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# Properties of intracellular calcium stores in pregnant rat myometrium

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**1** The properties of the $Ca^{2+}$ stores in myometrium of 21-day pregnant rats were studied by recording the contractile responses of saponin-treated skinned muscles.

**2** After accumulation of $Ca^{2+}$ into the stores in the presence of 5 mM $NaN_3$, inositol 1,4,5-trisphosphate ($InsP_3$) at concentrations exceeding 3 $\mu$M produced a contraction. The amplitude of this contraction was maximal at about 20 $\mu$M. A second application of 20 $\mu$M $InsP_3$ produced a smaller contraction than the first one. However after reloading the stores with $Ca^{2+}$, 20 $\mu$M $InsP_3$ produced a contraction of the same amplitude as the initial one.

**3** After application of 20 $\mu$M $InsP_3$, 1 $\mu$M A23187 still evoked a large contraction. If A23187 was applied first, the subsequent application of $InsP_3$ or A23187 no longer induced a contraction, even after $Ca^{2+}$ loading.

**4** Guanosine triphosphate (GTP) or arachidonic acid, both 100 $\mu$M neither evoked a contraction nor enhanced the subsequent contraction elicited by 20 $\mu$M $InsP_3$.

**5** Caffeine 25 mM did not induce a contraction nor did it affect the contraction elicited by 20 $\mu$M $InsP_3$.

**6** The results indicate that in pregnant rat myometrium $InsP_3$ releases $Ca^{2+}$ from intracellular stores as has been proposed in vascular smooth muscles.

## Introduction

Changes in the concentration of intracellular free $Ca^{2+}$ play a predominant role in regulating the contraction-relaxation cycle of uterine smooth muscle (Izumi, 1985). The activator $Ca^{2+}$ is either released from intracellular stores (mainly from sarcoplasmic reticulum), or extracellular $Ca^{2+}$ enters through the $Ca^{2+}$-channels of the plasma membrane. Because acetylcholine (ACh) elicits a phasic contraction of myometrium in $Ca^{2+}$-free solution containing EGTA, it has been proposed that in this tissue agonists can release $Ca^{2+}$ from intracellular stores (Mironneau *et al.*, 1984; Izumi, 1985). However, the precise mechanism of this $Ca^{2+}$ release remains obscure.

Recently, the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) induced by neurotransmitters and the release of diacylglycerol (DG) and inositol 1,4,5-trisphophate ($InsP_3$), have been been reported (Berridge & Irvine, 1984; Nishizuka, 1986). $InsP_3$ is the putative messenger for the mobilization of $Ca^{2+}$ from non-mitochondrial stores (Berridge & Irvine, 1984). Marc *et al.* (1986) propose that also in myometrial cells carbachol and oxytocin, through activation of their receptors, stimulate the generation of $InsP_3$ by breakdown of $PIP_2$. Carsten & Miller (1985) reported that $InsP_3$ releases $Ca^{2+}$ from $Ca^{2+}$-transporting microsomes derived from uterine sarcoplasmic reticulum. $InsP_3$ is therefore also thought to be a second messenger in myometrium.

It was recently suggested that guanosine triphosphate (GTP) could activate $Ca^{2+}$ release from endoplasmic reticulum in non-muscle cells (Chueh & Gill, 1986; Dawson *et al.*, 1987). In skinned vascular smooth muscle Saida & van Breemen (1987) reported that GTP enhances the $InsP_3$-induced $Ca^{2+}$ release from sarcoplasmic reticulum, and they suggested that guanine nucleotide-binding proteins are involved in the mechanism of $Ca^{2+}$-release. Also arachidonic acid would induce $Ca^{2+}$ release in skeletal muscle (Cheah, 1981) and in permeabilized pancreatic islet cells (Wolf *et al.*, 1986). A similar action of arachidonic acid has been described in rat liver microsomes (Chan & Turk, 1987).

It was therefore worthwhile to investigate whether $InsP_3$, GTP and arachidonic acid participate in the regulation of intracellular $Ca^{2+}$ concentration in myometrial cells. We have examined the effects of these agents on the release of $Ca^{2+}$ in saponin-treated skinned smooth-muscle cells of the longitudinal layer of myometrium of pregnant rats. We have

used the myometrium of pregnant rats, because the amplitude of the contraction induced by ACh in $Ca^{2+}$-free solution increases during the progress of gestation (Izumi, 1985). The skinning of the fibres allowed us to apply presumed second messengers for $Ca^{2+}$ release directly to the sarcoplasmic reticulum *in situ*.

## Methods

Pregnant Wistar rats at day 21 were anaesthetized by intraperitoneal injection of sodium pentobarbitone (120 mg $kg^{-1}$). The uteri were dissected free from the mesometrial side in longitudinal direction and the foetuses and placentas were removed. Longitudinal strips of myometrium were prepared from the uterine horns by carefully removing the circular muscle layer and the endometrium.

### *Recording of mechanical responses*

For tension recording from intact and skinned smooth-muscle cells, small strips were dissected from the longitudinal layer of myometrium. The dissection was carried out under a binocular microscope, with fine forceps and small knives made from pieces of razor blades. Finally a thin longitudinal muscle strip (0.3–0.5 mm in length, 0.05–0.08 mm in width and 0.03–0.05 mm in thickness) was isolated. Each end of the strip was knotted by a fine silk fibre which was then fixed to a piece (about 1 mm × 1 mm) of Scotch double-sided adhesive tape (3M Co., St. Paul, MN, U.S.A.). One tape was fixed at a side of the chamber and the other tape to a strain gauge (U-gauge, Shinko, Tokyo, Japan). The chamber had a volume of 0.9 ml. The solutions were rapidly injected by a syringe from one end of the chamber and simultaneously siphoned off from the other end. The tissue was not superfused continuously but remained exposed to the solution in the chamber till the next solution was injected.

After recording the contraction of the intact muscle induced by high $K^+$ solution or carbachol, the tissue was exposed to the relaxing solution. Then smooth-muscle cells were skinned by exposure to saponin (30 $\mu$g $ml^{-1}$) for 20 min in the relaxing solution (Izumi, 1985). The $Ca^{2+}$-induced contractions of these skinned tissues were determined at different concentrations of free $Ca^{2+}$, using EGTA buffer solutions (Itoh *et al.*, 1981). The intracellular stores were loaded with $Ca^{2+}$ by exposing the skinned fibres to a solution containing 0.6 $\mu$M free $Ca^{2+}$ (buffered with 4 mM EGTA). The effect of substances which were presumed to release $Ca^{2+}$ was investigated in a $Ca^{2+}$-free solution supplemented with 0.1 mM EGTA. In some experiments, nominal $Ca^{2+}$-free solutions without EGTA were used instead of solutions containing 0.1 mM EGTA. To avoid $Ca^{2+}$ uptake by the mitochondria of the skinned fibres, the mitochondrial inhibitor $NaN_3$ (5 mM) was present throughout the experiments. Calmodulin (0.1 $\mu$M) was added to the bathing solution to prevent the deterioration of the function of the contractile proteins. The experiments were performed at 25°C (Iino, 1981; Itoh *et al.*, 1981), because the skinned muscle fibres deteriorate more rapidly at a higher temperature. Under the above experimental conditions, the amplitude of contractions induced by 0.6 $\mu$M free $Ca^{2+}$ or by 20 $\mu$M $InsP_3$ remained stable for 4 or 5 trials.

### *Solutions*

The modified Krebs solution contained (mM): $Na^+$ 132.5, $K^+$ 5.9, $Mg^{2+}$ 1.2, $Ca^{2+}$ 1.5, $Cl^-$ 141.1, glucose 11.5 and HEPES 11.5. It was bubbled with $O_2$ and the pH was kept at 7.3. High $K^+$ solution was prepared by replacing NaCl with KCl. The relaxing solution contained (mM): K methanesulphonate (KMs) 114, Tris maleate 20, $Mg(Ms)_2$ 5.1, adenosine 5'-triphosphate (ATP) 5.2 (10.4 mM $Na^+$ as $Na_2ATP$) and ethyleneglycol *bis*-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 4. The free $Ca^{2+}$-concentration was changed by adding appropriate amounts of $Ca(Ms)_2$ to EGTA. The apparent binding constant of EGTA for $Ca^{2+}$ at 25°C and at pH 6.8 is $10^6$ $M^{-1}$ according to Itoh *et al.* (1981). For experiments in which $InsP_3$ or other presumed $Ca^{2+}$-releasers were used, the concentration of EGTA was reduced to 0.1 mM.

### *Drugs*

$InsP_3$ and arachidonic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), ATP and GTP from Boehringer (Mannheim, F.R.G.), A23187 as free acid from Calbiochemicals (CA, U.S.A.), saponin from ICN Pharmac. Inc. (Cleveland, OH, U.S.A.), caffeine from BDH Chemicals (Poole, Dorset, England), ethyleneglycol-*bis*-(β-aminoethylether)-N,N,N'N'-tetraacetic acid (EGTA) from Fluka (Switzerland), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) from Merck (Darmstadt, F.R.G.). Calmodulin was prepared from bovine brain according to Gopalakrishna & Anderson (1982).

## Results

### *Effects of $InsP_3$ on the stored $Ca^{2+}$ in skinned smooth muscle fibres*

After treating the fibres for 20 min with 30 $\mu$g $ml^{-1}$ saponin in relaxing solution, either 1 mM carbachol

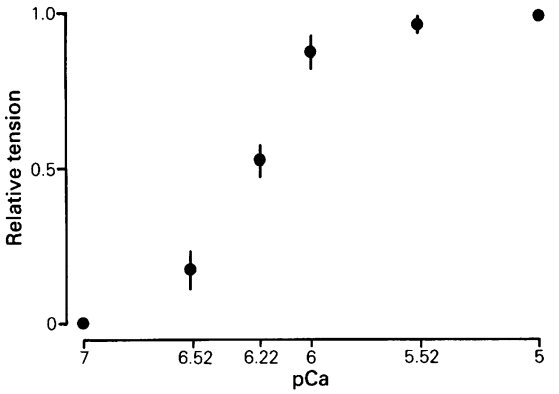


**Figure 1** The pCa-tension relationship observed in skinned rat myometrium muscles. The amplitude of the contraction evoked by 10 $\mu$M free $Ca^{2+}$ was normalized to 1.0. Vertical bars indicate s.d. ($n = 5$).

in Krebs solution or 138 mM $K^+$ solution was no longer able to induce any force development. However, a contraction appeared if 4 mM EGTA and 5.2 mM ATP were present and if the free $Ca^{2+}$ concentrations exceeded 0.3 $\mu$M. The contraction induced by 10 $\mu$M free $Ca^{2+}$ in skinned fibres was larger than that induced by 1 mM carbachol or 138 mM $K^+$ in the intact tissue. Figure 1 shows the pCa-tension relationship, which proceeds along a sigmoidal curve as shown previously by Izumi (1985).

In order to investigate the effects of $InsP_3$ on the $Ca^{2+}$ stores of saponin-treated skinned fibres, the following procedures were used. The intracellular stores were loaded with $Ca^{2+}$ by exposure of the tissues for 3 min to a solution containing 0.6 $\mu$M free $Ca^{2+}$ (buffered with 4 mM EGTA). Thereupon the tissues were rinsed successively for 2 min with the relaxing solution containing 0.1 mM EGTA supplemented with 6 mM $KH_2PO_4$ to accelerate the relaxation (Itoh *et al.*, 1986) and for 2 min with the usual relaxing solution containing 0.1 mM EGTA only. A transient contraction could then be elicited by a 3 min superfusion with a solution containing 0.1 mM EGTA and the addition of 20 $\mu$M $InsP_3$. A second application of 20 $\mu$M $InsP_3$ produced a smaller contraction than the first one (Figure 2a). After a 3 min reloading with $Ca^{2+}$, 20 $\mu$M $InsP_3$ again produced a contraction of the same amplitude as the first one (data not shown). Application of 5 $\mu$M $InsP_3$ evoked a smaller contraction and a subsequent 20 $\mu$M $InsP_3$ evoked a contraction that was appreciably smaller than that obtained by the first application of 20 $\mu$M $InsP_3$ (Figure 2b). Figure 3 shows the relation between the concentration of $InsP_3$ and the amplitude of force development for the same degree of $Ca^{2+}$ filling of the stores. The minimum concentra-

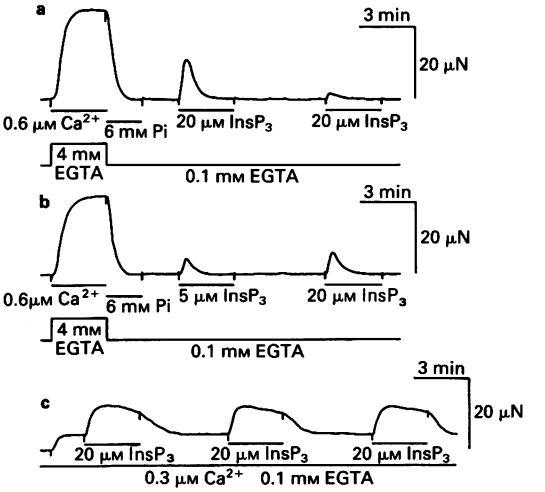


**Figure 2** Effects of inositol 1,4,5-trisphosphate ($InsP_3$) on the $Ca^{2+}$ store sites in saponin-treated skinned muscles. (a) In the saponin-treated skinned muscle, 0.6 $\mu$M free $Ca^{2+}$ (buffered with 4 mM EGTA) was applied for 3 min. The tissue was rinsed with relaxing solution containing 0.1 mM EGTA in the presence of 6 mM $KH_2PO_4$ (Pi) for 2 min and was rinsed again with solution containing 0.1 mM EGTA in the absence of Pi. $InsP_3$ (20 $\mu$M) was applied twice for 3 min with an interval of 5 min. (b) $InsP_3$ (5 $\mu$M) was applied for 3 min, then $InsP_3$ (20 $\mu$M) was applied after a 5 min interval. (c) $InsP_3$ (20 $\mu$M) was applied three times at intervals of 5 min after the contraction induced by 0.3 $\mu$M free $Ca^{2+}$ buffered with 0.1 mM EGTA had reached a steady amplitude. (a), (b) and (c) were obtained from same strip. Four experiments were performed giving identical results.

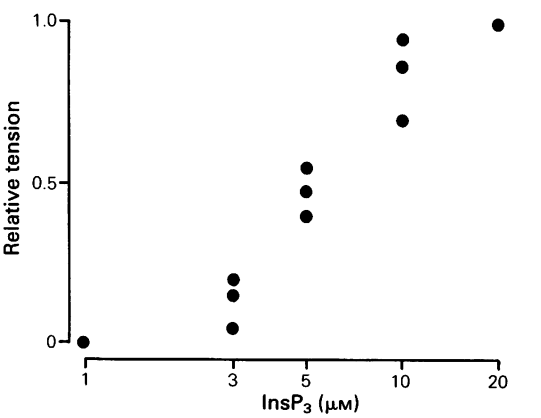


**Figure 3** Dose-response relation of the inositol 1,4,5-trisphosphate ($InsP_3$)-induced contraction. Various concentrations of $InsP_3$ were applied after a standard loading of the stores with $Ca^{2+}$ (0.6 $\mu$M free $Ca^{2+}$ buffered with 4 mM EGTA for 3 min). The amplitude of the 20 $\mu$M $InsP_3$-induced contraction was normalized as 1.0 ($n = 3$).

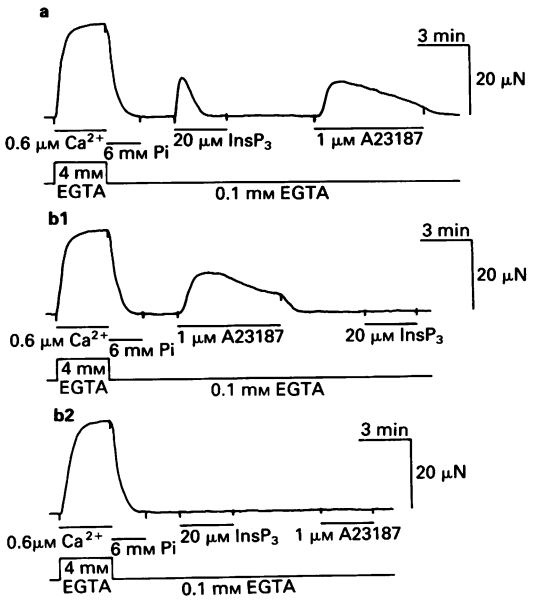


**Figure 4** Effects of A23187 on the $Ca^{2+}$ stores in saponin-treated skinned muscles. (a) After a 20 $\mu$M inositol 1,4,5-trisphosphate ($InsP_3$)-induced contraction, 1 $\mu$M A23187 was subsequently applied for 6 min. Other procedures were the same as described in Figure 2a. (b1) After inducing a contraction with 1 $\mu$M A23187, 20 $\mu$M $InsP_3$ was subsequently applied; (b2) after application of A23187 in (b1), $InsP_3$ and A23187 were successively applied following a loading of the stores with $Ca^{2+}$. (b1) and (b2) are continuous recordings from the same strip, but (a) was obtained from a different strip. These results are typical for 4 experiments.

tion of $InsP_3$ required to produce a contraction was 3 $\mu$M and the maximal contraction was reached at 20 $\mu$M $InsP_3$.

An application of 20 $\mu$M $InsP_3$ to skinned fibres in a solution with 0.3 $\mu$M free $Ca^{2+}$ buffered with 0.1 mM EGTA produced a small sustained contraction and repeated addition of 20 $\mu$M $InsP_3$ to the above solution at 5 min intervals elicited contractions of constant amplitude (Figure 2c).

*Effects of caffeine and A23187 on the $Ca^{2+}$ stores in skinned smooth-muscle fibres*

In vascular tissues caffeine is known to release $Ca^{2+}$ from the intracellular stores, which are also affected by noradrenaline. Itoh *et al.* (1983) observed that in skinned muscles of vascular tissues, high concentrations of caffeine produce one single contraction and they therefore proposed that these caffeine-induced contractions can be used to estimate the amount of stored $Ca^{2+}$ in skinned fibres of vascular tissues.

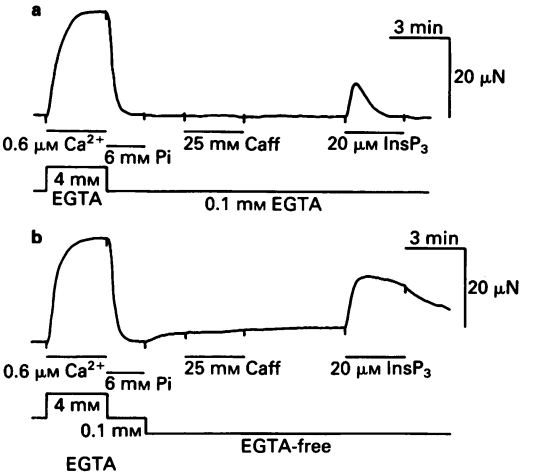


**Figure 5** Effects of caffeine on the $Ca^{2+}$ stores in saponin-treated skinned muscles. (a) Before application of 20 $\mu$M inositol 1,4,5-trisphosphate ($InsP_3$), 25 mM caffeine (Caff) was applied for 3 min. Other procedures were the same as those described in Figure 2a. (b) After loading the stores with $Ca^{2+}$, caffeine, and $InsP_3$ were applied in the absence of EGTA. (a) and (b) were obtained from the same strip. These results are typical for 4 experiments.

Also the Ca ionophore A23187 is used as a $Ca^{2+}$ releasing agent in non-muscle cells (Hirata *et al.*, 1984), and in skinned muscles of the rabbit mesenteric artery (Itoh *et al.*, 1985a). It was therefore important to investigate also the effects of caffeine and A23187 on saponin-treated smooth muscles of myometrium.

After application of 20 $\mu$M $InsP_3$ to the skinned fibres, a contraction of almost the same amplitude as that elicited by 20 $\mu$M $InsP_3$ could still be evoked by 1 $\mu$M A23187 (Figure 4a). However, after a preceding exposure of the skinned fibres to 1 $\mu$M A23187, 20 $\mu$M $InsP_3$ was unable to induce a contraction. After this exposure to 1 $\mu$M A23187 a renewed loading for 3 min in a solution with 0.6 $\mu$M $Ca^{2+}$ was not followed by a contraction on applying 20 $\mu$M $InsP_3$ or 1 $\mu$M A23187 (Figure 4b). These findings indicate that application of 1 $\mu$M A23187 disturbs the functions of the intracellular $Ca^{2+}$ compartments and that A23187 releases $Ca^{2+}$ by a mechanism that is different from that of $InsP_3$.

In contrast to $InsP_3$ and A23187, 25 mM caffeine did not induce a contraction in skinned myometrial fibres nor did it affect a subsequent contraction elicited by 20 $\mu$M $InsP_3$ (Figure 5a). In a relaxing solution without EGTA, the contaminating $Ca^{2+}$ directly activates the contractile proteins, and supplementary $Ca^{2+}$ released from the stores by e.g. $InsP_3$ elicits a larger contraction than that obtained

in the solution containing 0.1 mM EGTA. Therefore we investigated the effect of 25 mM caffeine during exposure to a solution without EGTA. However, under these conditions 25 mM caffeine was still unable to induce a contraction (Figure 5b).

*Effects of GTP and arachidonic acid on the $InsP_3$-induced contraction in skinned smooth-muscle fibres*

Both GTP and arachidonic acid have recently been proposed as intracellular second messengers for $Ca^{2+}$ release. Skinned muscle fibres were bathed in the loading solution for 3 min. The subsequent exposure to a solution containing 100 $\mu$M GTP did not evoke a contraction nor did it enhance the subsequent contraction induced by 20 $\mu$M $InsP_3$ (Figure 6b). A similar negative result has been obtained for 100 $\mu$M arachidonic acid (Figure 6c). A study of the effects of these agents during exposure of the fibres to a solution without EGTA, also showed no activity of these substances (data not shown).

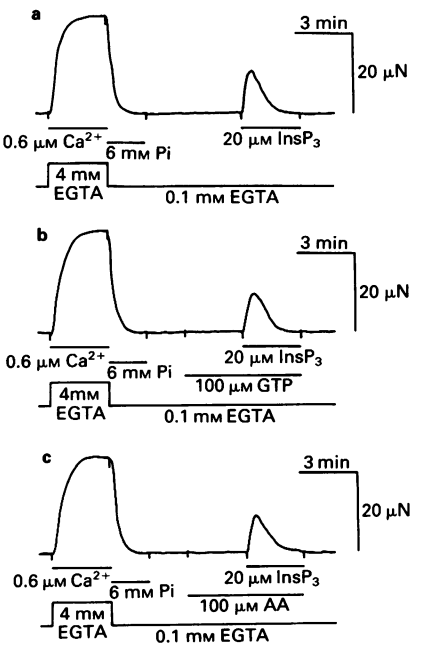


**Figure 6** Effects of guanosine triphosphate (GTP) and arachidonic acid on the inositol 1,4,5-trisphosphate ($InsP_3$)-induced contractions of saponin-treated skinned muscles: (a) control; (b) 100 $\mu$M GTP was applied for 3 min and then $InsP_3$ with GTP was applied; (c) 100 $\mu$M arachidonic acid (AA) was applied for 3 min and then $InsP_3$ was applied together with AA. (a), (b) and (c) were obtained from the same strip and are typical for 4 experiments.

## Discussion

Agonists such as noradrenaline and acetylcholine release $Ca^{2+}$ from intracellular stores in smooth muscle which is responsible for part of the pharmacomechanical coupling (Somlyo & Somlyo, 1968; Itoh *et al.*, 1983). These intracellular $Ca^{2+}$ stores are thought to correspond to the sarcoplasmic reticulum. However, only recently it became clear that the activation of receptors by agonists induces a $Ca^{2+}$ release from sarcoplasmic reticulum through $InsP_3$, which is a hydrolytic product of $PIP_2$. Evidence has been brought forward that $InsP_3$ also exerts this function in vascular smooth muscle (Suematsu *et al.*, 1984; Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986). Recently, Walker *et al.* (1987), using caged $InsP_3$, reported that $InsP_3$ releases $Ca^{2+}$ from stores of the rabbit main pulmonary artery permeabilized by saponin at a concentration and with an activation rate compatible with the *in vivo* physiological response. These results provide strong support for the hypothesis that $InsP_3$ is a second messenger for releasing $Ca^{2+}$ from the sarcoplasmic reticulum of vascular smooth muscle. In order to explain the $Ca^{2+}$ release by $InsP_3$, the existence of an $InsP_3$ receptor on the intracellular stores has been proposed (Spät *et al.*, 1986).

Oxytocin and acetylcholine have been shown to induce repetitive contractions of rat myometrium in $Ca^{2+}$-free solution (Mironneau *et al.*, 1984; Ashoori *et al.*, 1985). It has also been suggested that hydrolysis of $PIP_2$ might play a role in transferring the activation of muscarinic and oxytocin receptors to the sarcoplasmic reticulum in human and guinea-pig myometrium (Schrey *et al.*, 1986; Marc *et al.*, 1986), and $InsP_3$ was reported to release $Ca^{2+}$ from microsomes from the sarcoplasmic reticulum of bovine pregnant uteri (Carsten & Miller, 1985).

The present results support the view that $InsP_3$ is a second messenger for the release of $Ca^{2+}$ in myometrial cells. The characteristics of the $Ca^{2+}$-release in the myometrium by $InsP_3$ are almost the same as those in the rabbit mesenteric artery. $InsP_3$ induced a $Ca^{2+}$ release in tissues which were exposed to relaxing solutions or to solutions containing 0.3 $\mu$M free $Ca^{2+}$ (Hashimoto *et al.*, 1986). Because our experiments were done in the presence of $NaN_3$, it can be excluded that the $Ca^{2+}$ stores affected by $InsP_3$ corresponded to the mitochondria. They are most likely part of the sarcoplasmic reticulum. However, the sensitivity of the myometrium for $InsP_3$ was less than that of vascular tissues. This difference may be explained by tissues or species differences. It is also possible that the purity of $InsP_3$ used in these experiments is lower than that of others. After eliciting a maximum contraction with 20 $\mu$M

$InsP_3$, 1 $\mu$M A23187 could still produce a large sustained contraction suggesting that there are $Ca^{2+}$ stores which do not respond to $InsP_3$.

Recent studies have revealed that GTP and arachidonic acid can also release $Ca^{2+}$ from intracellular stores (Chueh & Gill, 1986; Wolf *et al.*, 1986). GTP has also been reported to enhance the $InsP_3$-induced $Ca^{2+}$ release in skinned muscles of the rabbit mesenteric artery (Saida & van Breemen, 1987). However, in the present experiments on myometrium we could not see any effect of GTP or of arachidonic acid, although the concentrations used in the experiments were higher than those used in the above reports.

Caffeine is known to cause a transient contraction of intact vascular smooth muscle in $Ca^{2+}$-free solution and to release $Ca^{2+}$ from intracellular stores in saponin-treated skinned vascular smooth muscles (Itoh *et al.*, 1983). However, Osa (1973) and Ashoori *et al.* (1985) reported that caffeine inhibits the contractions induced by agonists or by high potassium solution in the pregnant and non-pregnant myometrium. Caffeine is also known to increase the concentration of cyclic AMP due to the inhibition of phosphodiesterase, and the increased cyclic AMP inhibits the contractile proteins of skinned smooth muscles in the presence of cyclic AMP-dependent protein kinase (Itoh *et al.*, 1985b). However, this inhibitory effect on contractile proteins is not complete. Therefore, our results indicate that caffeine does not release $Ca^{2+}$ from intracellular stores in myometrium.

In conclusion, $InsP_3$ releases $Ca^{2+}$ from intracellular stores in the skinned muscles of pregnant rat myometrium, as in vascular smooth muscles and non-muscle cells. Caffeine does not release $Ca^{2+}$ in myometrium; in this respect, myometrium may have intracellular $Ca^{2+}$ stores that differ from those of vascular smooth muscles. Other proposed second messengers such as GTP and arachidonic acid do not act on intracellular $Ca^{2+}$ stores of myometrial cells.

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