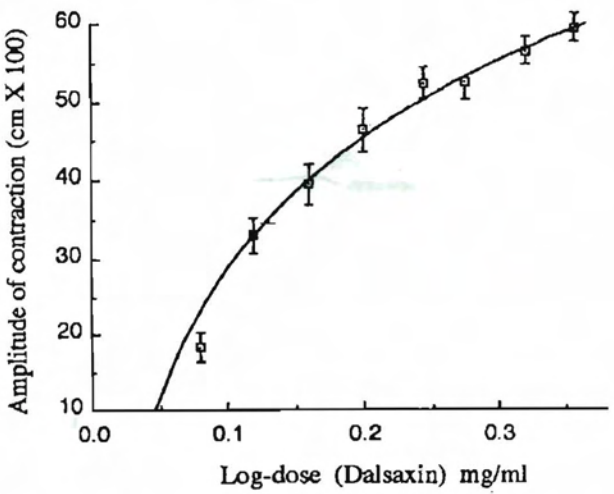

Fig. 1 : Concentration-dependent effects of dalsaxin on rat uterine muscle contractions.
Data represent the mean ± SEM (n=5).

Effect of dalsaxin on uterine force in Ca^{2+}–free medium and with verapamil hydrochloride :

Removal of Ca^{2+} from the incubation medium caused a significant decrease ($P<0.01$; $n = 6$) in myometrial contraction and in the tissue responses to 0.21 mg/ml dalsaxin (Fig. 2a). The onset of responses to dalsaxin was immediate and characterized by a small but slow plateau that decayed towards the baseline after 45

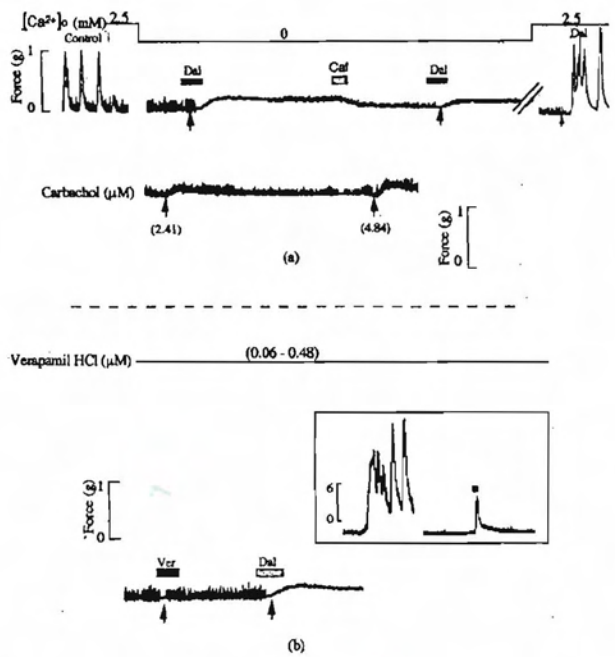

Fig. 2 : Effects of (a) extracellular calcium and (b) verapamil (0.06–0.48) μ mol) on dalsaxin (0.21 mg/ml) induced myometrial contraction. Block (•) in inset shows contractile response to carbachol (4.8 μ mol) in physiological solution containing verapamil (30 μ mol). Note the relaxation of tension caused by caffeine (caf) in (a) above. (n=6).

sec. Addition of caffeine further suppressed this contractile response to dalsaxin in Ca^{2+}–free physiological solution. Responses to carbachol (2.41–4.84 μ mol) in the absence of $[Ca^{2+}]_0$ were similarly attenuated ($P<0.01$) and characterized by one brief but small contraction presumably due to Ca^{2+} release from intracellular storage sites. (Figs. 2a and b).

In an attempt to obtain further evidence for the involvement of $[Ca^{2+}]_0$ in dalsaxin-mediated excitation-contraction coupling, varying doses of verapamil (0.06–0.38 μ mol) were used as voltage-dependent Ca^{2+} channel blockers. As in the experiments in Ca^{2+}–free medium, verapamil significantly suppressed (90%; $P<0.01$) uterine muscle responses to dalsaxin (0.21 mg/ml; Fig. 2b) regardless of the dose of verapamil used. The extent of change in uterine muscle contractility with verapamil was comparable to the activity in Ca^{2+}–free medium. The optimal concentration needed to suppress dalsaxin activity was 0.06 μ mol which was found ineffective in carbachol induced contractile force. However, responses to carbachol (4.8 μ mol) were significantly inhibited (52.9%; P,0.05) when a superthreshold concentration of verapamil (30 μ mol) was used. The tissue responded with a transient single contraction (block (■) in inset, Fig. 2b). This dose of verapamil had been reported to completely block K^+–induced Ca^{2+} mobilization in smooth muscle preparations (5).

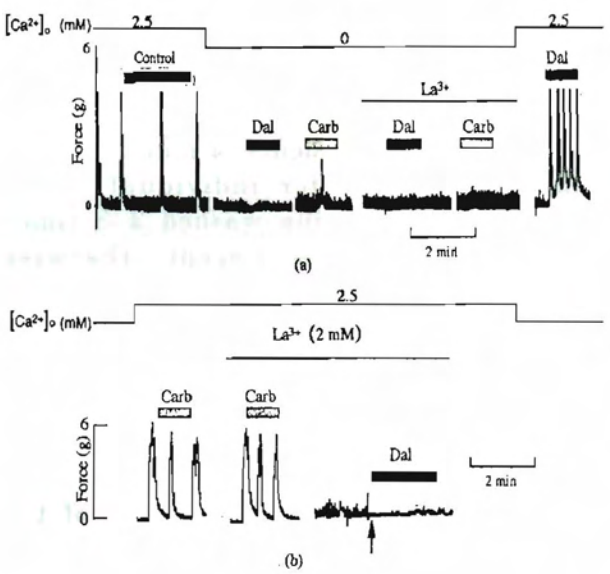

Fig. 3: Effect of extracellular calcium and La^{3+} on dalsaxin (0.21 mg/ml) and carbachol (2.4 μ mol) induced myometrial contraction. Arrow indicates the point at which dalsaxin was applied. (n=5).

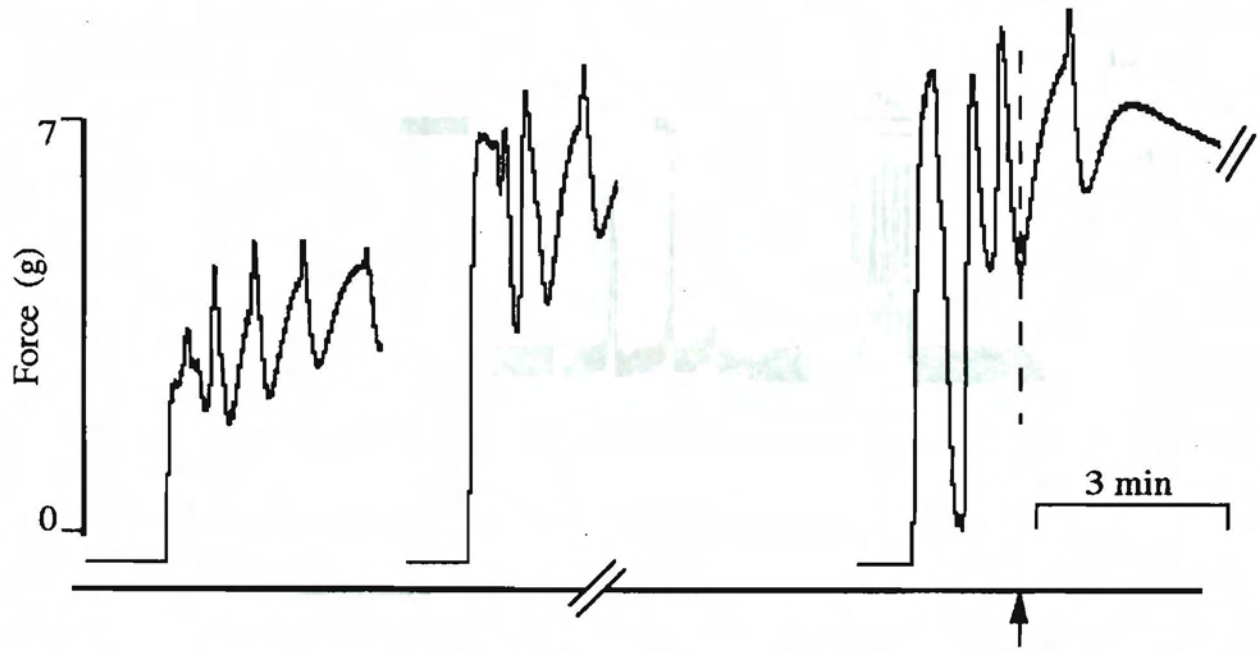

Fig. 4 : Uterine muscle preparation depolarized with excess KCl (140 mmol) followed by dalsaxin (0.24 mg/ml). Dalsaxin was applied at the point indicated by the arrow after the 4th depolarization with excess K^+. The duration of the contracture after dalsaxin is indicated under the horizontal bar (2 min). (n=5.).

Responses to Lanthanum Chloride (La^{3+}) :

In the presence of La^{3+} (2 mmol) to block surface membrane Ca^{2+} efflux, carbachol (4.8 µ mol) and dalsaxin (0.21 mg/ml) were unable to elicit any contractile activity in Ca^{2+}–free physiological solution. However, the transient contraction with carbachol in Ca^{2+}–free solution was restored when La^{3+} was removed (Fig. 3a). On return to Ca^{2+}–containing medium, contractile response to carbachol in the presence of La^{3+} was not significantly different from the control experiment (that is, minus La^{3+}). This was in sharp contrast to complete loss of uterine motility seen with dalsaxin (Fig. 3b).

Effect of high K^+ depolarization :

Amplitude of contraction following repeated high K^+ depolarization in Ca^{2+}–containing medium was sustained and repetitive with each application. This fast-phasic contraction was replaced by a sustained plateau contracture which lasted for approximately 2 min when a submaximal concentration of dalsaxin (0.24 mg/ml) was added without wash (Fig. 4).

Responses to amiloride :

In experiments in which amiloride (50–83 µ mol) was used to inhibit Na^+/H^+ exchanger, no significant alteration in

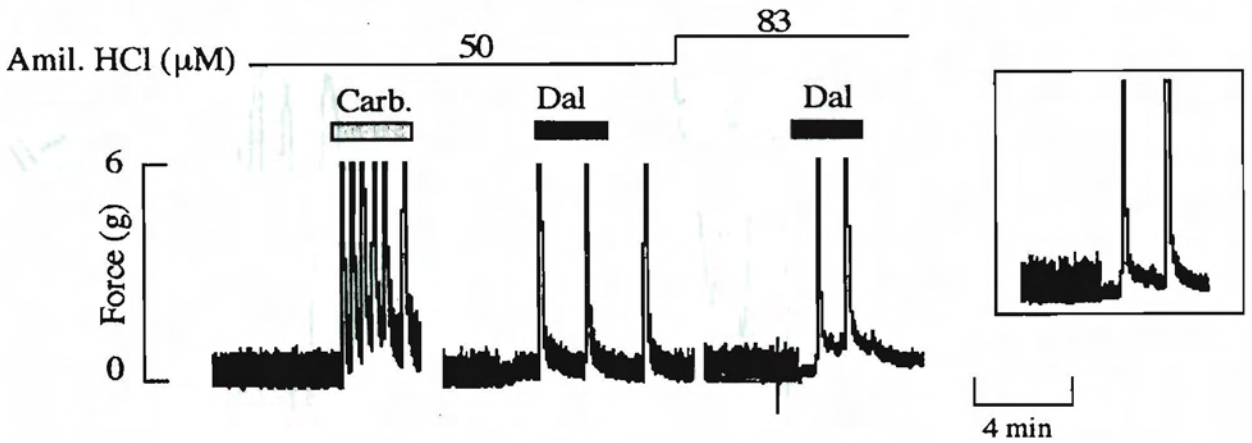

Fig. 5 : Effect of amiloride (50–80 μ mol) on carbachol (2.4 μ mol) and dalsaxin (0.21 mg/ml) induced uterine force. Inset is dalsaxin control contraction in the absence of amiloride.(n=5).

contractile responses to carbachol and dalsaxin were observed (Fig. 5).

DISCUSSION

In this study, it was established that dalsaxin-induced uterine force is mediated predominantly through Ca^{2+} influx from the extracellular compartment since these contractile responses were significantly suppressed ($P<0.01$) in the absence of $[Ca^{2+}]_o$. The very small transient contraction seen in Ca^{2+}–free medium and in solutions containing the voltage-sensitive Ca^{2+} channel blocker, verapamil could be attributed to Ca^{2+} mobilization from intracellular storage sites such as the sarcoplasmic reticulum and the mitochondria (6, 7) (Fig. 2a,b).

Unlike dalsaxin, carbachol was found to elicit uterine muscle contraction in Ca^{2+}–free medium even when a superthreshold concentration of verapamil (30 μ mol) was used [block (■) in inset, Fig. 2b). The contractile response to verapamil was small and transient but was 63% greater ($P<0.05$) than that observed with dalsaxin, suggesting strongly that the degree to which these uterine contractants can mobilize stored Ca^{2+} varies and that this intracellular Ca^{2+} pool is small and easily depleted. A number of uterine muscle contractants including oxytocin, acetylcholine and carbachol are known to generate inositol, 1, 4, 5–trisphosphate (IP_3) which evokes Ca^{2+} release from intracellular storage sites in the rat and guinea pig smooth muscle cells (8,9) and thus elicit contractions in these preparations. It is likely that dalsaxin also utilizes IP_3 to mobilize Ca^{2+} from the intracellular storate sites or may alternatively act directly on the sarcoplasmic membrane to release stored Ca^{2+}. However, there is no direct evidence from the present study to substantiate these claims.

The ability of caffeine to release Ca^{2+} from the uterine muscle cells was similarly investigated in this study. As illustrated in

Fig. 2a, caffeine (2 mmol) was unable to elicit any contractions in the preparations in a Ca^{2+}–free physiological solution. This is not surprising since a specific Ca^{2+} release mechanism activated by caffeine has yet to be identified in uterine muscle cells. Indeed, it was found that the uterine muscle response to dalsaxin (0.21 mg/ml) in a Ca^{2+}–free medium was further suppressed when caffeine was added. This result and that of other investigators in pregnant (10) and non-pregnant (11) rat myometrium suggest that caffeine suppresses uterine muscle contractions presumably by acting as a phosphodiesterase inhibitor (12) and elevating cAMP concentration since this relaxation effect was mimicked by cAMP in skinned (13) and intact muscle strips and by isoprenaline and theophiline which are known to increase cAMP concentration in myometrial cells (14).

Previous work in ovariectomized, estrogen treated rats showed that when La^{3+} is used to block surface membrane Ca^{2+} transport in uterine muscle cells, all action potential activity is abolished (15). This effect was associated with complete inhibition of net transient inward current and decreased steady-state current under Ca^{2+} regenerative, voltage clamp conditions. This reported effect of La^{3+} agrees well with the results of the present study where this trivalent cation repeatedly blocked contractions stimulated by dalsaxin (0.21 mg/ml) in Ca^{2+}–fee and Ca^{2+}–containing media (Fig. 3a,b). The fact that La^{3+} blocked carbachol-induced uterine force in Ca^{2+}–free, but not in Ca^{2+}–containing solution suggests the involvement of two distinct $[Ca^{2+}]_o$ entry mechanism for these contractants. Unlike carbachol, it is conceivable that Ca^{2+} for dalsaxin stimulated uterine force resides primarily on the outer cell surface membrane. La^{3+} therefore acted as a 'competitive' inhibitor of this membrane Ca^{2+} conductance.

Extracellular solutions containing high concentrations of K^+ depolarize the cell membrane and increase $[Ca^{2+}]_i$, primarily by influx of Ca^{2+} through VOC. This Ca^{2+} influx is blocked by the aminopyridine derivatives (16) as confirmed in the present study with verapamil. As shown in Fig. 4, repeated exposure to high K^+ depolarization gave rise to fast-phasic type contractions which were replaced by a sustained tonic component when 0.24 mg/ml dalsaxin was added. This result confirms and extends the observation that dalsaxin mobilizes Ca^{2+} and stimulates uterine muscle contraction through mechanisms other than a change in membrane potential. However, contribution from these sources must be very negligible.

That the contractile response to dalsaxin in independent on Na^+/H^+ exchanger system is evident from the experiments with amiloride hydrochloride. Amiloride was unable to cause any observable change in uterine force mediated by dalsaxin (Fig. 5), although contributions of Na^+/H^+ exchanger system to Ca^{2+} extrusion and uterine muscle relaxation is negligible under most physiological conditions (17, 18).

In conclusion, the work reported herein has shown clearly that dalsaxin stimulates uterine muscle contraction by mobilizing Ca^{2+} primarily from the extracellular milieu through a voltage-gated Ca^{2+} channel. The contribution of stored Ca^{2+} to the overall

cytosolic-free Ca^{2+} pool and therefore myometrial contraction mediated by dalsaxin is very minimal. Structural elucidation of this uterine muscle contractant as well as investigations on its *in vivo* effects on gestational age will be worthwhile as the compound may well prove useful as a template for yet another synthetic uterotonic principle of clinical significance.

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