



Extracellular Calcium and Induction of Uterine Muscle Contraction by Aqueous Ethanolic Leaf Extract of *Mucuna pruriens*

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ABSTRACT: Calcium (Ca²⁺) serves as an essential signaling molecule in biological systems, regulating a wide range of cellular processes of which uterine smooth muscle contraction is among. The present study was designed to evaluate the involvement of Ca²⁺ on isolated uterine muscle contraction induced by aqueous ethanolic leaf extract of *Mucuna pruriens* (*M. pruriens*). Uterine muscle contraction induced by the extract was concentration-dependent and was completely abolished (100%; P<0.05) in nominally Ca²⁺-free physiological salt solution and in solutions containing (EGTA 1.5 mmol), lanthanum chloride (1.5 and 3 mmol), caffeine (3 and 4.4 mmol) and verapamil (0.007-0.14 μmol). It is concluded that the inability of the extract to produce contractions in Ca²⁺-free media, indicates that it lacks the ability to mobilize calcium from intracellular storage sites. Hence, its uterine stimulatory property is therefore solely dependent on extracellular Ca²⁺.

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Regulation of Ca²⁺ flux across the plasma membrane is essential in skeletal and smooth muscle contractile activities. Regardless of the stimulus, smooth muscle cells use Ca²⁺ as a second messenger signaling molecule to initiate cross-bridge cycling between actin and myosin to develop force (Webb, 2003). This is referred to as excitation-contraction coupling (Bilge *et al.*, 2013). Elevation of cytosolic Ca²⁺ is through Ca²⁺ release from intracellular stores such as the sarcoplasmic reticulum (Izumi, 1994), as well as entry from the extracellular compartment in response to specific stimuli elicited by different agonists or by electrical depolarization (Webb, 2003). The action potential embodied by membrane depolarization initiates many intracellular events seen in all excitable tissues. This is also the case in smooth muscle cells where plasma membrane depolarization leads to the entry of extracellular Ca²⁺ leading to the elevation of cytosolic Ca²⁺ (Wray *et al.*, 2003). *Mucuna pruriens* belongs to the Family- Fabaceae (Sharma *et al.*, 2012). It is a twinning and tropical legume known as velvet bean with other common names such as Agbara (Igbo), Yerepe (Yoruba), Karara (Hausa), itchy bean and many others (Eze *et al.*, 2012). All parts of *M. pruriens* possess valuable medicinal properties (Adepoju and Odubena, 2009). Roots, leaves and seeds of the plant are used to treat different ailments

such as impotence, snake bite, diabetes, cancer and Parkinsonism (Dhanasekaran *et al.*, 2010). In a previous study, it was established that the aqueous ethanolic leaf extract of *Mucuna pruriens* has ability to cause uterine smooth muscle contraction (Francis *et al.*, 2019). In the present study, the role of Ca²⁺ on *M. pruriens*-induced uterine smooth muscle contraction in albino rat was investigated.

MATERIALS AND METHODS

Extraction studies: Fresh leaves of *Mucuna pruriens* were collected and identified by a taxonomist at Bioresource Development and Conservation Center (BDCC), Aku road Nsukka, Enugu state of Nigeria with voucher No-INTERCEDD/1569. The leaves were air dried and pulverised into fine powder, using a conventional hammer mill. This was subjected to cold extraction initially using petroleum ether (70-90) for 72 hours and later with 70% aqueous ethanol for 48 hours with intermittent shaking at two (2) hours interval. The extracts were allowed to dry at room temperature and subsequently stored in the refrigerator at 4°C.

Animals: Non-pregnant Albino rats of breeding age, weighing between 180 g and 250 g were used for the *in vitro* bioassay studies. The rats were supplied by a

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breeder and kept at the Laboratory Animal Unit of the Department of Veterinary Physiology, Michael Okpara University of Agriculture, Umudike, Nigeria. Standard commercial pelleted feed (Vital feeds, Nigeria) and clean drinking water were given to the animals *ad libitum*. Each of the rats received 0.1mg/kg stilboestrol in paraffin oil administered subcutaneously 24 hours prior to the experiments.

Tissue Preparation and Isometric Contraction studies: The animals were sacrificed by stunning and decapitation. About 12 mm segment of uterine horn was removed and attached by ligatures at one end to a specimen holder and at the other to an isometric force displacement transducer (Forte transducer Medicaid, India) connected to a physiograph. This was suspended vertically in a 35 ml conventional organ bath containing physiological salt solution of the following composition (mmol): KCl (4.7); NaCl (118); KH_2PO_4 (1.2); NaHCO_3 (2.5); CaCl_2 (2.5); MgSO_4 (1.2); and glucose (11); and perfused continuously with a gas mixture of 95% O_2 and 5% CO_2 , maintained at 37°C. All uterine strips were put under a little amount of tension and experiments started within 40 minutes following development of stable rhythmic, spontaneous uterine contractions. To obtain evidence for involvement of extracellular Ca^{2+} in *M. pruriens* -mediated uterine smooth muscle contraction, Ca^{2+} -free physiological salt solution was prepared by replacing CaCl_2 with EGTA (1.5 mmol incubated for five minutes for maximum chelation of extracellular Ca^{2+}). Ethylene glycol bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was omitted

in experiments involving Lanthanum Chloride (LaCl_3) and caffeine; hence such solutions were considered nominally Ca^{2+} -free. Caffeine was used to study the ability of the extract to release Ca^{2+} from the sarcoplasmic reticulum via Ca^{2+} -induced, Ca^{2+} -release (CICR) mechanism.

The uterine tissues were also exposed to the voltage-dependent Ca^{2+} channel blocker- verapamil in physiological salt solution containing CaCl_2 . In all the experiments, a minimum of one (1) minute was allowed for tissue contact following which the tissue was washed three (3) times with the physiological salt solution in preparation for subsequent experiment. Concentrations of the test substances presented in the body of this work represent the final nutrient bath concentrations.

Statistical Analysis of Data: Data generated were analyzed using one-way Analysis of Variance (ANOVA). Results are presented as mean \pm standard error of the mean (SEM). Differences between the means of the control and experimental groups were considered significant where $P < 0.05$.

RESULTS AND DISCUSSION

Uterine muscle response in Ca^{2+} -free physiological salt solution: In nominally Ca^{2+} -free physiological salt solution, *M. pruriens* (0.29 – 2.86 mg/ml) was unable to elicit any observable uterine contraction, while uterine response to carbachol (3 μmol) was significantly reduced ($P < 0.05$) Table 1

Table 1: Uterine smooth muscle response to carbachol (3 μmol) and graded concentrations of *M. pruriens* in nominally Ca^{2+} -free and calcium containing physiological salt solutions

Extract/Drug	Uterine muscle responses (mm)	
	Ca^{2+} free media	Ca^{2+} containing media
Carbachol (3 μmol) n=3	3.00 \pm 0.57	7.33 \pm 1.45
<i>M. pruriens</i> (0.29 mg/ml) n=4	0.00	5.00 \pm 1.73
<i>M. pruriens</i> (0.57 mg/ml) n=4	0.00	6.50 \pm 0.95
<i>M. pruriens</i> (1.14 mg/ml) n=4	0.00	4.80 \pm 1.60
<i>M. pruriens</i> (2.29 mg/ml) n=4	0.00	3.80 \pm 1.55
<i>M. pruriens</i> (2.86 mg/ml) n=4	0.00	3.30 \pm 0.63

Moreover, in Ca^{2+} -free physiological salt solution containing EGTA (1.5 mmol), responses to carbachol were further suppressed and subsequently abolished in a fresh Ca^{2+} -free medium (Fig 1a). In contrast, *M. pruriens* was unable to elicit any contraction in physiological salt solution containing the chelator, EGTA (Fig. 1b). This has shown that carbachol was able to elicit myometrial contraction in Ca^{2+} -free media unlike the contraction stimulated by *M. pruriens* which was solely dependent on extracellular Ca^{2+} since the contraction was completely abolished in nominally Ca^{2+} -free media and in Ca^{2+} -free media

containing the chelator EGTA. The residual contraction observed with carbachol in the Ca^{2+} -free media seem to suggest that this uterine muscle contractant, unlike the extract was able to access Ca^{2+} from storage sites in the cell cytosol. That this internally stored Ca^{2+} is small and could easily be depleted was evident from the comparatively lower amplitude and its abolition following wash (Fig. 1a). *M. pruriens* was still unable to elicit any uterine smooth muscle contraction in nominally Ca^{2+} -free physiological salt solution containing caffeine (4.4 mmol) Fig. 2a. On the other hand, carbachol (3 μmol)

elicited uterine muscle response characterized by a small contraction that decayed towards the baseline in 10 seconds (Fig. 2b). The inability of *M. pruriens* to produce any uterine contraction in nominally Ca^{2+} -free physiological salt solution containing caffeine (Fig. 2a) is most likely due to the inability of the extract to utilize the CICR (calcium-release calcium-induced) mechanism to access calcium in the intracellular storage site. Previous studies on the response of rat uterine smooth muscle to caffeine also showed that caffeine lacks the ability to elicit contraction in Ca^{2+} -free physiological salt solution (Uchendu, 1999) unlike the visceral and vascular smooth muscles (Itoh, 1981). Moreover, caffeine failed to elevate intracellular Ca^{2+} in cultured uterine cells (Lynn *et al.*,

1993; Arnaudeau *et al.*, 1994; Holda *et al.*, 1996) and did not increase force of permeabilized or intact fibres (Savineau *et al.*, 1990). When lanthanum chloride (1.5 mmol) was used to block Ca^{2+} efflux in nominally Ca^{2+} -free physiological salt solution, no contraction was elicited by *M. pruriens* (0.86 mg/ml) (Fig. 3a.). However, carbachol (3 μmol) was able to elicit contraction in the media containing LaCl_3 , though no visible difference was observed in the amplitude by the different concentrations of LaCl_3 (1.5 and 3 mmol) used (Fig. 3b). When compared, it was discovered that the uterine response stimulated by carbachol in the presence of La^{3+} was significantly higher ($P < 0.05$) than that stimulated in the presence of caffeine and EGTA (Table 2).

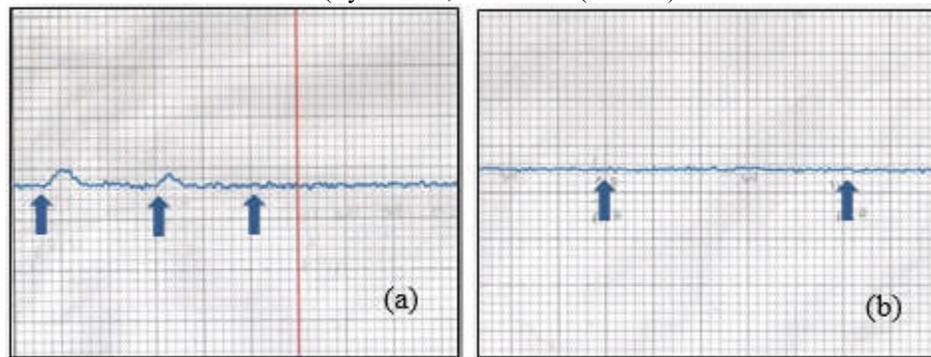


Fig. 1: Uterine smooth muscle response to (a) carbachol (3 μmol) and (b) *M. pruriens* (0.86 mg/ml) in Ca^{2+} -free physiological salt solution. Arrows indicate point of introduction of carbachol or extract.

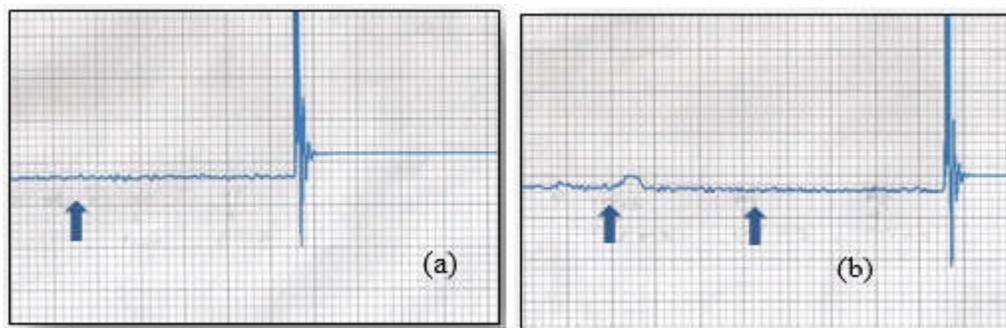


Fig 2: Uterine response to (a) *M. pruriens* (0.86mg/ml) and (b) carbachol (3 μmol) in physiological salt solution containing caffeine (4.4 mmol). Arrows indicate point of addition of extract or carbachol.

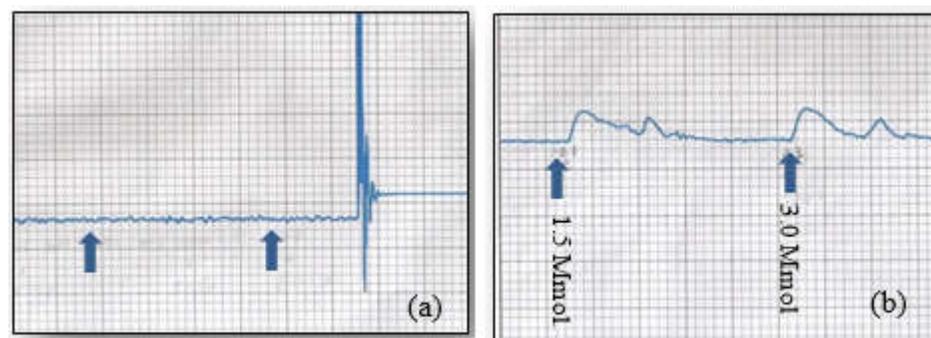


Fig. 3: Uterine smooth muscle response to (a) *M. pruriens* (0.86 mg/ml) and (b) carbachol (3 μmol) in physiological salt solution containing lanthanum chloride. Arrows indicate point of application of *M. pruriens* or carbachol.

The contractile response to carbachol in the presence of LaCl_3 which was much more sustained and of longer duration suggests an increase in the Ca^{2+} pool and by extrapolation, the contractile proteins within the cell cytosol resulting from inhibition of Ca^{2+} extrusion and Ca^{2+} release from intracellular storage sites. It has been suggested that the release of Ca^{2+} from sarcoplasmic reticulum is usually accompanied by Ca^{2+} extrusion and thus irreversible loss of this cation to the extracellular compartment (Leijten and Van, 1986). In addition, La^{3+} is also thought to affect cellular Ca^{2+} homeostasis by actions such as releasing Ca^{2+} from Golgi complexes, thus contributing further to the intracellular Ca^{2+} pool (Zha and Morrison, 1995).

Table 2: Effects of EGTA (1.5 mmol), Caffeine (4.4 mmol) and LaCl_3 (3 mmol) on uterine smooth muscle force induced by carbachol in nominally Ca^{2+} - free physiological solution

Drug	Amplitude (mm)
Carbachol + EGTA (n=3)	2.33 ± 0.33
Carbachol + Caffeine (n=3)	1.67 ± 0.88
Carbachol + La^{3+} (n=3)	$*6.30 \pm 0.33$

*Value significantly different at $P < 0.05$

Effect of verapamil on M. pruriens and Carbachol induced uterine contractions: Varying concentrations of verapamil (0.007 – 0.14 μmol) in physiological salt solution containing CaCl_2 was used as a voltage dependent Ca^{2+} channel blocker. *M. pruriens* did not elicit any uterine smooth muscle contraction even at the lowest concentration of verapamil used (0.07 μmol). However, verapamil suppressed carbachol-induced (3 μmol) contraction in a concentration - dependent manner; with maximal suppression achieved by 0.14 μmol concentration of verapamil ($P < 0.05$) Fig. 4.

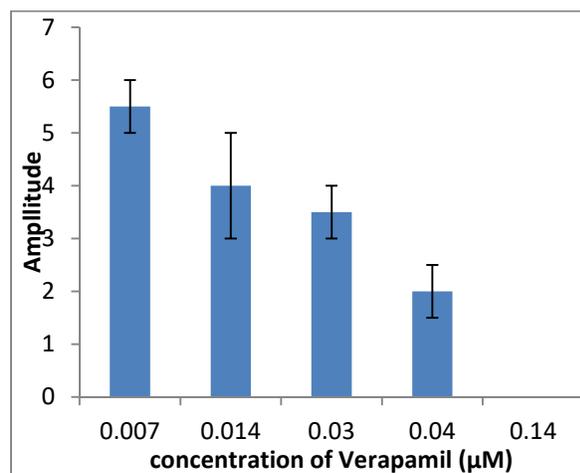


Fig. 4: Effect of graded concentrations of verapamil (0.007- 0.14 μmol) on carbachol (3 μmol) -induced uterine smooth muscle contraction.

Verapamil is a calcium-channel blocking drug that acts by blocking the L-type calcium channels. In those phasic smooth muscles, such as the uterus where action potentials occur, depolarisation and consequent opening of L-type Ca^{2+} channels make this the major source of Ca^{2+} for contraction (Matthew *et al.*, 2004). That *M. pruriens* was unable to access Ca^{2+} in verapamil- containing physiological salt solution is suggestive of the fact that the extract causes calcium entry through the L-type calcium channels alone.

Conclusion: In conclusion, the inability of the extract to produce contractions in Ca^{2+} - free and in nominally Ca^{2+} - free media shows that it lacks the ability to mobilize intracellular calcium. Its uterine stimulatory activity is solely dependent on extracellular Ca^{2+} and therefore, functions through surface receptors.

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