Characterization of Contractile Activity and Intracellular Ca^{2+} Signalling in Mouse Myometrium

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OBJECTIVE: *To characterize the contractile responses of mouse myometrium, the associated calcium (Ca^{2+}) changes and the role of the sarcoplasmic reticulum (SR), and to better understand excitation contraction coupling in this tissue.*

METHODS: *Strips of longitudinal myometrium were used, and Ca^{2+} was measured after loading with Indo-1.*

RESULTS: *Intracellular Ca^{2+} transients, produced by Ca^{2+} entry, preceded phasic spontaneous contractions. Depolarization with high potassium concentration significantly increased the amplitude of the contractions and transformed the pattern of activity from phasic to tonic, with accompanying changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Oxytocin significantly stimulated contractile activity and $[Ca^{2+}]_i$ above the level occurring spontaneously. Thus all forms of contractile activity were closely correlated with Ca^{2+}. When the SR was emptied using a blocker of the SR calcium-adenosinetriphosphatase, cyclopiazonic acid, spontaneous Ca^{2+} and force transients increased greatly in frequency and amplitude. Ryanodine, a blocker of Ca^{2+}-induced Ca^{2+} release (CICR), did not impair activity. In the absence of external Ca^{2+}, oxytocin was able to release Ca^{2+} from the SR through IP_3 but produced only a small increase in force, demonstrating a requirement for Ca^{2+} entry as part of the mechanism of agonist action.*

CONCLUSION: *Mouse myometrium, (1) produces contractile activity reflecting changes in $[Ca^{2+}]_i$ irrespective of the stimulus, (2) has a significant SR Ca^{2+} content releasable by agonists but not CICR, (3) has an SR acting to inhibit spontaneous activity, and (4) behaves qualitatively similarly to human and rat myometrium in major aspects of excitation contraction coupling and is therefore a useful model tissue. (J Soc Gynecol Investig 2004;11:207–12)* Copyright © 2004 by the Society for Gynecologic Investigation.

KEY WORDS: Smooth muscle, SR Ca^{2+} release, agonists, sarcoplasmic reticulum.

Much has been learned about uterine physiology from in vitro studies of animal and human myometrium. In particular, recent work has greatly increased our knowledge of excitation-contraction coupling.[1,2] Such work may eventually point the way to the prevention or better treatment of preterm labor or problematic term labor. Furthermore, insights may be predicted from the sequencing of the human genome and our ability to alter gene function in knock-out (KO) mice. Thousands of KO mice have been made and many physiologic processes studied, including, for example, parturition.[3–5] To date, however, few studies using KO mice have been conducted in the area of excitation-contraction coupling. One potential drawback of such studies is the lack of basic data on normal, wild-type mouse myometrium, especially concerning calcium (Ca^{2+}) signalling, compared with rats and human, limiting the usefulness of such studies. The mouse has also been developed as a good model system to study the immune system and infection-induced preterm labor.[6]

Early investigations by Osa and colleagues[7–9] described in vitro contractions from murine tissue. The influence of gestation,[10,11] estrus,[12] and phosphatase inhibition[13] have subsequently been reported. However, no measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) have been made so far in mouse myometrium, nor has the effect of manipulation of the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), been investigated. Thus the mechanisms underlying contractility changes are incompletely understood. The aim of this work was therefore to answer fundamental questions concerning contractility, the SR, and $[Ca^{2+}]_i$ in the mouse myometrium. In addition, such data can be compared with those already available for human and rat myometrium. In this way

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insight will be gained into which responses and mechanisms may be considered fundamental and which are open to modification.

In mouse myometrium we therefore (1) characterized spontaneous Ca^{2+} transients and contractile activity and its dependence on Ca^{2+} entry and SR Ca^{2+} release; (2) determined the changes in contractile activity produced by agonist stimulation and high potassium (K^+) depolarization, along with the changes occurring in $[Ca^{2+}]_i$; and (3) investigated the role of the SR in force production. A preliminary account of this work has been presented (Matthew AJG, Wray S. A study of calcium, force and the sarcoplasmic reticulum (SR) in mouse myometrium. J Soc Gynecol Investig 2003;10(2):128A; Matthew AJG, Wray S. The sarcoplasmic reticulum and related ion channels in the mouse myometrium. Pflugers Archive 2002;443[2]:P41).

METHODS

Tissue

The CD-1 strain of mouse was chosen as this strain is commonly used in laboratories producing KO mice. The nonpregnant mice were used at 6–8 weeks old and killed humanely by cervical dislocation under carbon dioxide anesthesia. The uterine horns were removed, cleared of fat and mesentery, and opened out, and thin sheets of longitudinal myometrium were dissected (3 × 1 mm). The strips were kept for up to 24 hours in physiologic saline (see below) at 4C until used for study or loaded with Indo-1 for Ca^{2+} measurements.

INDO-1 LOADING. The acetoxy-methyl (AM) form of Indo-1 was used for loading the strips. Preliminary experiments were performed to determine the optimal loading concentrations, times, and temperature for cytosolic loading of the indicator into mouse myometrium. Optimal loading was that which (1) did not compromise tissue function, as assessed by comparing activity before and after loading; (2) gave good signal-to-noise ratios, as seen in the figures throughout; (3) produced even loading in the cytoplasm and throughout the multicellular preparation when viewed microscopically; and (4) did not buffer the $[Ca^{2+}]_i$, as judged by comparing Ca^{2+} transients obtained at lower Indo-1 concentrations. From these preliminary studies the following protocol was adopted: strips were loaded with 12.5 μM Indo-1 dissolved in a preprepared solution of 50 mg pluronic and 200 μL dimethyl sulfoxide (DMSO) added to 2 mL Krebs solution for 3 hours at 20C with gentle agitation on a blood tube rotating table (Stuart Scientific Ltd, Loughborough, United Kingdom). The tissue was irradiated with 350 nm light, and a ratio of the emissions recorded at 400 and 500 nm light was used to estimate the changes in $[Ca^{2+}]_i$.

FORCE MEASUREMENTS. Both loaded and unloaded preparations were transferred to small organ baths and secured with thin aluminium clips with one end attached to a tension transducer (Swema, Linton Instruments, Diss, United Kingdom). The strips were superfused with physiologic saline at 35C and 4 mL/minute and left to equilibrate for at least 60 minutes. Force was quantified by measuring contraction amplitude, frequency, duration, and area under the curve in unit time, usually 10 minutes. Ca^{2+} measurements were obtained from the ratio of the Indo-1 emissions at 400 and 500 nm, after excitation at 350 nm, with a 75-W Xenon lamp and using a ×10 fluor objective as described elsewhere.[14,15] Fluorescence emissions were detected using photomultiplier tubes (Electron Tubes, Ruislip, United Kingdom), and signals were filtered at 10 or 100 Hz.

SOLUTIONS. Physiologic saline solution was composed of 154 mM sodium chloride; 5.6 mM potassium chloride; 0.12 mM magnesium sulfate; 10.9 mM HEPES; 11.7 mM glucose; and 2 mM calcium chloride, pH 7.4. In some experiments K^+ was elevated to 40–60 mM, by isosmotic substitution of sodium chloride, and in others calcium chloride was omitted and 1 mM EGTA was added. In some experiments the SR was emptied by using cyclopiazonic acid (CPA) to inhibit the SR calcium adenosinetriphosphatase.[16] The CPA was dissolved in DMSO and diluted in external solutions at volumes less than 0.1%. L-type Ca^{2+} channels were inhibited with nifedipine, dissolved in ethanol, and maintained at a final concentration of 0.1 or 1 μM. Oxytocin (10 or 100 nM) was used in some experiments to stimulate the tissues, as stated. All chemicals were from Sigma (Poole, Dorset, United Kingdom), except Indo-1 (Molecular Probes, Eugene, OR).

Statistics

Throughout, n is the number of animals, and mean ± standard error of the mean values are given. Statistical significance was tested using the relevant t test, and significance was assumed for P values less than .05.

RESULTS

Spontaneous Activity

Phasic contractions commenced within 1 hour of tissues being placed in the perfusing chamber in approximately 70% of all preparations. These spontaneous contractions were usually highly regular in appearance and typically lasted for up to 3 hours. The mean contraction frequency was around 1 per minute (1.03 ± 0.09 contractions/minute, n = 20), and a typical record is shown in Figure 1A.

This activity depended completely upon the presence of external Ca^{2+}, as shown in Figure 2A. When Ca^{2+} was absent from the perfusion solution (EGTA present), the spontaneous contractions ceased abruptly, and no activity was apparent (n = 15). Phasic activity was rapidly (ie, within 5 minutes) restored upon readmission of Ca^{2+}.

To determine whether the required Ca^{2+} entry was through L-type channels, nifedipine, a blocker of such channels, was used at 0.1 μM and 1 μM (n = 4). Nifedipine rapidly abolishes spontaneous contractions and Ca^{2+} transients, as shown in Figure 2B. Taken together these data indicate that spontaneous uterine activity in the mouse is dependent upon external Ca^{2+}

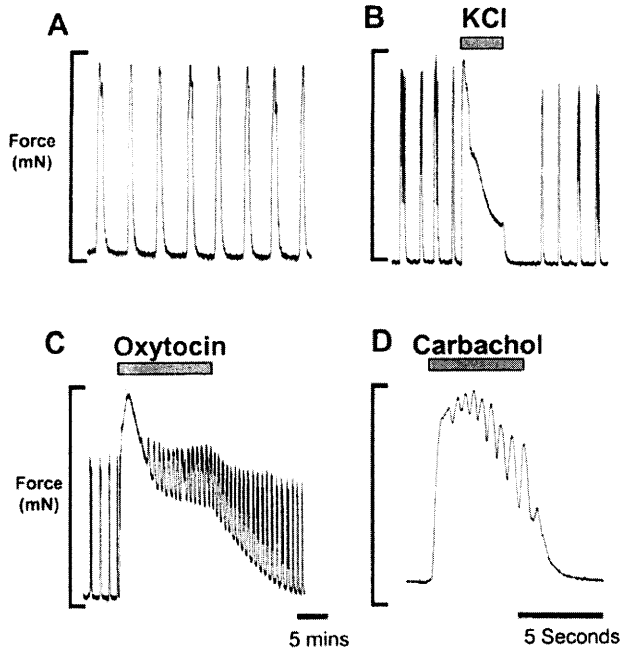

Figure 1. Contractile responses of mouse myometrium. A) Spontaneous activity. B) In response to K^+ (60 mM) depolarization. C) Application of oxytocin (100 nM). D) Application of carbachol (100 μM). The *solid bars* here, and elsewhere, indicate the period in which the drug was applied.

entry through voltage-gated, L-type Ca^{2+} channels and occurs at a rate of one contraction and Ca^{2+} transient every minute.

High Potassium Depolarization

The normal phasic activity of the mouse myometrium was transformed to a more or less maintained (tonic) contraction upon depolarization with 40 or 60 mM K^+ (n = 6, Figure 1B and 3A). The initial peak force was significantly greater in amplitude (21 ± 4%) than the amplitude of the preceding spontaneous contractions. The contraction was maintained but at a reduced level compared with peak, for as long as the high K^+ was present (up to 20 minutes), and 40 mM K^+ was

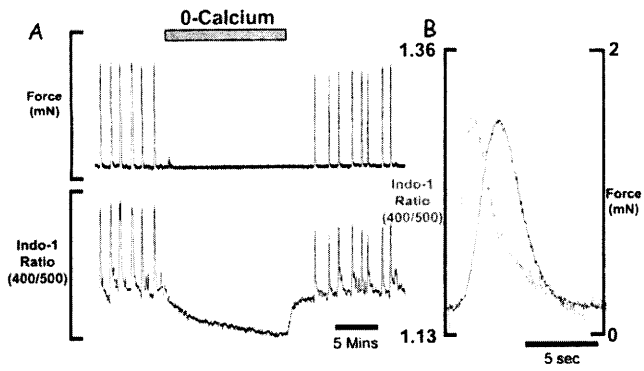

Figure 2. Simultaneous force and $[Ca^{2+}]_i$ measurements. A) Spontaneous force (*top trace*) and Ca^{2+} transients (Indo-1, *bottom trace*). B) The effect of nifedipine (100 nM) on spontaneous force and Ca^{2+} transients.

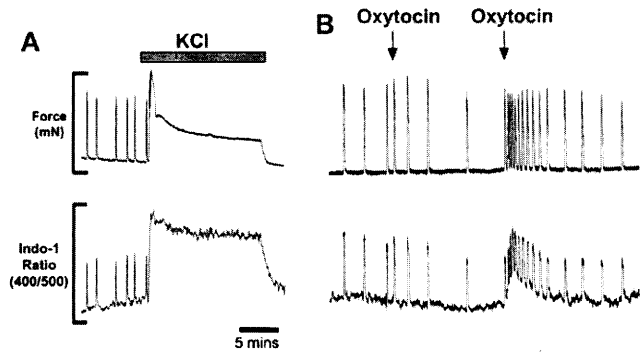

Figure 3. Effects of stimulation. Simultaneous force (*top trace*) and Ca^{2+} (*bottom traces*) records from mouse myometrium stimulated with high (40 mM) K^+ (A) and oxytocin (5-second application) at 10 and 100 nM (B).

sufficient to give maximal response in the tissue. Upon return to control solution, spontaneous phasic contractions reappeared after a delay of approximately 5 minutes, as shown in Figure 1B.

Oxytocin Stimulation

Oxytocin was applied at 10 and 100 nM to the mouse myometrium (n = 7). As shown in Figure 1C and 3B, it produced a marked stimulation of contraction compared with control activity, with an initial fusing of contractile responses seen with 100 nM. Oxytocin at 10 nM significantly increased contractile amplitude (135 ± 7%, n = 7) compared with control spontaneous contractions. The area under the curve was significantly increased by oxytocin (494 ± 24% and 509 ± 9%, force and Ca^{2+}, respectively, 100 nM).

Having therefore established the contractile responses of the mouse myometrium to a variety of stimuli, the next question to be addressed was whether these changes were a reflection of changes in $[Ca^{2+}]_i$.

Changes in $[Ca^{2+}]_i$ With Contraction

SPONTANEOUS ACTIVITY. Simultaneous force and $[Ca^{2+}]_i$ measurements showed that Ca^{2+} transients accompanied each spontaneous contraction and that these were as regular as the contractions (n = 20, Figure 2A). Normalization and superimposition of the force and Ca^{2+} records revealed that the Ca^{2+} transients increased and peaked significantly before the force transients (not shown), and the decrease in $[Ca^{2+}]_i$ also preceded the decrease of force (inset Figure 2). The average duration of the Ca^{2+} transients was 6.59 ± 0.30 seconds and that of force was 7.01 ± 0.28 seconds (n = 9). These values are significantly different from each other. It can also be seen in Figure 2 that there was a close correspondence between the changes in $[Ca^{2+}]_i$ and the elicited change in force.

HIGH K^+ DEPOLARIZATION. The rapid elevation of force was accompanied by an equally rapid increase in $[Ca^{2+}]_i$, and the plateau in the force produced was also accompanied by

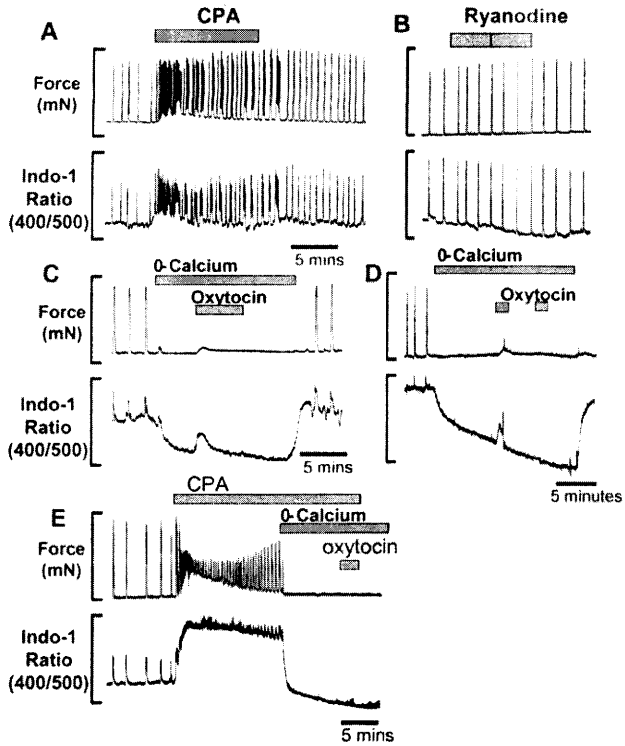

Figure 4. Contribution of the sarcoplasmic reticulum. A) Cyclopiazonic acid (CPA, 10 μM) applied to spontaneously active mouse myometrium. B) Application of ryanodine at 10 μM (first 20 minutes) and then at 50 μM (20 minutes) produced no significant effect on force or Ca^{2+}. C) Release of SR Ca^{2+} by oxytocin (100 nM) in Ca^{2+}-free (EGTA-containing) solution. D) Two applications of oxytocin in Ca^{2+}-free solution. Note no effect of second application. E) Oxytocin application subsequent to CPA; note no Ca^{2+} release occurred.

an elevated level of $[Ca^{2+}]_i$ (Figure 3A). However, whereas the increase in the area under the curve for force produced was 261 ± 65%, the $[Ca^{2+}]_i$ increased 660 ± 96% compared with control period (100%) (n = 4). The decline in tension from peak was also accompanied by a decrease in peak $[Ca^{2+}]_i$.

OXYTOCIN. The changes in $[Ca^{2+}]_i$ in the mouse myometrium elicited by oxytocin (n = 5) were reflected in the measured changes in $[Ca^{2+}]_i$. Figure 3B shows simultaneous force and Ca^{2+} records when the myometrium was stimulated by 10 nM and then 100 nM for 5 seconds.

Role of the Sarcoplasmic Reticulum and Effects of Cyclopiazonic Acid

The SR calcium adenotriphosphatase (Ca-ATPase) was blocked using cyclopiazonic acid (CPA, 10 μM), and the SR Ca^{2+} store dissipated because of the passive leak of Ca^{2+}, which occurs into the cytoplasm.[16] The effects of CPA on force and Ca^{2+} can be seen in Figure 4A. CPA produced a profound stimulation of spontaneous activity (n = 12). The frequency of the contractions and Ca^{2+} transients was so increased that the responses became almost tonic, and incomplete relaxation between transients was apparent. There was also a consistent increase (62 ± 26%, n = 12) in the level of basal $[Ca^{2+}]$ upon addition of CPA, as seen in Figure 4A. This was reflected in basal tension (42 ± 16%, n = 12). We next investigated whether the SR Ca^{2+} release was through ryanodine or IP_3 channels.

Ryanodine

Ryanodine at high concentration prevents Ca^{2+} release from the SR through Ca^{2+}-induced Ca^{2+} release (CICR), by either holding the release channels open or blocking them. Thus, if CICR is augmenting spontaneous uterine activity, then ryanodine application should produce a reduction in force. In mouse myometrium, ryanodine at either 10 or 50 μM (n = 6) did not reduce any aspect of spontaneous activity (Figure 4B). On the contrary, its application stimulated the frequency of contractions in three of six preparations, and the area under the tension curve increased (from 64.06 ± 20 to 108 ± 29 arbitrary units), although this increase did not achieve statistical significance.

The Effect of CPA on Agonist Releasable SR Ca^{2+}

Oxytocin (100 nM) was added to the mouse myometrium in 0-Ca^{2+} (EGTA) solution to determine whether Ca^{2+} and force could be produced. The oxytocin was applied 5 minutes after changing to 0-Ca^{2+} solution, to ensure that Ca^{2+} from the interstitial spaces had been removed but that the SR Ca^{2+} store had not run down. As shown in Figure 4C (typical of three others) an increase in Ca^{2+} and force occurred upon oxytocin stimulation in 0-Ca^{2+} solution. Compared with preceding spontaneous Ca^{2+} and contraction changes (100%), the amplitude of the Ca^{2+} responses was comparable (140 ± 19%), but the force response was very much reduced (7.6 ± 3.0%). This single application of oxytocin for 5 minutes was sufficient to release all the available Ca^{2+} from the SR, as a subsequent application of oxytocin failed to increase Ca^{2+} or force (n = 3, Figure 4D).

When that protocol was repeated, but after 15 minutes of incubation with CPA, no release of Ca^{2+} or force production occurred (n = 3, Figure 4E).

These data indicate that CPA does indeed empty the SR, and (1) the effects seen in Figure 4A are a result of this; (2) the contribution of the SR to the effects of oxytocin to force production are small compared with those of extracellular Ca^{2+} (compare Figures 1C and 3B with 4C); and (3) an IP_3 Ca^{2+} release mechanism is present in mouse myometrium.

DISCUSSION

These data confirm earlier observations that the mouse myometrium is spontaneously active and responds to changes in contractility upon stimulation with agonists. We have made the first measurements of intracellular Ca^{2+} in the mouse myometrium and found that changes in $[Ca^{2+}]$ underlie all forms of contractile activity in mouse myometrium. We also demonstrated an absolute dependence of Ca^{2+} transients, and hence spontaneous contractions, on external Ca^{2+} entry. An agonist-releasable internal Ca^{2+} store is also demonstrated, but

this was rapidly depleted and produced only transient elevations of force. When the SR was emptied, spontaneous force was enhanced not reduced, suggesting that the SR does not augment spontaneous activity. Ryanodine, when used to block SR Ca^{2+} release channels, did not reduce force, indicating that CICR is not present in mouse myometrium. This is consistent with CPA not reducing force. The mechanisms underlying these effects and their physiologic significance and relation to data obtained on human myometrium are discussed below.

Calcium Measurements in Mouse Myometrium

It can be seen in the figures throughout that AM loading of Indo-1 into the longitudinal murine myometrium provides good measurements of $[Ca^{2+}]_i$ changes. The optimal conditions for loading mouse myometrium with Indo-1 (ie, temperature, concentration, and duration) are similar to those reported in human[17] and rat[18] myometrium. The Ca^{2+} transients preceded spontaneous activity and generally mirrored the force changes, as also described in rat[19] and human[17] myometrium.

Spontaneous Activity

The frequency of spontaneous activity found (ie, around one every minute) is similar to that previously reported (8.4 every 10 minutes[11] and eight every 10 minutes[4]). It is faster than the rates we find under similar experimental conditions in adult rat and human myometrium (Wray, unpublished observation). Our data clearly show that the phasic contractions were preceded by Ca^{2+} transients and that there was a close correlation between the two. The phasic activity was completely dependent on external Ca^{2+} entry, and as shown by the use of nifedipine, this entry occurs through L-type Ca^{2+} channels. This absolute dependence of spontaneous activity on voltage-gated Ca^{2+} entry is consistent with data obtained in rat[19] and human[20] myometrium. Thus, during spontaneous phasic contractions there appears to be no significant contribution from SR Ca^{2+} release, which is in agreement with the CPA and ryanodine data (see below).

Agonist-Induced and Depolarization-Induced Activity

Although no previous measurements of $[Ca^{2+}]_i$ have been made, the mouse myometrium has been shown to respond with contraction to a variety of agonists.[21–23] We report the responses to the important physiologic stimulus, oxytocin. Our data suggest that the changes in contractile activity brought about by oxytocin are secondary to the alterations in $[Ca^{2+}]_i$. There is no obvious effect of Ca^{2+} sensitization, as this would be expected to produce a dissociation between $[Ca^{2+}]_i$ and force. Such effects are most prominent in tonic rather than phasic smooth muscles.[24] These data are also in keeping with data obtained in the rat and human uterus using Y-27632, a drug used to inhibit rho kinase and thereby prevent one of the main Ca^{2+}-sensitizing pathways.[25] This has little effect on phasic activity, whether it occurs spontaneously or as a result of oxytocin.[19] Some small effects attributed to Ca^{2+} sensitization have been reported in human myometrium during oxytocin stimulation.[26] With high K^+ depolarization, there was some decrease in force produced in comparison to the maintained increase in $[Ca^{2+}]_i$.

We conclude that in the mouse myometrium, force production, irrespective of the mechanism used to stimulate the uterus, occurs in response to changes in $[Ca^{2+}]_i$, and that these changes closely mirror the functional effects, although some small role for Ca^{2+}-sensitization pathways may be present.

Role of the Sarcoplasmic Reticulum

An intense stimulation of force and Ca^{2+} activity occurred when CPA was used to empty the SR. There was a slow increase in basal Ca^{2+}, suggesting that the SR had released its Ca^{2+} by passive leakage, which could not be countered by the SR Ca-ATPase, as CPA was inhibiting it. In turn, this suggests that uptake of Ca^{2+} into the SR is part of the normal Ca^{2+} homeostatic mechanism maintaining $[Ca^{2+}]_i$. The most marked effect of CPA, however, was on the frequency of Ca^{2+} and force transients: both were greatly increased.

Thus, functionally disabling the SR promotes contractile activity; this suggests that the SR when it contains Ca^{2+} must be acting to decrease contractility. Similar conclusions have been drawn from studies on rat[19] and human[25] myometrium. Comparing the effect of CPA on the three species, however, reveals that the effects are most marked in the mouse. They are however comparable to those found in neonatal rat,[15] where it was concluded that there was an enhanced role of the SR compared with that in adult myometrium. It is not currently known whether this represents a structural difference in the size of the SR or some other reason. It has been speculated that the SR acts normally to spontaneously release Ca^{2+} targeted to K^+ (BK) channels on the surface membrane.[26] Thus, the K^+ channels are no longer stimulated when the SR is emptied, and the membrane will tend to depolarize and facilitate voltage-gated Ca^{2+} entry. It may be therefore that the BK channels are more numerous or active in mouse (and neonatal rat) uterus compared with human or adult rat. It is also possible that emptying the SR stimulates a capacitative Ca^{2+} entry mechanism.[27]

IP_3 but Not Ca^{2+} Induced Ca^{2+} Release

That the SR in mouse myometrium was able to release its Ca^{2+} in response to oxytocin stimulation was demonstrated by the data obtained from 0-Ca^{2+} solutions. The amount of Ca^{2+} released is finite, as demonstrated by the inability of a second oxytocin application to produce a subsequent release. The Ca^{2+} released had very little effect on force production; it was only 7% of a spontaneous contraction. It is not clear why the released SR Ca^{2+} was unable to stimulate contraction, although it is possible that it was not directed or available to the myofilaments. The store is emptied during the first 1 or 2 minutes of oxytocin application, demonstrating the need for external Ca^{2+} entry for oxytocin to have its full physiologic response (cf Figures 3B with 4C). Compared with data obtained in rat[19] and human,[25] the size of the Ca^{2+} release from the SR was larger in the mouse (when normalized and com-

pared with preceding contractions in Ca^{2+}-containing solutions). This again suggests, as CPA did, that the SR in the mouse may be structurally larger or physiologically more important than that of the other two species. This release is suggested to occur entirely through IP_3 receptors, for the reasons described next.

Ryanodine had no detrimental effects on spontaneous contractions. This is consistent with recent data[28] which showed that the nonpregnant mouse myometrium expresses only type 3 ryanodine receptors, and these were insensitive to increases in $[Ca^{2+}]_i$ under normal conditions. Furthermore the same authors saw no spontaneous or triggered Ca^{2+} sparks in the single cells. Our data in intact tissue are therefore consistent with these findings and lead us to suggest that CICR plays little or no role in the mouse myometrium.

In summary, these data provide measurements of $[Ca^{2+}]_i$ in mouse myometrium. Changes in force were found to mirror those of the $[Ca^{2+}]_i$ changes. Although there were quantitative differences in some of the responses found in the mouse, we conclude that several aspects of Ca^{2+} signaling appear to be the same as those reported for rat and human tissue. Thus, we conclude that (1) the functional role of the SR is to feed back and limit contractions; (2) the SR makes only a limited contribution to agonist-induced effects; and (3) external Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels is essential for uterine contractility irrespective of how it is produced. These data point to a common dominant role for Ca-dependent, force-producing pathways in the uterus compared with possible calcium-independent paths. The mouse appears to be a good model for studying Ca^{2+}signaling and contraction, for example, in relation to premature labor and use of KO animals.

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