Abstract—The effects of high potassium, carbachol and oxytocin on \(^{40}\text{Ca}\sim^{45}\text{Ca}\) exchange in the estradiol-treated rat uterine longitudinal muscle were compared with those in the ovariectomized one using the modified “Lanthanum Method.”” The treatment of estrogenized or ovariectomized myometrium with 60 mM K\(^+\) significantly increased the \(^{45}\text{Ca}\) space at 5-, 10-, 30- and 60-min incubation times. The increase in the \(^{45}\text{Ca}\) by high-K\(^+\) in the estrogenized myometrium was much greater than that in the ovariectomized one. Carbachol (2\times10^{-6} \text{ M}) had little influence on the \(^{45}\text{Ca}\) space at all incubation periods with the exception of the 10-min one in both uteri. The higher concentration of carbachol (2\times10^{-4} \text{ M}) in the estrogenized myometrium produced a marked increase in the \(^{45}\text{Ca}\) space after the 10-min incubation, while in the ovariectomized uterus, a slight increase in the \(^{45}\text{Ca}\) space was observed at the 10- and 30-min incubation times. The increase in the \(^{45}\text{Ca}\) space by 2\times10^{-4} \text{ M} carbachol in the estrogenized myometrium was greater than that in the ovariectomized one. Oxytocin (10^{-4} and 10^{-3} \text{ units/ml}) increased the \(^{45}\text{Ca}\) space at and after the 10-min incubation times in the estrogenized uterus, while in the ovariectomized uterus, oxytocin (10^{-3} and 10^{-2} \text{ units/ml}) showed little influence on the increase in the \(^{45}\text{Ca}\) space. The increase in \(^{45}\text{Ca}\) space by carbachol and oxytocin was inhibited by papaverine. These results suggest that differences in dependence upon Ca\(^{2+}\) in the medium may exist in contractions induced by high-K\(^+\), carbachol and oxytocin in the uterine smooth muscle and that the contractions in the estradiol-treated myometrium may be more dependent upon extracellular Ca\(^{2+}\) than those in the ovariectomized one.

It has been reported that the responses of rat uterus to sympathomimetic amines vary with the hormonal state of the individual (1−3), and permeabilities of ions in rat uterine smooth muscle may alter with changes in hormonal influences (4−6). Recently, Uruno et al. (7) presented evidence that the antioxytocin action of papaverine in the estrogenized rat uterus was antagonized by excess external Ca\(^{2+}\) (5−10 mM), but not antagonized in the ovariectomized one, and Ca exchangeability in the estrogenized myometrium was greater than that in the ovariectomized one. These results suggest that contractile agents may produce different effects on Ca exchangeability in the uterine smooth muscle of rats in different hormonal states.

The present study was undertaken to investigate the effects of high potassium, carbachol and oxytocin on \(^{40}\text{Ca}\sim^{45}\text{Ca}\) exchange in the estradiol-treated rat uterine longitudinal muscle, and their effects were compared with those in the ovariectomized one using the modified “Lanthanum Method.”
Materials and Methods

Virgin female Wistar rats weighing 150 to 250 g were primed by a subcutaneous injection of estradiol benzoate (1.0 mg/kg) dissolved in peanut oil two days before sacrifice, or they were ovariectomized by the dorsal route under ether anaesthesia and allowed to recover for more than a week before the experiments.

Rats were stunned by a blow on the head, the uterine horns were rapidly removed, and placed in Tris-buffered physiological salt solution. The Tris-buffered solution had the following millimolar composition: NaCl, 125; KCl, 2.7; MgCl₂, 1.2; CaCl₂, 1.8; glucose, 11.0; and tris (hydroxymethyl) aminomethane, 23.8. The pH was adjusted to 7.4 with 6N HCl.

For measurement of the ⁴⁵Ca tissue space, the excised uterine horns in Tris-buffered solution, bubbled continuously with 100% O₂ at room temperature, were freed from fat and connective tissue; and the myometrial longitudinal muscle layers were separated as described previously (7). After equilibration with the oxygenated Tris-buffered solution for 60 min which was kept at 32°C, the muscle layer was incubated in the oxygenated Tris-buffered solution (10 ml) containing ⁴⁵Ca (0.1 μCi/ml) or ⁴⁵Ca plus KCl (60 mM), carbachol (2×10⁻⁶ and 2×10⁻⁴ M) or oxytocin (10⁻⁴, 10⁻³ and 10⁻² units/ml) for given times (5, 15, 30 and 60 min) at 32°C. In some experiments, an antispasmodic, papaverine (3×10⁻⁵ M) or atropine (3×10⁻⁶ M), was allowed to remain in contact with the muscle layer 5 min before and throughout the period of the incubation. After the desired time interval, the muscle layer was transferred into a beaker (50 ml) containing the oxygenated La³⁺-Tris solution for 5 min. The La³⁺-Tris solution was made by replacing CaCl₂ in the Tris-buffered solution by 20 mM LaCl₃. The purpose of the La³⁺ treatment for 5 min was to minimize ⁴⁵Ca loss from smooth muscle cells during the relaxation since the contractile tissue was relaxed by exposure to La³⁺. The La³⁺-treated tissue was then removed from the La³⁺ solution, gently blotted on Whatman 42 filter paper, and weighed. After weighing the wet tissue was placed in 1 ml Protosol (New England Nuclear, Massachusetts) and incubated for 3 hr at 55°C to solubilize the tissue. Aliquots of this solution were used for the radioassay of the ⁴⁵Ca fraction resistant to displacement by 20 mM La³⁺ (8) by liquid scintillation counting. The ⁴⁵Ca tissue space was calculated as the ratio between the radioactivity (cpm) of the muscle and that of the bathing medium (9).

All experiments were carried out on a pair basis: i.e. one longitudinal muscle layer always served as the control for the other layer. The data were analyzed by means of the Student's t-test, and statistical significance was set at P<0.05.

The drugs and chemicals used were: carbachol (Sigma Chemical Co. Ltd., St. Louis), oxytocin (Atonin-O, Teikoku Hormone MFG. Ltd., Tokyo), papaverine hydrochloride (Sigma Chemical Co. Ltd., St. Louis), atropine sulfate (E. Merck, Darmstadt), lanthanum chloride (Wako Pure Chemical Industries Ltd., Osaka), and ⁴⁵CaCl₂ (s.a.=16.6 mCi/mg Ca, New England Nuclear, Massachusetts). All the other chemicals were of reagent grade, and all solutions were made using reagent-grade water processed through Milli-Q systems (Millipore Corp., Bedford).

Results

The effects of KCl on the ⁴⁵Ca tissue space in the estradiol-treated or ovariectomized rat uterine longitudinal muscle layers are shown in Fig. 1. High-K⁺ depolarizing solution was made by substituting 60 mM KCl for 60 mM NaCl in the Tris-buffered solution. Potassium
was applied to the tissue by changing the bathing solution to the high-K⁺ medium. The treatment of estrogenized or ovariectomized myometrium with 60 mM K⁺ significantly increased the ⁴⁵Ca space at all incubation periods. The increase in the ⁴⁵Ca space by high-K⁺ in the estradiol-primed myometrium was much greater than that in the ovariectomized one. It should be noted that significant increase in the ⁴⁵Ca space was observed at the 5-min incubation time in both myometria (Fig. 1A and 1B).

Figure 2 illustrates the effects of carbachol on the ⁴⁶Ca space in the estrogenized or ovariectomized rat myometrium. The concentrations of carbachol, 2×10⁻⁶ M and 2×10⁻⁴ M, were chosen to give about 50% and maximal contractile responses to the uterine longitudinal smooth muscle strips, respectively. Carbachol at the concentration of 2×10⁻⁶ M had little influence on the ⁴⁵Ca space at all incubation periods except for the 10-min one in both estrogenized and ovariectomized myometrial strips (Fig. 2A and 2C). The higher concentration of carbachol (2×10⁻⁴ M) in the estrogenized myometrium produced a marked increase in the ⁴⁵Ca space at and after the 10-min incubation times, while in the ovariectomized tissue a slight increase in the ⁴⁵Ca space was observed at the 10- and 30-min incubation times (Fig. 2B and 2D). The increase in the ⁴⁵Ca tissue space by 2×10⁻⁴ M carbachol in the estradiol-treated myometrium was much greater in comparison with that in the ovariectomized one (Fig. 2B and 2D).

Figure 3 shows the effects of oxytocin on the ⁴⁵Ca space in the estrogenized or ovariectomized rat myometrial longitudinal smooth muscle layers. The oxytocin concentrations used were 10⁻⁴ and 10⁻³ units/ml in the estradiol-treated myometrium and 10⁻³ and 10⁻² units/ml in the ovariectomized one because the sensitivity was not the same for the estrogenized and ovariectomized preparations. The respective concentrations of oxytocin produced approximately 50% and maximal contractile responses in the respective tissues. Oxytocin increased the ⁴⁵Ca space at and after the 10-min incubation times in the estradiol-primed rat uterine longitudinal smooth muscle. However, the concentration-dependent increase was not observed (Fig. 3A and 3B). In the case of the ovariectomized myometrium, oxytocin at the concentration of 10⁻³ or 10⁻² units/ml
showed little influence on the increase in the 45Ca space (Fig. 3C and 3D).

In order to investigate whether the increase in 45Ca tissue space by carbachol or oxytocin may be related to its contractile response or not, the effects of antagonists in the presence or absence of each agonist on Ca exchange-ability were studied in the uterine longitudinal smooth muscle layers of estrogen-dominated rats. Figure 4 shows the effects of two different types of antispasmodics, papaverine and atropine, on the 45Ca space in the estradiol-treated myometria. Papaverine (3x10^-5 M), a non-specific antispasmodic, decreased the 45Ca space; but atropine (3x10^-6 M), a competitive antagonist for carbachol, had little influence on it. The concentration of papaverine used inhibited approximately 50% of the contraction induced by carbachol (2x10^-4 M) or oxytocin (10^-3 units/ml), and atropine (3x10^-6 M) almost completely inhibited the contraction induced by 2x10^-4 M carbachol in the estrogenized myometrial strips (data not shown). The effects of the antispasmodics in the presence of carbachol or oxytocin on the 45Ca space are illustrated in Fig. 5. Papaverine (3x10^-5 M) significantly decreased the 45Ca space increased by oxytocin (10^-3 units/ml) or carbachol (2x10^-4 M) at the 10- and 30-min incubation times (Fig. 5A and 5B). Atropine (3x10^-6 M) inhibited the increase in the 45Ca space by carbachol (2x10^-4 M) during the 10-, 30- and 60-min incubation times (Fig. 5C).
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Fig. 3. Effects of oxytocin on the $^{45}$Ca space in estradiol-treated and ovariectomized rat uterine longitudinal muscle layers. (A) and (B): Estradiol-treated, (C) and (D): Ovariectomized. (-○−), (-△−): Control, (-●−): $10^{-4}$ units/ml oxytocin in (A), $10^{-3}$ units/ml oxytocin in (C), (-■−): $10^{-3}$ units/ml oxytocin in (B), $10^{-2}$ units/ml oxytocin in (D). The bar above or below each point is the standard error for 6 different preparations. The S.E. of the mean is indicated if it is larger than the size of the symbols. *Significantly different from the control at P<0.05.

Discussion

The effects of KCl, carbachol and oxytocin on $^{45}$Ca fractions resistant to displacement by 20 mM LaCl$_3$ in the estradiol-treated rat uterine longitudinal smooth muscle layers were compared with those in the ovariectomized ones. Since lanthanum displaces...
extracellular bound Ca (10, 11), the 45Ca tissue space after treating with 20 mM lanthanum may be indicative of cellular 45Ca including intracellular 45Ca and less superficially bound 45Ca of the cell membrane. In the control experiments (Figs. 1-4), the time 45Ca space curves exhibited two phases, an initial phase within 10 min and a later one after 10 min. The initial rapid phase suggests the existence of the rapid 40Ca-45Ca exchange in a 45Ca fraction(s) little displaced by 20 mM-lanthanum treatment for 5 min.

The significant increases in the 45Ca space were observed in both the initial and later phase by isotonic 60 mM K+ in both estradiol-dominated and ovariectomized rat myometria (Fig. 1). The increase in the 45Ca space in the estrogenized myometrium, especially in the later phase, was much greater than that in the ovariectomized one. The greater increase in the 45Ca space by high-K+ in the later phase in the estrogenized uterus may be due to the fact that the Ca exchangeability in the estrogenized tissue is larger than that in the ovariectomized one (7).

The contractile responses of the rat longitudinal muscle strips to the contractile agents used here reached their maximum within 10 min (data not shown). This implies that the increase in 45Ca space in the initial phase under our experimental conditions may be mainly related to the increase in 45Ca influx, if the contraction is dependent upon extracellular Ca2+, while the increase in the later phase is related to the increased Ca exchangeability by the agonists. Therefore, our results suggest that high-K+ may increase Ca2+ influx and Ca exchangeability in the estradiol-treated rat myometrium and that in the ovariectomized uterus, high-K+ may increase Ca2+ influx in the initial phase and induce the smaller increase in Ca exchangeability in the later phase. Ogasawara et al. (12) reported that the amplitude of the tonic contraction induced by the isotonic K solution in the estradiol-treated rat uterine longitudinal muscle is larger than that in the ovariectomized one and that the dependence of the K-contracture on the external Ca concentration is most greatly influenced in the estrogen-treated uterus. These facts may be related, in part, to the increased Ca exchangeability in the estradiol-dominated rat uterus.

Atropine had little influence on the increase
in $^{45}$Ca space in the absence of an agonist, but papaverine significantly inhibited the increase in the $^{45}$Ca space (Fig. 4). Furthermore, papaverine inhibited the increase in the $^{45}$Ca space by carbachol or oxytocin, and atropine inhibited the increase in the $^{45}$Ca space by carbachol (Fig. 5). Since papaverine did not significantly inhibit the increased $^{45}$Ca space at the 60-min incubation time, it seems likely that papaverine slows Ca exchangeability or Ca influx from the extracellular space in the myometrium stimulated by the contractile agents. Our results obtained here suggest that the increase in the $^{45}$Ca space by the spasmogens may be related to the contractions induced by them. Papaverine, but not atropine, decreased the $^{45}$Ca space in the absence of the agonist (Fig. 4). This reduced exchangeability of Ca may partly participate in the relaxing or inhibitory action of papaverine in the myometrium. It has been suggested that one of the mechanisms of action of papaverine may be related to a reduction of Ca influx (13-15) and/or an increase in Ca efflux (13, 15-17) in smooth muscle.

Carbachol at the concentration of $2 \times 10^{-6}$ M had little influence upon the $^{45}$Ca space in both estrogenized and ovariectomized myometria (Fig. 2A and 2C). These results suggest that contraction induced by the lower concentration of carbachol may be less dependent upon Ca$^{2+}$ in the medium in both myometria. The higher concentration of carbachol ($2 \times 10^{-4}$ M) produced a larger increase in the $^{45}$Ca space after the 10-min incubation in the estrogenized myometrium than that in the ovariectomized one (Fig. 2B and 2D). These results suggest that the later phase of contraction by the higher concentration of carbachol may be partly dependent upon Ca$^{2+}$ from an extracellular source. In addition, our results show that the influx of Ca$^{2+}$ in the initial phase by carbachol is much smaller than that by high-K$^+$. Oxytocin increased the $^{45}$Ca space in both initial (at 10-min incubation time) and later phases in the estrogen-dominated myometrium, while it had little influence on the increase in the $^{45}$Ca space in the ovariectomized uterus (Fig. 3). These results suggest that the extracellular Ca$^{2+}$ may participate in contraction by oxytocin to a certain extent in the estrogenized uterus. In the ovariectomized uterus, when the muscle is contracted by oxytocin, the dependence upon Ca$^{2+}$ in the medium and the increase in Ca exchangeability in the later phase may be very small. Oxytocin did not increase the $^{45}$Ca space at the 5-min incubation time in contrast to high-K$^+$ in the estrogenized uterus (Fig. 3). This indicates that there may be some difference between K$^+$- and oxytocin-induced contractions and that the K$^+$-induced contraction may be more dependent upon extracellular Ca$^{2+}$ than the oxytocin-induced one in the estrogen-dominated uterus. Our results also indicate that contraction induced by oxytocin among the spasmogens used is exceedingly influenced by estrogen treatment in the rat uterine longitudinal smooth muscle. The concentration-dependent increase in the $^{45}$Ca space by oxytocin was not observed in the estrogenized uterus. This reason is at present unknown. This, however, raises the possibility that the increased $^{45}$Ca fraction by oxytocin might be partly derived from a mobile membrane-bound source which is affected by La$^{3+}$.

In the rat myometrium, several authors (18, 19) reported that it was not possible to demonstrate increased influx of $^{45}$Ca associated with contraction induced by contractile agents such as high KCl, acetylcholine or oxytocin. Hodgson and Daniel (19) used pregnant rats and measured $^{45}$Ca uptake after a 2 mM-Lanthanum wash, while we used the estrogen-dominated and ovariectomized longitudinal myometria of rats and treated the tissue by 20 mM La$^{3+}$.
for 5 min. Thus, there are some differences between their experimental conditions and procedures and ours. This makes it difficult to strictly compare our present results with theirs. The discrepancy between their results and ours might be due to the different hormonal states of animals and/or the possibility that the sites of exchange of $^{45}$Ca in the rat myometrium might be located in the cell membrane; therefore, an increased $^{45}$Ca fraction(s) could be time-dependently displaced by lanthanum in a Ca$^{2+}$-free, La$^{3+}$ solution. Marshall and Kroeger (9) found an increase in tissue calcium content as measured by the lanthanum technique during K contraction and a decrease when papaverine relaxed the K contracture. Our results with regard to high-K$^+$ and papaverine favored their observations.

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