Physiological Evidence for Multiple Calcium Sites in Smooth Muscle

GEORGE B. WEISS and LEON HURWITZ

From the Department of Pharmacology, School of Medicine, Vanderbilt University, Nashville. Dr. Weiss' present address is the Department of Pharmacology, Schools of Medicine, University of Pennsylvania, Philadelphia

ABSTRACT   Inherent smooth muscle tone and acetylcholine-induced contractions of the isolated longitudinal muscle from guinea pig ileum are inhibited by 1.2 M ethanol. The inhibitions are antagonized by high concentrations of calcium ion in the external medium. Previous work has indicated that an acetylcholine-induced increase in potassium efflux from the ileal muscle is also inhibited by ethanol and reactivated by high concentrations of calcium ion. It was found in this study that, in addition to ethanol, a drastic reduction in the calcium ion concentration of the bathing medium appeared to produce a depression of this drug-induced increase in potassium efflux. Preincubating the muscle in a reduced calcium ion concentration also inhibited the increase in potassium efflux initiated by a high potassium medium. Conversely, the exposure of the muscle to 1.2 M ethanol did not depress the potassium-induced increase in potassium efflux. Increases in smooth muscle tone produced by a high potassium medium have been reported to be inhibited both by ethanol and by a depletion of external calcium. These data suggest that the calcium ions which activate or enhance a potassium-induced increase in potassium efflux are not bound to the same loci in the muscle fiber as those involved in an acetylcholine-induced increase in potassium efflux or those involved in a potassium-induced increase in smooth muscle tone.

INTRODUCTION

Ethanol has been reported to alter muscle tone and potassium efflux in isolated longitudinal smooth muscle from guinea pig ileum. Both the inherent tone of the muscle fibers and the sustained increase in tone invoked by a high potassium medium are depressed by the drug. Similarly, the large increase in potassium efflux elicited by acetylcholine is inhibited, but potassium efflux from the unexcited smooth muscle is elevated by ethanol. The ethanol inhibitions of potassium-induced increases in muscle tone and acetylcholine-induced

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increases in potassium efflux are reversed by raising the calcium ion concentration in the medium. Based on these observations and on the evidence that the maintenance of ileal smooth muscle tone requires the presence of external calcium ion, the suggestion has been made that, in this smooth muscle preparation, ethanol acts by blocking certain physiological effects of calcium ion. The elevation of potassium efflux by ethanol in unexcited smooth muscle fibers is also consistent with this hypothesis because a reduction in the external calcium ion concentration has also been shown to have a stimulatory effect on potassium efflux from unexcited ileal muscle (1).

The experiments presented in this report were designed to further characterize the interaction between ethanol and calcium ion in ileal smooth muscle. Previous findings are extended to include data on the effects of ethanol and/or calcium ion upon inherent tone, upon potassium-induced increases in potassium efflux, and upon increases in potassium efflux and smooth muscle tone elicited by acetylcholine.

During the course of this study we noted that increases in potassium efflux from smooth muscle cells brought about either by acetylcholine or by a medium containing a high potassium ion concentration appeared to require the presence of calcium ion. However, the flux changes stimulated by each of the two agents did not exhibit the same kind of dependence upon calcium ion. Modifications of the external calcium ion concentration and the application of ethanol helped reveal the complex relationship which exists between calcium ion and transmembrane potassium movement in ileal smooth muscle.

METHODS

The tissue employed in this study was the isolated longitudinal smooth muscle from guinea pig ileum. The separation of this relatively pure smooth muscle layer from the rest of the ileum and the histological methods employed to ascertain its purity have been described previously (2). In the potassium efflux experiments muscle preparations varying in purity from 50 to 80 per cent longitudinal fibers were employed. Tissues of less purity were often used in experiments in which only contractile responses were measured.

Muscles were normally incubated in a modified Tyrode solution of the following composition: NaCl 0.125 M, KCl 0.0027 M, $CaCl_2$ 0.0018 M, $MgCl_2$ 0.0005 M, $NaHCO_3$ 0.0238 M, and glucose 0.011 M. Two other bathing solutions were also used. One was a high potassium medium which was employed as an excitatory agent. Its composition was as follows: KCl 0.127 M, $CaCl_2$ 0.0018 M, $MgCl_2$ 0.0005 M, $KHCO_3$ 0.024 M, and glucose 0.011 M. The other was a bicarbonate-free solution in which large quantities of calcium ion were easily dissolved. Its composition was: tris (tris(hydroxymethyl) aminomethane, Sigma 121) 0.0238 M, NaCl 0.125 M, KCl 0.0027 M, $CaCl_2$ 0.0018 M, and glucose 0.011 M. The solution was adjusted to pH 7.6 with 6 N HCl. Introduction of a high concentration of $CaCl_2$ (0.054 M) did not affect the pH.

A gas mixture consisting of 95 per cent O_2 and CO_2 was constantly bubbled

through the bathing solutions containing bicarbonate ion, and 100 per cent O_2 was constantly bubbled through the bathing solution containing tris buffer. The temperature of the muscle bath was kept at 31.5–32.5°C. The tissues used in potassium efflux experiments were suspended in baths containing 10 ml of solution whereas contraction studies were performed in baths containing 40 ml of solution. The smooth muscles were attached to levers which recorded isotonic contractions of the longitudinal fibers on a kymograph. Tension on the muscle was approximately 0.35 gm.

A high potassium ion concentration or acetylcholine served as excitatory agent. Ethanol, the removal of calcium ion, or both the removal of calcium ion and the addition of EDTA was used to produce inhibitory effects. All solutions containing 1.2 M (7 per cent) ethanol were mixed to volume. Final concentrations of acetylcholine $(1.1 \times 10^{-5} \text{ M})$, of EDTA $(6.7 \times 10^{-5} \text{ M})$, and of greater than normal levels of calcium ion were obtained by introducing small volumes of concentrated stock solutions of these agents. Each of the additions increased the final volume of the bathing solution 2 per cent or less. Calcium-deficient solutions were identical in composition to Tyrode solution or to a high potassium solution except that the 0.0018 M $CaCl_2$ was omitted.

Potassium efflux was measured with the radioactive tracer K^{42}. The rate of outward movement of K^{42} from a muscle preparation which had been made radioactive previously was estimated by measuring the increase in number of counts per unit time that appeared in the bathing fluid. Details of this experimental procedure have been described elsewhere (3).

RESULTS

Effects of Ethanol and Calcium Ion on Acetylcholine-Induced Smooth Muscle Contractions Ethanol has been shown to inhibit the increase in smooth muscle tone induced by a high potassium medium. The inhibition is reversed by raising the concentration of calcium ion in the bathing medium (1). Similar results have been obtained using acetylcholine as the excitatory agent. When Tyrode solution containing 1.2 M ethanol is introduced into the muscle bath, the smooth muscle contraction normally obtained upon addition of acetylcholine is reversibly blocked (Fig. 1 A). This blockade is largely prevented by increasing the calcium ion concentration from 0.0018 to 0.0108 M (Fig. 1 B).

Effects of Ethanol and Calcium Ion on Inherent Tone of Unexcited Muscle In Fig. 2 the effects of ethanol and of a high calcium ion concentration on inherent smooth muscle tone are recorded. As noted previously (1), either the addition of a high calcium ion concentration (Fig. 2 A) or of ethanol (Fig. 2 B) results in an inhibition of smooth muscle tone. However, when both 1.2 M ethanol and 0.0558 M calcium ion are present in the muscle bath an increase in smooth muscle tone occurs (Fig. 2 A and B). The possibility that this response is due partly to the increase in osmotic pressure exerted by the high concentration of calcium ion has been excluded by adding 0.081 M NaCl instead of 0.054 M $CaCl_2$ to the bath. Under these conditions no contractile response was obtained whether ethanol was present or not.
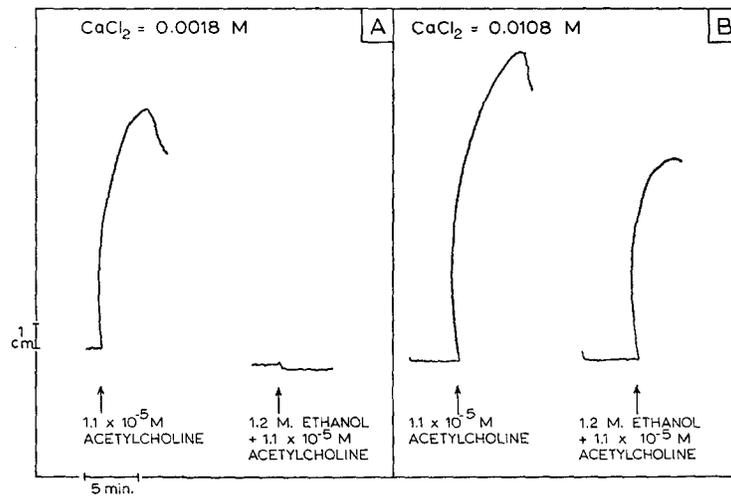
FIGURE 1. Isotonic changes in tone of isolated longitudinal smooth muscle. The bathing solutions used in the experiments shown in sections A and B contained the $CaCl_2$ concentrations indicated at the top of each section. Points at which designated substances were introduced into the muscle bath are indicated by arrows. The stated concentrations represent final molar concentrations in the bathing fluid.
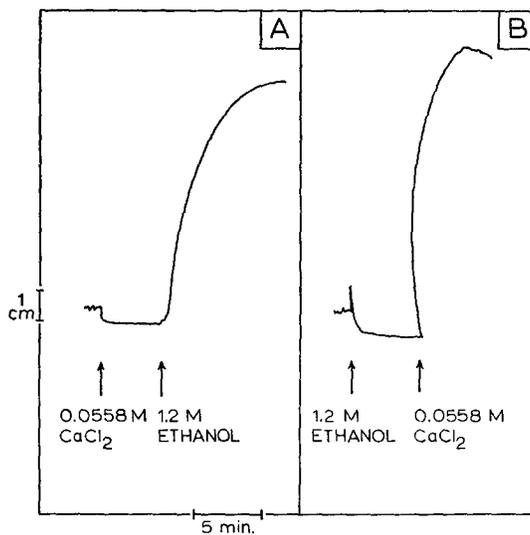

FIGURE 2. Isotonic changes in tone of isolated longitudinal smooth muscle. The bathing solution used in the experiments shown in this figure was the tris-buffered solution. Points at which designated substances were introduced into the muscle bath are indicated by arrows. The stated concentrations represent final molar concentrations in the bathing fluid.

These observations, as well as others mentioned in the introductory section, suggest that the effect of ethanol on smooth muscle function is similar to that produced by a reduction in the external concentration of calcium ion. A major objective of this study was to determine whether the similarity between ethanol and a reduced calcium ion concentration also applies to their effects on the elevated efflux of potassium ion invoked by acetylcholine and by a high po-

tassium medium. It has already been shown that the increase in potassium efflux elicited by acetylcholine is inhibited by ethanol and reactivated by high levels of calcium ion (1). Additional studies which were carried out to provide a basis for comparing the effects of ethanol and reduced calcium ion concentrations on potassium efflux are presented below.

Effect of Lowering Calcium Ion Concentration on the K^{42} Efflux Induced by a High Potassium Medium and by Acetylcholine   The efflux of potassium ion from an isolated ileal smooth muscle immersed in a modified Tyrode solution obeys the kinetics of a first order reaction. Thus, the logarithm of the rate of outward movement of potassium ion regresses linearly with time. The introduction of a high potassium medium or of a 1.1×10^{-5} M concentration of acetylcholine for a 5 minute period evokes a large increase in the K^{42} efflux. In ten experiments the increase in potassium efflux produced by the high potassium medium ranged from 303 to 703 per cent and averaged 403 per cent. In five experiments 1.1×10^{-5} M acetylcholine produced an increase in K^{42} efflux that ranged from 200 to 406 per cent and averaged 289 per cent. These results are illustrated by single representative experiments shown in Fig. 3 A and C.

If 0.0018 M $CaCl_2$, which is regularly added to all bathing solutions, is omitted from the high potassium medium, the resulting increase in K^{42} efflux is somewhat higher than normal. In ten experiments using paired tissues (tissues obtained from adjacent segments of the same guinea pig ileum) an average increase of 403 per cent was obtained in the tissues exposed to a high potassium medium and 519 per cent in the tissues exposed to a high potassium medium from which calcium ion was omitted. There is a statistically significant difference between the two averages ($p < 0.01$ by sign test).

It was found that the omission of calcium ion from the bathing solution for a 5 minute period prior to the introduction of the high potassium, calcium-deficient medium resulted in an opposite effect. This short preincubation period in which the muscle was deprived of a normal calcium ion concentration (0.0018 M) in its external environment was sufficient to cause an appreciable inhibition of the potassium-induced increase in potassium efflux (Fig. 3 A and B). In five experiments the average increase in potassium efflux was 242 per cent (range: 177 to 320 per cent), whereas in media which contained a normal level of calcium ion it averaged 403 per cent.

When acetylcholine was employed as the stimulatory agent the experimental findings were quite different. In this case, an ileal smooth muscle, incubated for a 5 minute period in a medium that did not contain the regularly added $CaCl_2$, exhibited a larger than normal increase in K^{42} efflux upon addition of acetylcholine (Fig. 3 C and D). The average increase observed in six experiments was 443 per cent (range: 324 to 636 per cent) as opposed to an average increase of 289 per cent induced by acetylcholine in normal bathing media.
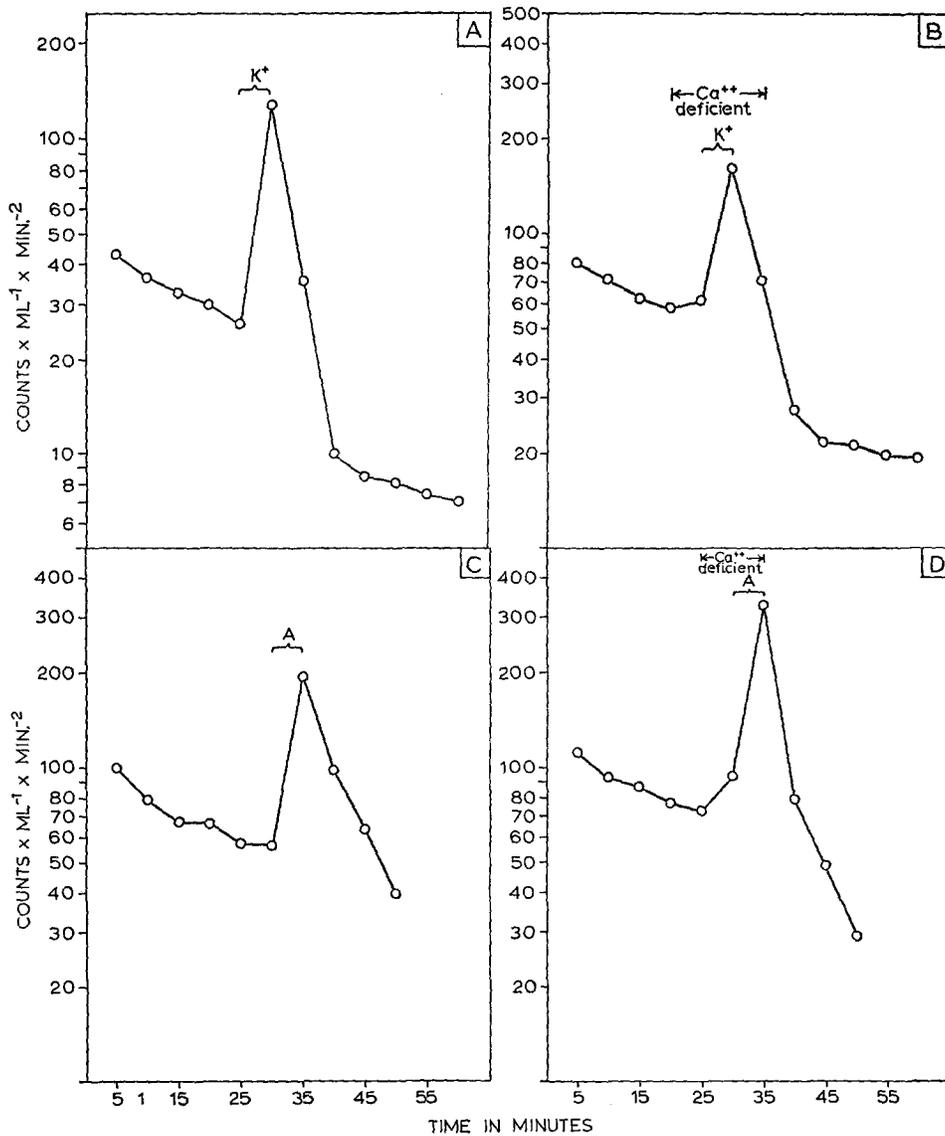
FIGURE 3.   Potassium efflux from isolated longitudinal smooth muscle. Calcium-deficient solutions (Ca^{++}-deficient) were used as bathing media for the periods of time indicated by the appropriate arrows. A final concentration of 1.1×10^{-5} M acetylcholine (A) or a high potassium medium (K^+) was introduced into the muscle bath for the 5 minute periods indicated by the braces.

All the above stated values of percentage increase in K^{42} efflux were determined in the same manner. As illustrated in figures presented in this paper, experimental data were plotted on a graph which showed, on a logarithmic scale, the average rate of loss of counts from the tissue for a 5 minute interval

(given as counts $\times$ ml^{-1} $\times$ min.$^{-2}$) measured at successive 5 minute intervals which were spaced on a linear scale. The best straight line was fitted to those points which denoted rate of loss of counts at various time periods during which the tissue was suspended in a normal bathing medium. The line was then extended to the point on the time scale that represented the period during which a stimulatory agent was present in the muscle bath. This was done to approximate the rate of loss of counts that would have been expected in that time period had normal conditions prevailed. The actual rate of loss of counts from the muscle as it was influenced by the changes made in experimental conditions was determined experimentally. The extrapolated value obtained by extension of the straight line and the experimentally determined value were used to calculate the percentage increase in K^{42} efflux resulting from altered experimental conditions; *i.e.*, addition of stimulatory agents and modifications of the external calcium ion concentration.

This method of determining percentage increase in K^{42} efflux gave values which were smaller than they should have been in those experiments in which the concentration of calcium ion was reduced 5 minutes before introducing the stimulatory agent. This is so because the method did not allow for the greater than normal loss of potassium ion from the tissue during its 5 minute preincubation in the calcium-deficient medium. For example, the 242 per cent increase induced by the high potassium medium, under these experimental conditions, was estimated to be in error by as much as 40 percentage points. Therefore, the real increase may have been as high as 282 per cent. These errors do not affect the interpretation of our results.

A second group of experiments was performed in which the changes in potassium efflux brought about by a high potassium medium and by acetylcholine were measured in tissues that previously had been exposed to calcium-deficient bathing media for 1 hour. The results are illustrated in Fig. 4 A and B. The high potassium medium produced an increase in potassium efflux which averaged 116 per cent (range: 100 to 133 per cent) in five experiments and 1.1×10^{-5} M acetylcholine produced an increase which averaged 240 per cent (range: 171 to 340 per cent) in five experiments.

The data given in Fig. 4 A and B show that the mere omission of CaCl$_2$ from Tyrode solution will enhance the efflux of potassium ion from the smooth muscle fibers. Therefore, values of percentage increase in potassium efflux calculated from the higher baseline, in these latter experiments, cannot be compared directly with values calculated from the lower baseline level of potassium efflux obtained from tissues immersed in normal media. However, drug-induced changes in K^{42} efflux arising from the same baseline can be compared.

It was observed that in tissues immersed in media that contained a normal calcium ion concentration (0.0018 M) the average increase in K^{42} efflux produced by the high potassium medium (403 per cent) was higher than that pro-

duced by acetylcholine (289 per cent). When the tissue was exposed to a calcium-deficient medium 5 minutes before, as well as during the interval of time that the stimulatory agents were present in the muscle bath, the action of acetylcholine on the efflux of potassium ion was enhanced, while the action of the high potassium medium was inhibited. Acetylcholine produced almost twice the increase in K^{42} efflux (443 per cent) that the high potassium medium
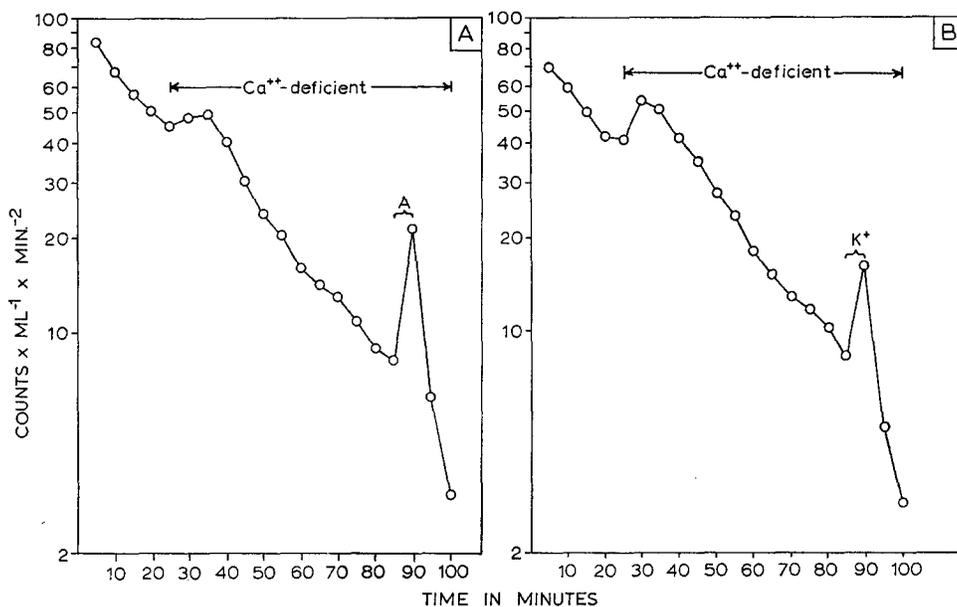

FIGURE 4.   Potassium efflux from isolated longitudinal smooth muscle. Calcium-deficient solutions (Ca^{++}-deficient) were used as bathing media during the periods designated by the appropriate arrows. A final concentration of 1.1×10^{-5} M acetylcholine (A) or a high potassium medium (K^+) was introduced into the muscle bath for the 5 minute periods indicated by the braces.

did (242 per cent). Similarly, in tissues that were incubated in calcium-deficient media for 1 hour prior to the introduction of a stimulatory agent, acetylcholine produced about twice as high a response (240 per cent) as did the high potassium medium (116 per cent).

This difference between the actions of the two stimulatory agents was not demonstrable on the contractile response of the smooth muscle preparation. The omission of 0.0018 M $CaCl_2$ from the bathing solution inhibited a sustained increase in smooth muscle tone whether it was initiated by acetylcholine or a high potassium medium.

The difference between the actions of the two stimulatory agents on potassium efflux was also found to be less distinguishable if calcium ions were more completely removed from solution by the inclusion of the calcium com-

plexor EDTA (ethylenediaminetetraacetic acid). Experiments were performed in which the smooth muscle tissue was suspended in a calcium-deficient solution for 1 hour before adding the stimulatory agent. EDTA (6.7×10^{-5} M) was introduced into the solution during the last 20 minutes of that hour. The muscle remained in the calcium-deficient solution which contained EDTA for the 5 minute period that the stimulatory agent was present in the bath and for
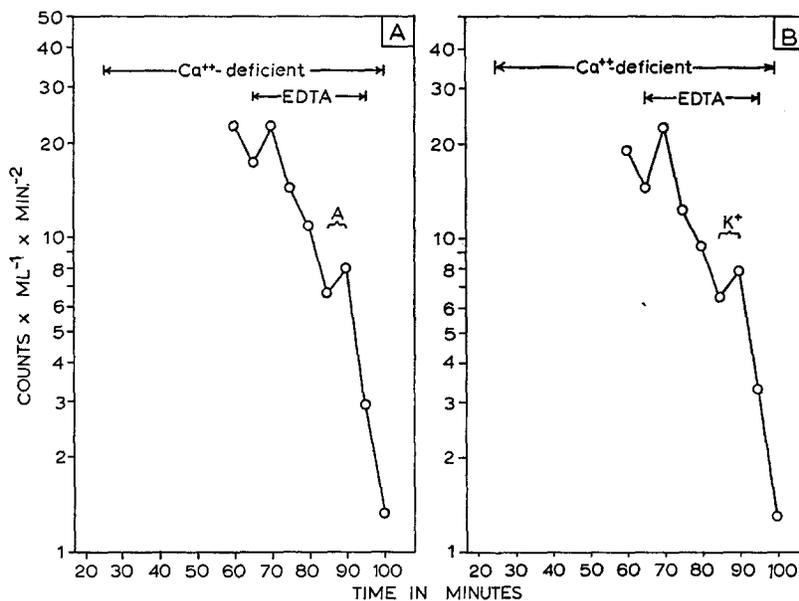

Figure 5. Potassium efflux from isolated longitudinal smooth muscle. Calcium-deficient solutions (Ca^{++}-deficient) were used as bathing media for the periods of time indicated by the appropriate arrows. A final concentration of 6.7×10^{-5} M EDTA was introduced into the muscle bath for the periods of time indicated by the appropriate arrows. A final concentration of 1.1×10^{-5} M acetylcholine (A) or a high potassium medium (K^+) was introduced into the muscle bath for the 5 minute periods indicated by the braces.

5 minutes after it was removed. The average increase in K^{42} efflux produced by acetylcholine was 63 per cent. Values ranged from 17 to 84 per cent in five experiments. The average increase in K^{42} efflux produced by the high potassium medium was 89 per cent. Values ranged from 49 to 123 per cent in five experiments. Typical experiments are presented in Fig. 5 A and B. It may be seen that the addition of EDTA alone increased the efflux of potassium from the smooth muscle fibers. A distinctive aspect of these experiments was the finding that acetylcholine induced either the same or a smaller increase in K^{42} efflux than did the high potassium medium. These experimental results are in sharp contrast to the findings mentioned previously that acetylcholine caused twice the increase in potassium efflux that the high potassium medium did in

muscles that were preincubated for 1 hour in calcium-deficient media. The data strongly suggest that a drastic reduction in the level of calcium ion in the external environment and perhaps in the smooth muscle fibers can depress the stimulatory action of acetylcholine on the efflux of potassium ion. An alternative interpretation may be that under conditions of severe calcium depletion the increases in K^{42} efflux elicited by both the high potassium medium and by acetylcholine are enhanced. This latter interpretation is a less probable one because the depletion of most of the calcium ion in the medium (by the use of calcium-deficient media, but not EDTA) resulted in a definite depression of the potassium-induced flux change, although the acetylcholine-induced flux change was increased.

Effect of Ethanol on K^{42} Efflux Induced by Acetylcholine and by a High Potassium Medium   A moderate reduction in calcium ion concentration does not inhibit the increase in K^{42} efflux initiated by acetylcholine, but it does enhance the inhibition of this transmembrane process by ethanol (1). Experiments showing the increase in K^{42} efflux elicited from the smooth muscle by acetylcholine in a solution containing one-fifth normal calcium ion concentration both in the absence and presence of ethanol have been presented previously (1). They are also shown in Fig. 6 A and B for the purpose of comparing them with analogous experiments in which a high potassium medium was employed as excitatory agent. In bathing solutions containing 0.00036 M $CaCl_2$, 1.2 M ethanol was reported to have caused a 71 per cent inhibition of the acetylcholine-induced increase in K^{42} efflux (average of five experiments).

Fig. 6 C and D illustrate the effect of ethanol on the increase in K^{42} efflux induced by a high potassium medium. Solutions containing one-fifth normal calcium ion concentration (0.00036 M) were used throughout. Under these conditions ethanol (1.2 M) appears to increase, or at least produce no change in the elevation of K^{42} efflux. In eleven experiments the average increase obtained in the absence of ethanol was 312 per cent. In six experiments the average increase obtained in the presence of 1.2 M ethanol was 342 per cent.

Thus, the effect of ethanol on the change in K^{42} efflux induced by a high potassium medium differs greatly from its effect on the change in K^{42} efflux induced by acetylcholine. In the former case there is either no effect or a slight increase in the induced potassium efflux, while in the latter case there is a substantial inhibition of the increased potassium movement. The accompanying smooth muscle contractions produced by acetylcholine or a high potassium medium are blocked by ethanol.

Contrast between Reduction of Calcium Ion Concentration and Addition of Ethanol   To compare the effect of preincubating the tissue in a reduced calcium ion concentration with that of preincubating it in ethanol, a series of experiments was performed in which ethanol was added for a 5 minute period prior to,
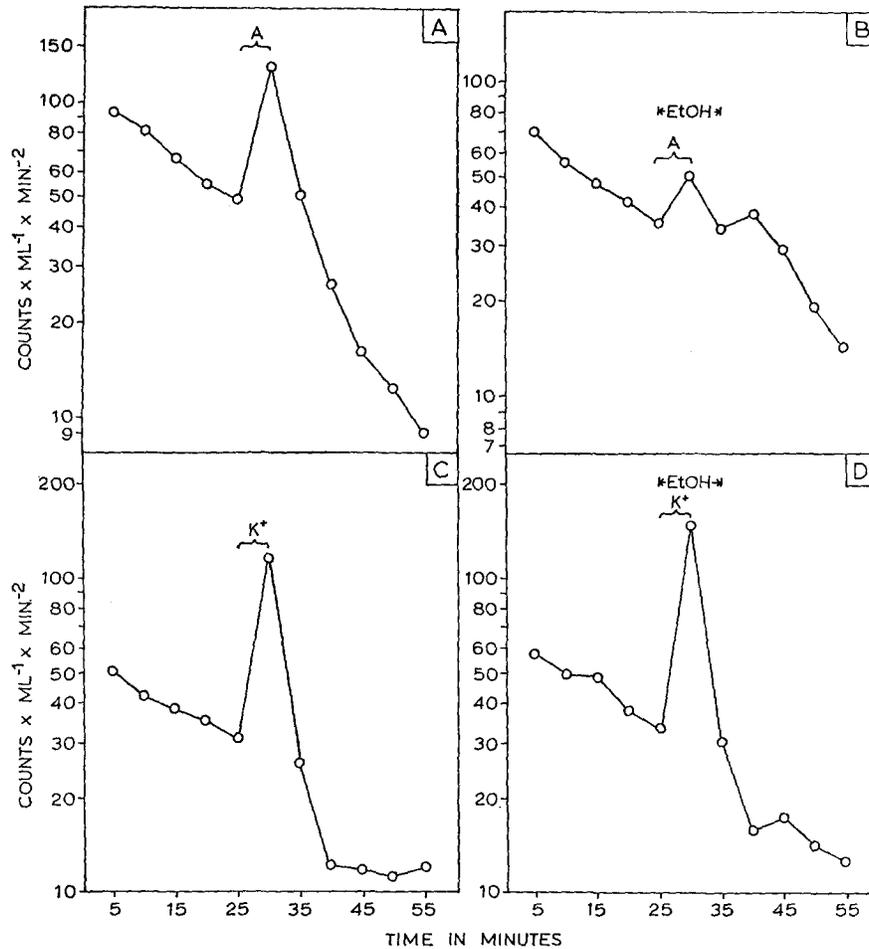
FIGURE 6. Potassium efflux from isolated longitudinal smooth muscle. The bathing solutions used in the experiments shown in this figure contained one-fifth normal $CaCl_2$ concentration (0.00036 M). A final concentration of 1.1 × 10^{-5} M acetylcholine (A) or a high potassium medium (K^+) was introduced into the muscle bath for the 5 minute periods indicated by the appropriate braces. A final concentration of 1.2 M ethanol (ETOH) was added for the periods of time indicated by the arrows.

during, and for a 5 minute period after the exposure of the smooth muscle preparation to a high potassium solution. As indicated above, the omission of calcium ion for a 5 minute period prior to the introduction of the high potassium medium was sufficient to cause a substantial inhibition of the increase in K^{42} efflux. On the other hand, preincubating the tissue in 1.2 M ethanol for the same period of time did not reduce the magnitude of the enhanced K^{42} efflux induced by a high potassium medium. In five experiments the increase in K^{42} efflux averaged 510 per cent.

Although the contractile response and the increase in K^{42} efflux obtained with either a high potassium medium or acetylcholine appear to be calcium-dependent, the results in this report indicate that calcium depletion by omission of calcium ion from the bathing medium and the postulated calcium blockade by ethanol do not have identical effects. The increase in K^{42} efflux induced by acetylcholine is more effectively blocked by ethanol than by calcium removal, while the corresponding increase in K^{42} efflux induced by a high potassium medium is inhibited by prior removal of calcium ion but not by the addition of ethanol.

DISCUSSION

Previously, calcium ion was postulated to have at least three different actions on the isolated longitudinal smooth muscle from guinea pig ileum (1). The first is an activation of a reaction which is essential for contraction and which occurs subsequent to membrane depolarization. The second is a depression of transmembrane ion movement which serves to stabilize the membrane. The third is an activation of a reaction which is involved in the acetylcholine-induced increase in potassium efflux from the smooth muscle fibers.

The last of these actions was inferred from the observations that ethanol inhibited the increase in potassium efflux elicited by acetylcholine and that the inhibition was reversed by increasing the calcium ion concentration in the bathing medium. Data obtained in this study have shown that the simple omission of $CaCl_2$ from the bathing solution does not reveal any necessity for having external calcium ions present to activate this induced outward movement of potassium. However, the inclusion of EDTA did appear to reduce the increased potassium efflux brought about by acetylcholine and thus supports the previous conclusion reached from experiments in which ethanol was employed. In both cases the data suggest that at least trace quantities of calcium ion must be present in the bathing medium to obtain an increase in potassium efflux with acetylcholine.

Additional evidence has been obtained for the contention that ethanol is a calcium antagonist. It has already been demonstrated that certain responses of the ileal smooth muscle are inhibited by ethanol and are reactivated by increasing the calcium ion concentration in the bathing medium (1). Records have been presented here showing that ethanol also inhibits the smooth muscle contraction produced by acetylcholine and that this inhibition too is reversed by high levels of calcium ion.

Judging from our previous experience with ethanol, the depression of inherent smooth muscle tone produced by the drug may be attributed to an antagonism toward calcium ion involved in the activation of the contractile mechanism rather than to an inhibition of membrane depolarization. This suggestion is based on the observation that ethanol induces an increase in

potassium efflux coincidentally with a depression of inherent smooth muscle tone (1). Furthermore, ethanol has been reported to cause membrane depolarization and to increase membrane conductance in other excitable tissues (4, 5). The depression of inherent tone which high levels of calcium ion produce seems more reasonably to be due to the stabilizing action of the divalent ion on the fiber membrane. Potassium efflux, as well as inherent tone of the longitudinal muscle, is inhibited by high calcium ion concentrations (6). The maintenance or enhancement of smooth muscle tone is not observed under these conditions, presumably because excessive stabilization of the fiber membrane by calcium ion prevents membrane changes which initiate the activating action of calcium on the contractile mechanism.

These considerations bring up the possibility that the combination of some particular inhibitory concentration of ethanol and of calcium ion may establish an experimental condition in which the ethanol counteracts the stabilizing effect of calcium ion on the membrane more effectively than it does the latent activating action of this divalent ion on muscle tone. The simultaneous presence of these two inhibitory agents may then promote an increase in smooth muscle tone. The records in Fig. 2 show that this is, in fact, the case.

Of particular interest is the finding that increases in the rate of outward movement of potassium ion from ileal smooth muscle elicited by different stimuli are not affected in the same way by ethanol. The response induced by acethylcholine is inhibited by ethanol, whereas the response induced by a high potassium medium is not. On the other hand, ethanol depresses smooth muscle contractions regardless of whether they are initiated by acetylcholine or a high potassium medium. Available evidence suggests that ethanol produces its inhibitory effects on smooth muscle function by indirectly antagonizing physiological actions of calcium ion (1). On the basis of these observations one is apt to assume that the increase in potassium efflux invoked by a high potassium medium does not require the presence of external calcium ion to be activated. In support of this conclusion is the finding that the introduction of a high potassium ion concentration accompanied by the simultaneous reduction in the calcium ion concentration enhances the resultant increase in potassium efflux. Other evidence, however, argues against this assumption. It can be shown that a significant inhibition of the induced increase in potassium efflux will occur if the smooth muscle is preincubated in a reduced calcium ion concentration for as little as 5 minutes prior to the addition of the high potassium (calcium-deficient) medium. Similar experimental procedures in which the addition of 1.2 M ethanol was substituted for the reduction in calcium ion concentration had no inhibitory effect on potassium movement. It is difficult, therefore, to avoid postulating that calcium ions associated with changes in potassium movement are not bound to a single homogeneous group of loci in the tissue. Tentatively, it would seem that the calcium ions bound to tissue

sites which restrict the outward movement of potassium ion from the muscle equilibrate rapidly with the external medium; the calcium ions bound to sites which activate or enhance a potassium-induced increase in potassium efflux equilibrate somewhat more slowly with the external medium and are insensitive to the inhibitory effect of ethanol, while the calcium ions bound to sites that are involved in an acetylcholine-induced increase in potassium efflux are very accessible to attack by ethanol.

Measurements of the rate of uptake and release of Ca^{45} in smooth muscle have suggested that this ion resides in several compartments of the muscle fiber (7-9). Our study of the physiological effects of ethanol and calcium ion on smooth muscle suggests a similar multiple compartment structure. It is quite possible, however, that any one of the several calcium compartments determined from the studies of calcium fluxes may ultimately be shown to be more than one compartment by the application of other techniques. For example, it would be reasonable to speculate that all the calcium involved in transmembrane potassium movement is located at or near the cell surface. Yet only part of this calcium is accessible to the inhibitory effects of ethanol.

In addition, there is an apparent separation between the calcium sites associated with a potassium-induced increase in smooth muscle tone and those associated with a potassium-induced increase in K^{42} efflux. Both the increase in tone and the increase in ion movement are inhibited by depleting calcium ions in the external medium. However, only the increase in smooth muscle tone is inhibited by ethanol; and this inhibition is rapidly reversed by adding an excess of calcium ions to the bathing medium.

It is, of course, possible that ethanol does not antagonize physiological actions of calcium ion in ileal smooth muscle. Calcium may reverse the inhibitory actions of ethanol in some non-specific manner. However, the experimental data indicate that in all but one of the smooth muscle responses studied the addition of ethanol and the depletion of calcium ion in the bathing medium produced qualitatively similar effects. Furthermore, depressions of smooth muscle functions induced by ethanol were invariably overcome by adding high concentrations of calcium ion to the bathing medium. Attempts have been made to reverse the ethanol inhibition of a potassium-induced increase in smooth muscle tone with high concentrations of $MgCl_2$. These attempts were unsuccessful (10). If ethanol does not block certain physiological actions of calcium ion in the isolated ileal smooth muscle, it would be of interest to learn why the stimulatory and inhibitory effects of ethanol and of a reduced calcium ion concentration are similar so frequently (but not in all cases) and why calcium ion is so effective in reversing the inhibitory actions of ethanol on smooth muscle function.

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