The Influence of High Potassium Depolarization and Acetylcholine on Calcium Exchange in the Rat Uterus

CASEY VAN BREEMEN and E. E. DANIEL

From the Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada. Dr. van Breemen's present address is the Department of General Physiology, University of Leicester, Leicester, England

ABSTRACT Net and radioactive calcium movements were studied in the rat uterus during stimulation with acetylcholine and high potassium solutions. High potassium did not affect the efflux of intracellular Ca^{45}, but was able to release Ca^{45} from a small parallel Ca fraction which was believed to be located in the cell membranes. High potassium did markedly slow the influx of Ca^{45} and caused a net calcium efflux. Acetylcholine had no effect on calcium movements in polarized myometrium, but it increased the Ca^{45} influx in depolarized uteri. Ca^{45} taken up during contraction exchanged more slowly during subsequent efflux than Ca^{45} taken up at rest. The results were interpreted as supporting the hypothesis that myometrial contraction is induced by a release of calcium from the inside of the cell membrane and the endoplasmic reticulum, and relaxation follows the removal of ionic cytoplasmic calcium by these same structures.

INTRODUCTION

Heilbrunn and Wiercinski in 1947 (1) were the first to demonstrate by injecting various ions into single skeletal muscle fibers that of the physiologically important ions only Ca activated the contractile mechanism when its myoplasmic concentration was raised. Recently Portzehl, Caldwell, and Ruegg (2) injected Ca-EDTA buffers into crab muscle fibers and found the threshold cytoplasmic Ca ion concentration above which contracture was initiated to be $5–10 \cdot 10^{-7}$ M. The same Ca ion concentration threshold has been found for the shortening of isolated myofibrils, for the ATPase activity of actomyosin, and for the shortening of glycerinated muscle (3, 4). Thus it is generally accepted that an increase in ionic Ca concentration in the cytoplasm to a critical level, which appears to be the same in skeletal and in smooth muscle, triggers the contractile mechanism. From the above it follows that the initiation of contraction will be accompanied by the movement of Ca ions into the cytoplasm either from the extracellular space by increased

membrane permeability or from a bound or localized cellular Ca fraction. In smooth muscle it has been postulated that contraction is initiated by an increased Ca influx (5–7), whereas others have provided convincing evidence that bound Ca is released for contraction (8–11). Most recently evidence has been forthcoming which indicates that a superficial rather loosely bound Ca fraction supplies the Ca for high potassium–induced contractions and that a more tightly bound, also called sequestered, Ca fraction supports drug-induced smooth muscle contractions (12, 13). The main objectives of the experiments reported here were to test the first postulate of net Ca entry and to find evidence for bound Ca fractions participating in Ca movements associated with contraction by measuring Ca^{45} and net fluxes during high potassium– and acetylcholine-induced contractions of the rat uterus.

METHODS

Tissues Isolated uterine horns weighing 25 to 50 mg were prepared from estrogen-pretreated immature female rats as described in the preceding paper (14).

Solutions Krebs-Ringer bicarbonate solution (K.R.): NaCl 115 mM, KCl 4.63 mM, $CaCl_2$ 1.5 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 21.9 mM, NaH_2PO_4 1.16 mM, glucose 50 mM, pH 7.4, bubbled with 95% O_2 and 5% CO_2.

High K depolarizing solution (191 K): 93 mM K_2SO_4 was substituted for 115 mM NaCl in K.R., making the total K concentration 186 + 4.6 or 191 mM.

Medium K depolarizing solution (40 K): 40 mM KCl was substituted for 40 mM NaCl in K.R.

Drugs Acetylcholine bromide, ethylene bis-glycol(β-aminoethylether)tetra-acetic acid (EGTA).

Analytical techniques were the same as described in the preceding report (14).

Procedures The Ca^{45} influx and efflux experiments were carried out in the same manner as before (14). The different sequences of influx and efflux solutions will be given in the results.

RESULTS

Effect of 191 Millimolar K on Ca^{45} Efflux

Uterine horns were incubated in Ca^{45}-labeled K.R. for 2 to 3 hours before starting the efflux in K.R. This period is sufficient to label practically all exchangeable Ca with Ca^{45} (14). After effluxing in K.R. for the first 20 minutes the tissues were transferred to tubes containing 191 K for a total of 30 minutes, after which time they were again passed through a series of tubes containing K.R. Whenever the uteri were exposed to 191 K they contracted vigorously and remained contracted for the entire high K exposure period. Fig. 1 shows the typical result of this procedure, namely, that high K depolarization has no detectable effect on the early part of the Ca^{45} efflux. High K depolarization causes a rise in the rate of efflux only if the depolariz-

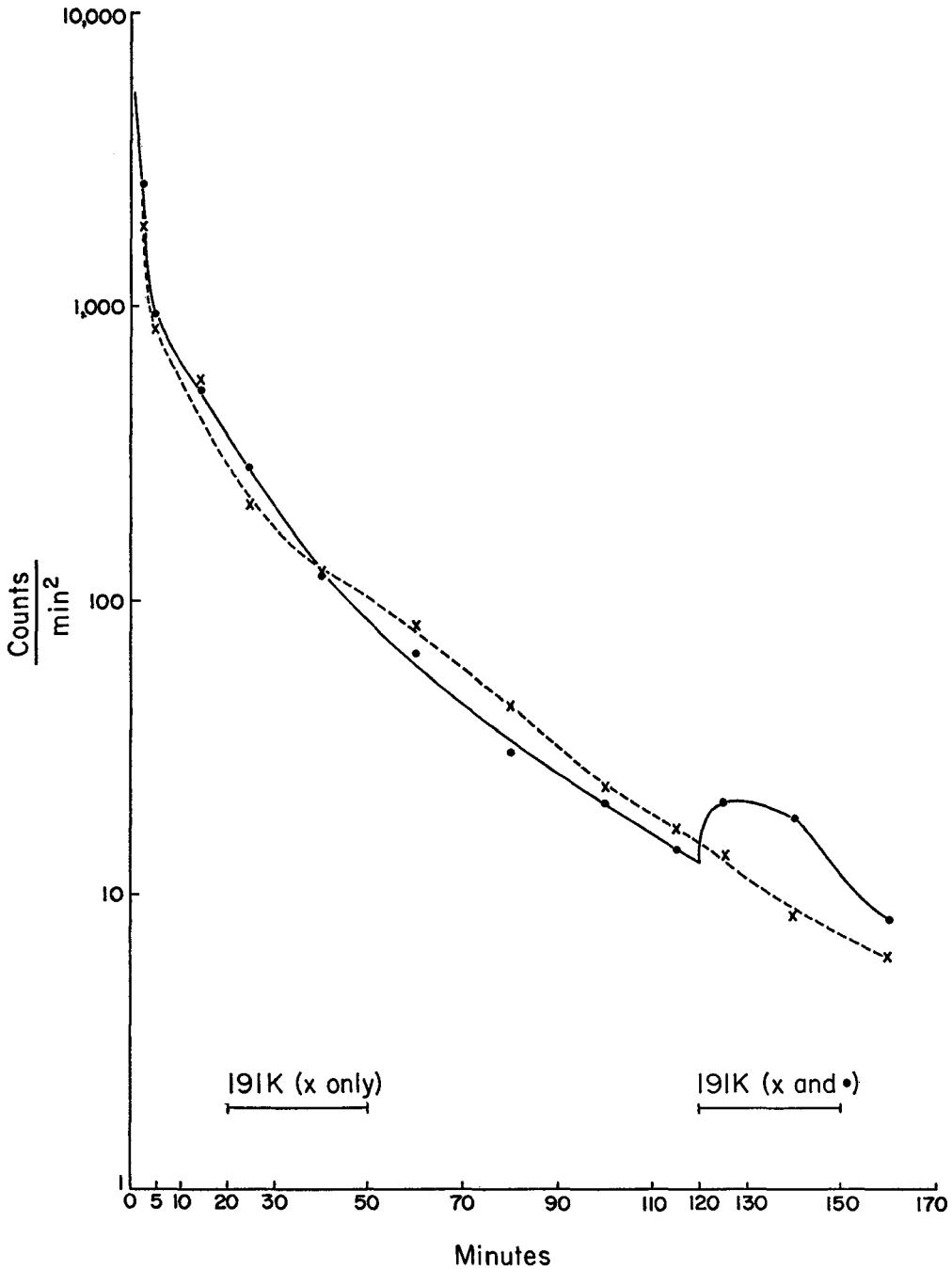

FIGURE 1. Effect of high K depolarization (191 K) on the Ca^{45} efflux from rat uteri previously loaded with Ca^{45} for 2 to 3 hours. The high K solution was substituted for K.R. as efflux medium during the times indicated by both horizontal bars for one uterine horn (dashed curve, crosses), and for the period indicated by the second horizontal bar for the other uterine horn from the same rat (solid curve, dots). The curves show that high K concentration causes a detectable rise in the rate of efflux only late in the efflux period, and that this rise is prevented by a previous depolarization early during the efflux period, as shown by the dashed curve.

ing solution is applied after the tissues have been allowed to efflux into K.R. for about 2 hours. This result is also illustrated in Fig. 1.

The observed increase in efflux is not likely to be due to an increased membrane permeability for Ca, or easier membrane permeation outward due to reversal of the electrical driving force on Ca ions. Under such conditions the efflux of Ca^{45} should have been enhanced also after 20 minutes of efflux into K.R., since at this time nearly all the extracellular Ca should have exchanged and most of the observed efflux would be from cellular Ca^{45}. In fact, however, the effect of high K depolarization was not observable until the efflux of cellular Ca^{45} had fallen to a very low level.

When the endometrium plus most of the circular smooth muscle is stripped away from the longitudinal smooth muscle, both parts of the uterus exhibit the high K–induced increased efflux to a more marked extent then does the whole uterus. In a number of experiments performed on dissected longitudinal muscle, the high K–induced increased efflux was very large (up to 10 times the control values) and variable. Owing to the probability of a contribution to this effect by the extra Ca associated with tissue damage (14), only the results obtained from whole uteri will be presented in the further analysis of the phenomenon under discussion.

At this point it is most convenient to postulate a working hypothesis for the source of the Ca involved in the high K–induced increase in Ca^{45} efflux. Results of experiments designed to test the hypothesis will then be presented. It is proposed that the high K depolarization releases a small fraction of Ca from the cell surfaces, and that this superficial Ca fraction is in parallel with the intracellular Ca, and exchanges more slowly than most of the remaining cellular Ca. Such a fraction would have the following properties: (*a*) Owing to the small size of the fraction, the release of Ca^{45} from it would be obscured by the large efflux of intracellular Ca^{45} during the early part of the efflux period. (*b*) The release could be observed during the latter part of the efflux period, since the specific activity of the intracellular Ca would decrease more rapidly than that of the superficial fraction in question. (*c*) As this superficial fraction is in parallel with the cellular Ca, once its Ca^{45} had been released it would not be restored by exchange with Ca^{45} leaving the cells. (*d*) Its Ca should be depleted by the use of an extracellular Ca chelating agent such as EGTA. (*e*) Its presence should be demonstrated in the isolated smooth muscle preparation. (*f*) Owing to the slow exchange of this fraction, a short incubation period should be insufficient to load it with Ca^{45}.

The experimental results illustrated in Fig. 1 demonstrate the first three properties. The dashed curve is from a uterus which was exposed to 191 K after 20 minutes and again for another 30 minute period after 2 hours. Although the first exposure to 191 K did not appreciably lower the Ca^{45} content below that of the control uterus, it abolished the increased efflux on the second

exposure. The uterus completely relaxed after the first exposure and contracted again on the second one.

In the experiment illustrated in Fig. 2 the effects of an initial 20 minute exposure to Ca-free K.R. containing 0.7 mM EGTA on the Ca^{45} efflux and the final depolarization were tested. This procedure was compared with a normal efflux followed by late application of 191 K, done on the paired uterine horns from the same rats. The results are shown as Ca^{45} in the tissues vs. time in the upper curves, and as the rate of efflux vs. time in the lower curves. The Ca concentration of K.R. and 191 K used in this experiment was raised to 2.5 mM.[1]

The results show that chelation of the extracellular Ca by EGTA increases the rate of Ca^{45} efflux,[2] and that it also removes the Ca which is involved in the increased efflux induced by high K depolarization. The rate curves show that although immediately before depolarization the efflux rate of the experimental horns is close to that of the controls, the depolarization has no effect on the rate of efflux in the EGTA-treated uteri. Finally, the point *f* was tested by incubating uteri in Ca^{45}-labeled K.R. for only 10 minutes. The tissues were then effluxed into K.R. for 30 minutes, into 191 K for 15 minutes, and for a final 10 minutes into K.R. Fig. 3 shows the average results of 3 such experiments. In spite of the short incubation period, high K was still able to produce a brief small increase in Ca^{45} efflux, which indicates that some Ca in the fraction affected by K depolarization exchanges in 10 minutes when exchange of cellular Ca is minimal (14).

The evidence presented above thus indicates that high K depolarization liberates some superficial Ca, most of which exchanges very slowly and which is in parallel with the remainder of the cellular exchangeable Ca.

Effect of Acetylcholine on Ca^{45} Efflux

The fact that high K depolarization releases some Ca from the cell surfaces suggested that this Ca might be involved in stabilizing the cell membranes and that its release might accompany increased membrane permeability and depolarization. If this superficial Ca fraction had such a specific function, it would be expected that acetylcholine, which increases membrane permeability and causes depolarization, would also liberate Ca from this fraction.

This theory was tested by adding acetylcholine to the media late during the efflux period. The tissues were loaded for the usual 2 to 3 hours in Ca^{45}-labeled K.R. The effects on efflux of acetylcholine concentrations of 3, 10, and 100 μg/ml were so tested. None of these concentrations increased Ca^{45}

[1] At 2.5 mM Ca the response to 191 K was more uniform than at the usual concentration of 1.5 mM, at which concentration it occasionally was so small as to be difficult to analyze.

[2] The effects of EGTA on efflux resemble those of EDTA previously described (14).

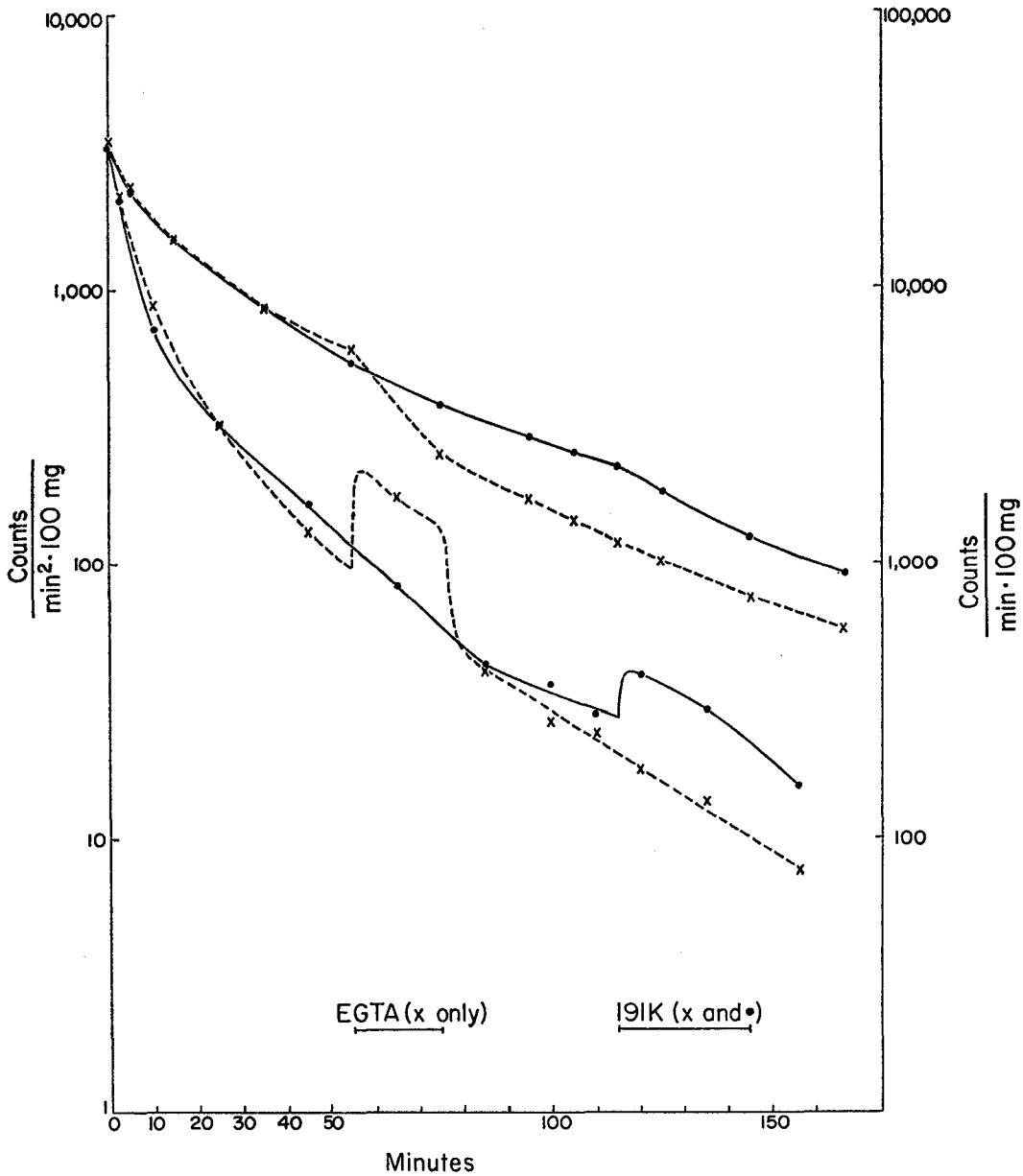

FIGURE 2. The dashed curves (crosses) show the Ca^{45} efflux from uterine horns for successive periods into K.R., into Ca-free K.R. containing 0.7 mM EGTA, into K.R., into 191 K, and finally into K.R. Opposite horns from the same rats were effluxed in K.R. and 191 K only (solid curves, dots). The times of EGTA and high K exposure are indicated by the horizontal bars. The upper curves indicate the loss of radioactivity and the lower curves the rate of loss of radioactivity from the tissues. Each point is an average of 5 determinations.

efflux or had any marked effect, as illustrated in Fig. 4 for 100 μg acetylcholine per ml K.R.

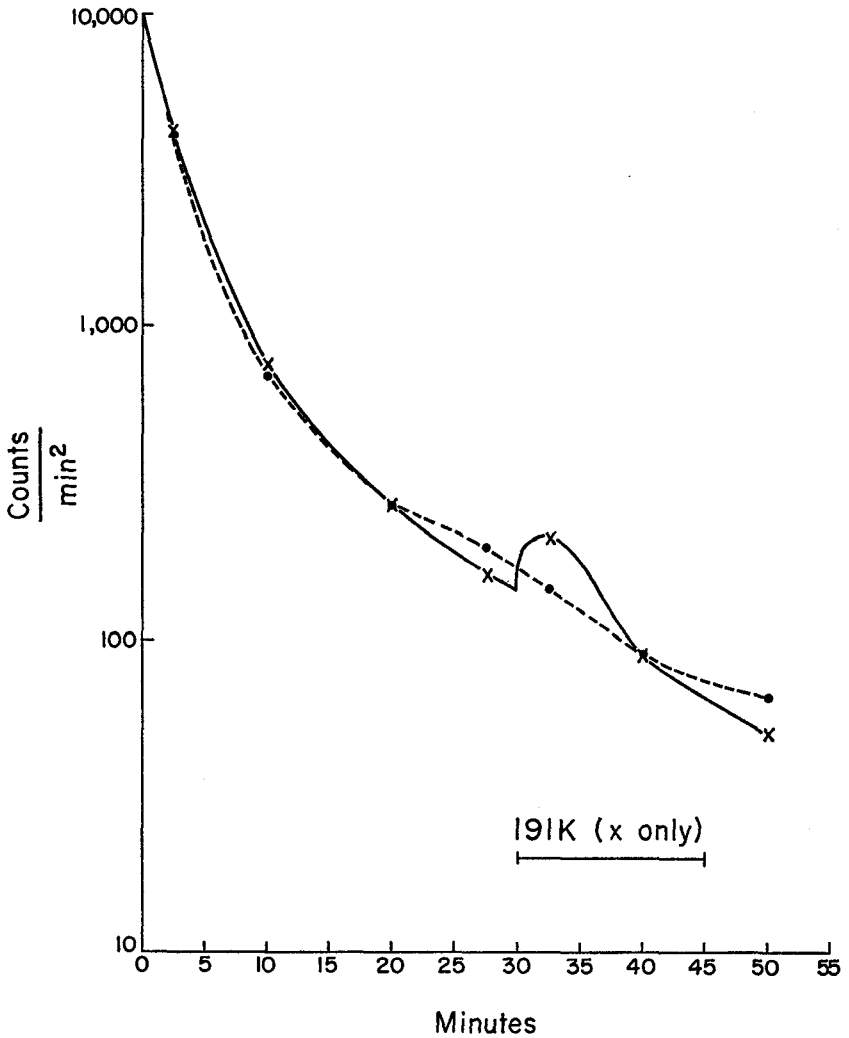

FIGURE 3. Rate of efflux of Ca^{45} from rat uteri which were loaded in Ca^{45}-labeled K.R. for 10 minutes. Efflux medium was changed from K.R. to 191 K for the period indicated by the horizontal bar in the case of the experimental uteri (crosses). Each point is an average of 3 determinations. Controls, dots.

Although acetylcholine did not release Ca^{45} into the efflux media, it was still possible that a release from a superficial fraction occurred, but that the released Ca^{45} entered the cells or was undetected. Since the Ca in the superficial fraction is in parallel with the intracellular Ca, this possibility could be

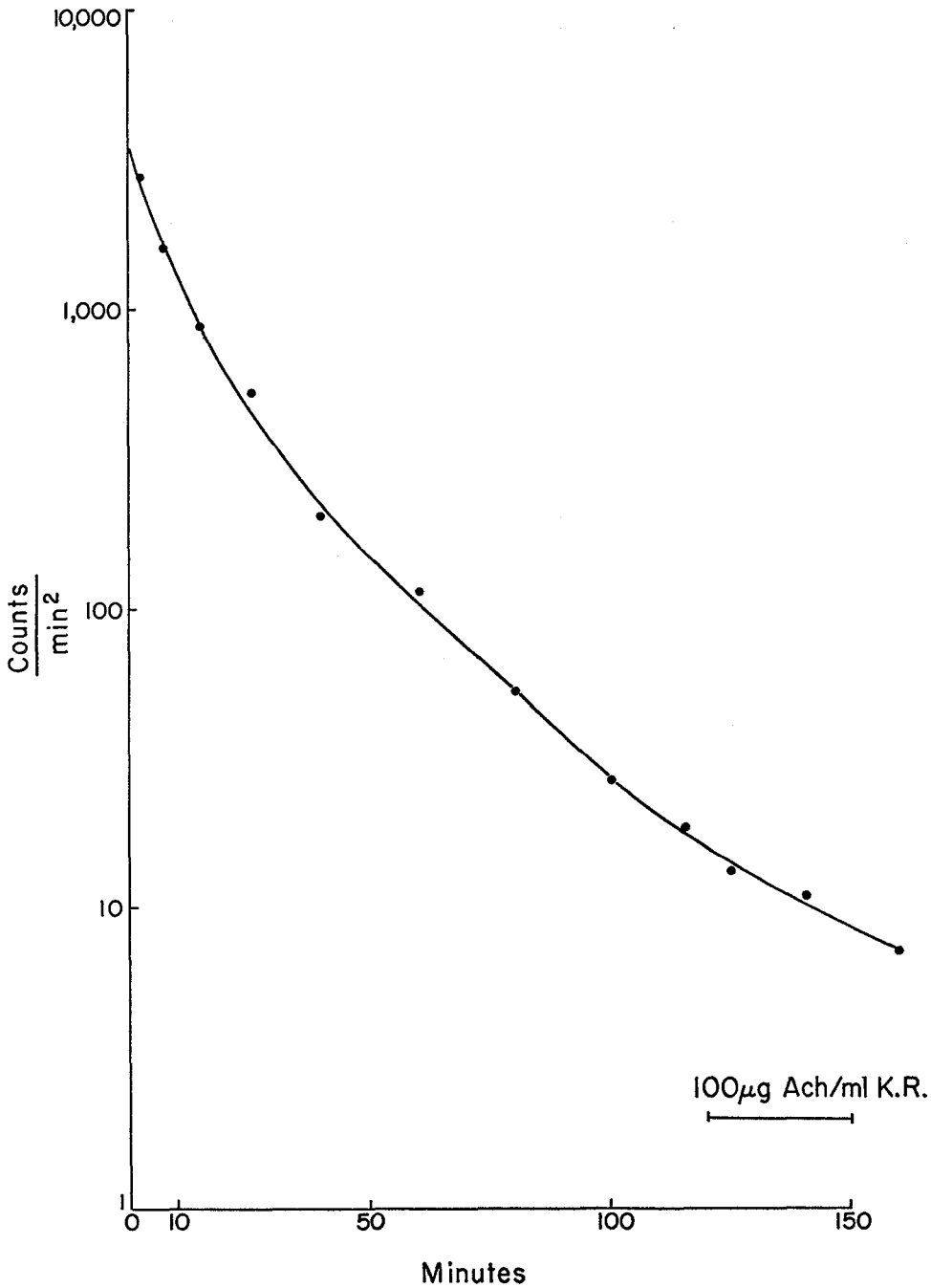

FIGURE 4. Rate of efflux from a uterine horn loaded in Ca^{45}-labeled K.R. for 2 hours. At the time indicated by the horizontal bar 100 μg acetylcholine (Ach) per ml K.R. was added.

tested for by determining the effect of early acetylcholine exposure on the high K–induced increased Ca^{45} efflux at a later time. Fig. 5 gives the average results of 4 such experiments. Even a 40 minute exposure to acetylcholine did not release Ca^{45} from the superficial fraction. The rate of efflux from the acetylcholine-treated horns is seen to be slightly higher than that from the controls. This could be due to higher Ca^{45} content of the experimental horns, as shown in the inset of Fig. 5. These results show that acetylcholine does not affect the slowly exchanging Ca which is released by high K depolarization.

The effect of acetylcholine on the Ca^{45} efflux in two modified media was also tested. Acetylcholine does not affect the loss of Ca^{45} into 191 K. As will be shown later, this is in contrast to its effect on influx from 191 K, in which case it appears to increase the permeability for Ca moving inward.

Acetylcholine also does not affect the efflux of Ca^{45} into Ca-free K.R. This finding does not support the hypothesis proposed by Edman and Schild (8), that acetylcholine increases the rate of loss of contractility in Ca-free media by increasing the rate of loss of Ca from the tissues.

Effects of High Potassium Depolarization and Acetylcholine on Ca^{45} and Total Ca Uptake

It is a widely held view that contraction of smooth muscle is initiated by an increased Ca influx resulting in a net increase in tissue Ca (5–7, 15). Other investigators have obtained evidence that the involvement of Ca is more complex and that the release of bound Ca is also necessary for contraction (8, 9, 11, 12, 16).

The most direct way of testing the theory that contraction is induced by a net Ca influx is to measure the influx of Ca^{45} and net Ca movement during a contraction. Fig. 6 compares the influx of labeled Ca under four different conditions causing contractions with the control influx. At time zero the uteri were placed in the labeled solution which is indicated beside its respective curve, and they were removed and analyzed after 10, 30, and 60 minutes. In all solutions except the control K.R. the uteri were observed to be contracted throughout the hour of incubation. Nevertheless only the uteri in K.R. containing acetylcholine had an uptake similar to that of the controls, and those in the high K depolarizing solutions had a markedly slower influx of labeled Ca than the controls.

The decrease in Ca^{45} uptake during the first 10 minutes in the contracting uteri can best be explained by the fact that contraction of smooth muscle can greatly diminish the extracellular space (17, 19). This explanation is also in accordance with the observation from Fig. 6 that addition of acetylcholine seems to further diminish the initial Ca^{45} uptake, since acetylcholine has been shown to potentiate a high K contraction (8). The observation that the initial uptake from acetylcholine-containing K.R. is larger than that from the high

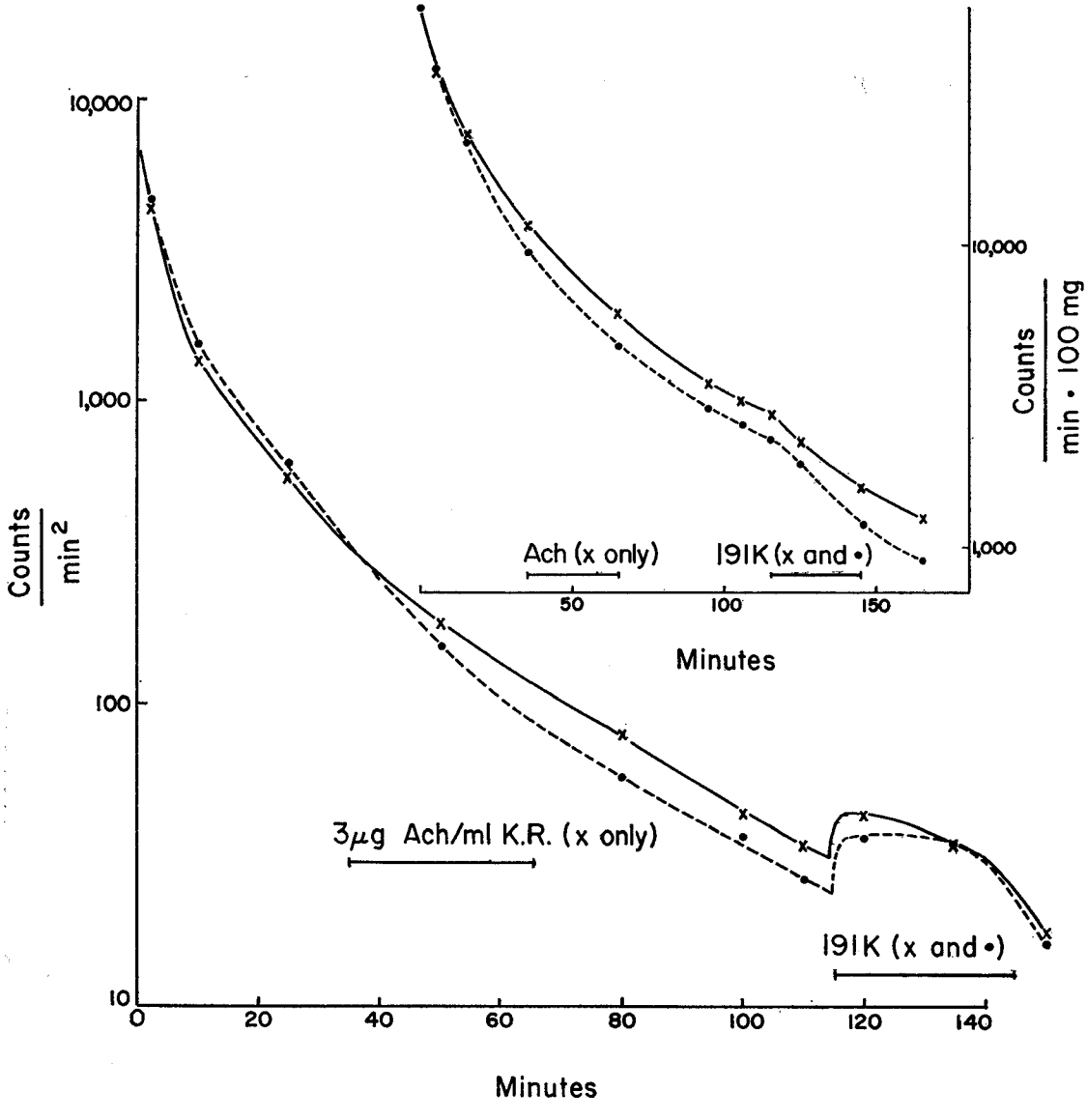

FIGURE 5. Uterine horns loaded with Ca^{45} for 2 hours before efflux into K.R. and 191 K. All uterine horns were exposed to 191 K for the time indicated by the second horizontal bar, and the experimental horns (crosses) were also exposed to 3 μg acetylcholine (Ach) per ml K.R. during the time indicated by the first horizontal bar. The curves in the inset show the loss of Ca^{45} and the lower curves show the rate of loss of Ca^{45}. The experimental (crosses) and control (dots) horns came from the same rats. Each point is the average of 4 determinations.

K solutions can also be explained by considering the effect of contraction on the extracellular space. At 37°C high K induces a contracture which is maintained at a perfectly steady level for as long as the K concentration remains elevated (15). This means that any mixing effect of the contraction to increase

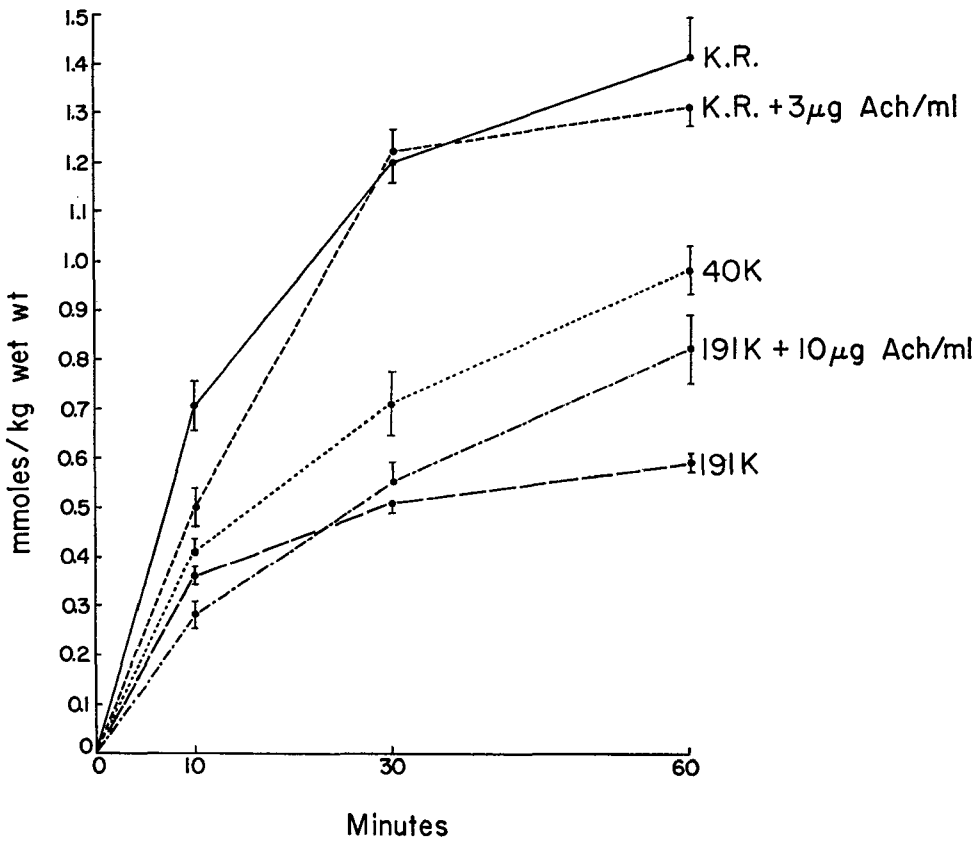

FIGURE 6. Ca^{45} uptake by rat uterine horns from different Ca^{45}-labeled media (as indicated by the respective uptake curves). Points of three lower curves are averages of 5 separate determinations; of two upper curves, averages of 4 separate determinations. Vertical bars equal twice SE. Bars projecting from one side of point equal SE.

the rate of exchange of extracellular Ca^{45} is likely to be of little importance, since the extracellular fluid is merely initially squeezed out. On the other hand, acetylcholine increases the frequency of action potentials and the contractions are rhythmic, so that the mixing effect on Ca^{45} in the extracellular space would be considerable, thus speeding up the early uptake of Ca^{45}.

Durbin and Jenkinson (5) showed that acetylcholine causes an increase in nonspecific membrane permeability in depolarized smooth muscle of the guinea pig taenia coli and causes a small increase in Ca^{45} uptake during 7

minutes' exposure to Ca^{45} high K depolarizing solution. Comparison of the curves for 191 K and 191 K plus acetylcholine in Fig. 6 also shows such an acetylcholine-induced Ca^{45} uptake in the rat uterus. Since the rate of Ca^{45} uptake becomes faster in the acetylcholine-treated depolarized uteri only after 10 minutes (the time required for exchange of extracellular Ca) and since the site of acetylcholine action is believed to be at the cell membrane (5), this acetylcholine-induced increased Ca uptake is taken to be an increased rate of Ca entry into the cells.

Finally, Fig. 6 demonstrates that the amount of decrease of Ca influx is related to the degree of depolarization. Casteels and Kuriyama report (18) that at elevated external K concentrations a tenfold increase in K concentration causes a 32 mv change in the membrane potential of the nonpregnant rat uterus. Utilizing this figure and taking the internal K concentration as 181 mM (19), an external K concentration of 40 mM depolarizes the membrane to about −21 mv (inside minus outside), and an external K concentration of 191 mM completely abolishes or slightly reverses the membrane potential. Thus as the inward electrical driving force for Ca ions is diminished to slightly less than one-half, the Ca influx is markedly reduced, and when the driving force is abolished the influx of Ca is reduced even further. These results could be most easily explained by assuming that, at least during one phase of membrane permeation, Ca penetrates the cell membranes in ionic form or as part of a positively charged complex.

Although these results very strongly suggest that high K–induced contractions are not accompanied by a net Ca influx in the uterine smooth muscle, more complete evidence is necessary to prove this point. Such evidence was obtained for the depolarization contraction induced by 191 mM K. For a net influx to take place during this procedure, it can be calculated from data in Fig. 6 that the efflux would have to decrease at least to one-third of its control value. However, Fig. 1 shows that the first depolarization had no discernible effect on the Ca^{45} efflux. This suggests that the high K depolarization was accompanied by a net Ca efflux instead of an increased net influx. This was verified by measuring the effects of 191 K on the total Ca concentration and on the total exchangeable Ca concentration. For the first determination, uterine horns were simply left for 2 hours in 191 K, while other horns serving as controls were left for 2 hours in K.R. The depolarized uteri had an average total Ca concentration of 1.8 ± 0.08 (4) mmoles/kg wet weight and the controls 2.2 ± 0.09 (4) mmoles/kg wet weight. The effect of 191 K on the total exchangeable Ca was determined by allowing it to become labeled by incubating the uterine horns for 140 minutes in Ca^{45}-labeled K.R. and then transferring them to 191 K, labeled with Ca^{45} at the same specific activity as the labeled K.R. for 1 hour. The controls were left in labeled K.R. for 200 minutes. The total amount of exchangeable Ca in the depolarized horns was 1.12 ± 0.10 (4) mmoles/kg wet weight, and in the controls was 1.67 ±

0.047 (4) mmoles/kg wet weight. In both measurements the depolarized uteri had significantly less total Ca or Ca^{45} than the controls ($P < 0.01$). Thus the high K–induced contraction was accompanied by a net efflux of about 0.5 mmole/kg wet weight. This is not due to a loss of ionic extracellular Ca by a reduced extracellular space during contraction, since the exchangeable cellular Ca concentration is about 2.9 mmoles/liter of cell water and the extracellular Ca concentration is 1.5 mM. Thus loss of extracellular fluid would slightly raise the total exchangeable tissue calcium expressed per unit weight.

If for convenience the approximating assumption is made that after the first 10 minutes the influx illustrated in Fig. 6 is due to penetration of Ca^{45} into the cells, then the Ca influx into the cells during 1 hour is 0.47 mmole/kg wet weight greater in the uteri incubated in K.R. than in those incubated in 191 K. Thus the decreased Ca uptake in 191 K accounts almost entirely for the net Ca loss induced by this procedure. This also confirms the finding that the efflux of cellular Ca^{45} was not significantly altered by depolarization.

The above results prove that the contraction induced by 191 mM K is not caused by a net inward movement of Ca.[3] They are also evidence against such a hypothesis for contractions induced by other K concentrations and acetylcholine.

The results presented in Fig. 6 suffer from the fact that the changes in extracellular space and the mixing effect of periodic contractions prevented a direct comparison between the Ca uptake during contraction and that during rest. In order to make a direct comparison possible, the following experimental procedures were carried out. Uterine horns were mounted as for the efflux experiments, and incubated for 10 minutes in Ca^{45}-labeled K.R. They were then transferred to a tube containing the Ca^{45}-labeled experimental test solution for a time T, after which they were washed in 5 tubes containing Ca^{45}-labeled K.R. for a total time of 10 minutes. The tissues were then effluxed for an appropriate length of time, blotted, weighed, ashed, and analyzed for Ca^{45}. The control uteri were treated in exactly the same way except that the test solution was Ca^{45}-labeled K.R. The specific activities of the labeled test solutions and labeled K.R. were made to be the same so that the tissue counts could be compared directly. The initial 10 minutes in labeled K.R. served to equilibrate the extracellular space, and the final 10 minutes of washing in labeled K.R. served to relax the contracted uteri and thus restore the ratio between extra- and intracellular spaces.

When the test solution was K.R. containing 3 μg acetylcholine per ml,

[3] Although the decrease in influx brought about by 191 K was too great to have been produced by even an absence of influx in the endometrium only, a check was made with carefully dissected longitudinal muscle. In an average of 3 experiments the labeled Ca gain between 10 and 30 minutes after the onset of Ca^{45} influx was 0.33 mmole/kg wet weight in the controls but only 0.14 mmole/kg wet weight in the depolarized uteri.

and T was 10 minutes, the uptake expressed in mmoles per kg wet weight was 1.32 ± 0.05 (5) for the experimental and 1.33 ± 0.48 (5) for the control uteri. Fig. 7 shows the results obtained when the test solutions were 191 K and 191 K containing 10 μg acetylcholine per ml, and time T was 30 minutes. It is evident that although 191 mM K induced an observable contraction,

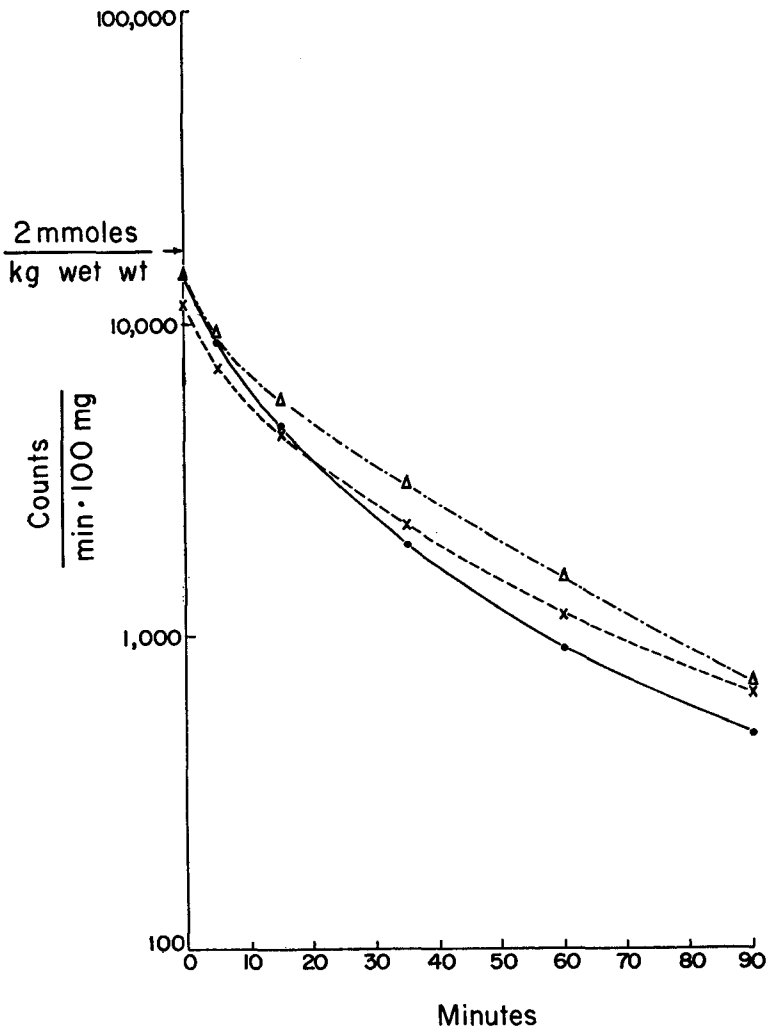

FIGURE 7. Rat uterine horns were incubated in Ca^{45}-labeled K.R. for 10 minutes and then for a further 30 minutes in Ca^{45}-labeled K.R. (dots), in Ca^{45}-labeled 191 K (crosses), or in Ca^{45}-labeled 191 K containing 10 μg acetylcholine per ml (triangles). The horns were then washed in Ca^{45}-labeled K.R. for another 10 minutes and effluxed into K.R. for 90 minutes. Each point is the average of 5 determinations. "2 mmoles/kg wet wt" indicates the tissue count which would be equivalent to 2 mmoles of labeled Ca (at specific activity of external solution) per kg tissue.

the uptake of Ca^{45} was less than in the controls. The addition of acetylcholine to the depolarizing solution increased the Ca influx, bringing it to a value which equals the control uptake.

Fig. 7 also demonstrates what could be an important phenomenon of Ca exchange during contraction, namely, that the Ca^{45} which was taken up during contraction is released more slowly than the Ca^{45} taken up by the tissues at rest. This can be seen by the crossing of the 191 K curve above the control curve after 20 minutes, and by the fact that after 10 minutes the control efflux curve falls below the 191 K plus acetylcholine efflux curve. These results could be explained by postulating that contraction is not necessarily dependent upon a movement of Ca from the outside into the cells, but is accompanied or preceded by a movement of Ca from a more readily exchangeable cellular fraction into a more slowly exchangeable fraction. The meaning of such a possible Ca movement accompanying contraction will be explored in the "Discussion."

In experiments of the same type using as the test solution either K.R. containing 10 μg acetylcholine per ml (4 experiments) or 191 K containing 10 μg acetylcholine per ml (3 experiments), the same results were obtained. The contraction during influx decreased the Ca^{45} uptake slightly, but the efflux of Ca^{45} taken up during contraction was slower.

To test the possibility that the lower efflux rate following Ca^{45} loading during contraction was caused by an uptake into the inexchangeable fraction, the efflux curves were compared after loading in Ca^{45}-labeled K.R. containing 3 μg acetylcholine per ml for 3 hours on the one hand, and loading for 3 hours in labeled K.R. on the other hand. If the acetylcholine-induced contraction causes a greater amount of Ca^{45} to be taken up into a fraction which is very slowly exchangeable or inexchangeable in K.R., then the efflux curve of the horns loaded during acetylcholine treatment should be above the control efflux curve. There was no difference in 5 experiments between the efflux curves obtained following the two procedures, thus indicating that the inexchangeable Ca is not involved.

DISCUSSION

The main conclusion from the Ca fluxes observed during contractions of the myometrium induced by acetylcholine and high K is that cytoplasmic Ca ions necessary to induce contraction are not supplied by an increased influx of extracellular Ca ions, but must be supplied from an intracellular source. The absence of increased Ca^{45} influx in the presence of acetylcholine agrees with Schatzmann's finding (28) that acetylcholine did not affect Ca^{45} uptake in the taenia coli of the guinea pig. An actual decrease of Ca^{45} influx and net Ca efflux during high K depolarization as observed in the rat myometrium has not been observed in other smooth muscle preparations. In

fact, an increased Ca^{45} influx during high K depolarization has been demonstrated in smooth muscle of cat intestine (21), rabbit aorta (22), and guinea pig taenia coli (15). However, the results of Urakawa and Holland (15) in taenia coli could also be explained as the consequence of a shift of Ca from a more rapidly exchanging cellular fraction to a more slowly exchanging one during depolarization. They washed the smooth muscle in inactive solution for a period of 4 minutes after the test influx. Bauer *et al.* (6) have shown this interval to be long enough to exchange almost 90% of the tissue Ca. Thus it appears that the residual Ca^{45} after the 4 minute wash could have resulted from a slower efflux during the wash period. The small net Ca gain on depolarization reported by Urakawa and Holland does not rule out the above explanation, since it could also have been caused by a squeezing out of extracellular fluid, which had a lower Ca concentration than the cells, with a resulting increase in calcium concentration expressed as mEq/kg wet tissue. A shift of Ca to a more slowly exchangeable fraction during contraction was demonstrated in the rat uterus by a slower efflux of Ca^{45} into K.R. from uteri stimulated to contract during influx than from the control uteri which were not exposed to high K or acetylcholine during loading with Ca^{45}.

In the previous communication (14) it was shown that active Ca accumulation into some cellular Ca fraction was necessary in order to maintain the ionic cytoplasmic Ca concentration at 10^{-7} M. In skeletal muscle the endoplasmic reticulum is responsible for the active accumulation of Ca ions from the sarcoplasm and also for the release of Ca during contraction (20, 3, 23–25). Analogously, it is likely that the structures which actively accumulate Ca in the myometrium in order to maintain relaxation also release Ca to induce contraction during stimulation, and it is postulated that these structures are the endoplasmic reticulum and the internal surface of the cell membrane. On the basis of this postulate, it would be predicted that contraction would increase the exchange between Ca associated with cellular membranes and Ca associated with actomyosin. Accordingly, the slower Ca^{45} efflux after incomplete loading of the tissue with Ca^{45} during contraction would be expected if the membrane- and reticulum-bound Ca fractions exchanged at faster rates than the actomyosin-bound fraction. That the actomyosin-bound Ca exchanges slowly is suggested by the fact that in skeletal muscle it is partly inextractable with Ca-free solutions (20).

The manner in which high K depolarization affects Ca exchange is not clear. The observation that the Ca influx was related to the degree of depolarization suggests that the decreased Ca influx upon depolarization resulted from a diminished inward electrical driving force on Ca ions in the membrane phase. These Ca ions would be in equilibrium with the Ca bound to binding sites in the uterine cell membranes (14). However, an expected comparable increase in Ca efflux did not occur. This may be a consequence

of the postulate (above) that depolarization releases some Ca from the inside of the membrane, if, as does not seem unreasonable, binding to the inside of the membrane is a necessary step in outward Ca movement.

Some experimental evidence other than that reported here also indicates that depolarization indeed decreases membrane affinity for Ca (8, 9). The reduced negative charge on the inside of the membrane may be responsible for the loss of affinity for the Ca cations. Calculations based on estimates of the change in surface-to-volume ratio if the smooth muscle cells shortened to half their length show that the absence of an increased Ca^{45} efflux during depolarization could not be simply accounted for by a decreased surface area for outward Ca movement. The absence of appreciable effects of acetylcholine-induced contractions on Ca^{45} exchange across the cell membranes also indicates that effects on Ca exchange produced by a decrease in surface-to-volume ratio tend to be minimal.

Finally, these results may be due to the presence of different pathways for Ca influx and efflux and/or an increased binding of Ca by the actomyosin during contraction.

The effects of high K depolarization in increasing exchange in a small fraction of the Ca^{45} efflux can be explained by assuming that high K depolarization decreases the affinity of membrane sites for Ca and that the sites which release Ca into the extracellular space rebind Ca from the extracellular space upon repolarization. The high K depolarization would at the same time release Ca from the inside of the membrane into the cytoplasm, causing contraction, and these sites would rebind cytoplasmic Ca upon repolarization, but this Ca released into the cytoplasm would not be detected in the efflux experiments. Thus after the external sites were labeled with Ca^{45}, exposure to high K solution or to Ca chelating agents, which are able to remove superficially bound Ca, during efflux would cause these sites to release their labeled Ca. Upon reexposure to K.R. during efflux these sites would not be relabeled, since they would now rebind unlabeled Ca from the efflux solution. Since membrane-bound Ca stabilizes the membrane (see reviews by Daniel (11), Shanes (26), Brink (27), and Schatzmann (28)) and since the Ca fraction demonstrated here has at least two properties compatible with such a function, namely, a superficial location and decreased Ca affinity upon depolarization, it is plausible to conclude that the Ca fraction which releases Ca into a high K solution is part of the fraction which regulates membrane excitability.

The failure to demonstrate a release of Ca from the same fraction when the uterus was stimulated with acetylcholine does not rule out such a regulatory function, since continual depolarization rather than the very transitory depolarization during acetylcholine-induced action potentials may be necessary for appearance of released Ca^{45} in the extracellular fluid. Acetylcholine

was also unable to affect the Ca^{45} efflux from rat uteri into high K and Ca-free media. Schatzmann (28) also found a difference between the actions of high K and acetylcholine on Ca^{45} efflux from taenia coli. In this smooth muscle both agents increased the rate of Ca^{45} loss, but the effect of acetylcholine was sustained, whereas high K caused a transient increase in efflux. The different effects of acetylcholine and high K appear to be in agreement with the postulate that drug stimulation releases Ca for contraction from the sequestered sites, whereas K depolarization initiates contraction by releasing Ca from the superficial sites (12).

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