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THE INFLUENCE OF BROMIDE IONS ON EXCITATION–CONTRACTION COUPLING IN FROG'S SKELETAL MUSCLE

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The substitution of bromide for chloride ions in the solution bathing a skeletal muscle results in a marked increase in the twitch tension produced by a single supramaximal stimulus but is without effect on the maximum tetanic tension of the same muscle (Kahn & Sandow, 1955). This property is shared by other anions of the lyotropic series below chloride in their ability to salt out proteins from solution, notably nitrate, iodide, and thiocyanate (Kahn & Sandow, 1950, 1955; Lubin, 1957). The speed of onset of this effect of anion substitution in intact muscles (Kahn & Sandow, 1950; Hill & Macpherson, 1954) and in single, isolated muscle fibres (Horowicz & Hodgkin, 1956) has led to the conclusion that it is due to an action of the anions at the surface of the muscle fibres. It has been shown that the increase in the twitch tension is due to an increased duration of the active state of the contractile mechanism within the muscle fibres (Hill & Macpherson, 1954; Ritchie, 1954). Intracellular micro-electrode studies have shown that replacing chloride with these anions results in an increase in the amplitude and duration of the negative after-potential (Etzensperger & Bretonneau, 1956; Lubin, 1957). However, this increased depolarization does not seem sufficient to account for the increase in the mechanical response, and it has been suggested that the increased mechanical response results from some other action of these anions on the cell membrane (Lubin, 1957; Shanes, 1958).

Evidence has recently been presented supporting the hypothesis of Heilbrunn & Wiercinski (1947) and Sandow (1952) that an increased influx of calcium ions during depolarization may represent at least one step of excitation–contraction coupling (Frank, 1958, 1960). The present study was undertaken to investigate the possibility that the effects of anion substitution on contraction might be due to an effect of the anions on the calcium entry mechanism. This concept is supported by the results of the experiments presented below.

METHODS

The extensor longus digiti IV muscle of the frog *Rana pipiens* was used in all experiments, which were performed at room temperature and at all seasons of the year.

Isometric responses were recorded with the muscle mounted vertically in a bath containing the appropriate solution. The lower end of the muscle was fixed and the upper end was attached to the free end of a lever above the bath by means of a nylon thread. Strain gauges were mounted on the lever near its fixed end and responses were recorded with a Grass Model 5 polygraph. For further details of the preparation see Frank (1960).

For electrical recording the muscle was mounted horizontally in a two-compartment bath (Frank, 1958). The compartments were isolated by means of a petroleum jelly gap 8 mm in length. Potential changes were induced by changing the composition of the solution in one of the compartments.

The primary solution had the following composition (mM): choline chloride, 111·8; KCl, 2·47; $CaCl_2$, 1·08; $NaHCO_3$, 2·38; NaH_2PO_4, 0·087; glucose, 11·1. Except for the absence of the $CaCl_2$, the composition of the calcium-free solution was identical. The isotonic potassium chloride solution contained 123 mM-KCl with or without the addition of 1·08 mM-$CaCl_2$. The 25 mM potassium solution was made by the addition of solid KCl to the primary solution. All other solutions with elevated potassium concentrations were made by mixing appropriate amounts of the primary and isotonic potassium chloride solutions. The bromide solutions were identical in composition, with choline bromide substituted for choline chloride and potassium bromide substituted for potassium chloride. Caffeine (Kahlbaum) was dissolved in a small quantity of solution with the aid of a few drops of concentrated HCl and diluted to the desired concentration. The resulting caffeine solution was buffered to pH 7·2 by the addition of solid $NaHCO_3$. In some of the experiments D-tubocurarine 10^{-4} g/ml. was added to the solutions or a gas mixture of 0·5 % CO_2 in O_2 bubbled through the solutions. Neither of these procedures modified the results obtained. Details of the precautions taken in preparing the water for the solutions, the selection of reagents and in other ways preventing contamination with calcium from any source have been described elsewhere (Frank, 1960).

The details of the general procedure used in most of the experiments were described previously (Frank, 1960). Briefly, this consisted of determining the response of the muscle to an elevated potassium concentration before and after treating the muscle with a solution of modified composition. Following each test with elevated potassium the muscle was kept in a solution containing 2·47 mM potassium for at least 10 min before another test was performed. Before each test with an altered solution a control response of the muscle was obtained.

RESULTS

Effects of substituting bromide for chloride ions on potassium-induced contractures

In studies using a single muscle fibre preparation, Horowicz & Hodgkin (1956) noted that substitution of nitrate for chloride ions increased the tension induced by low concentrations of potassium but had little effect on tensions induced by high concentrations of potassium. A similar effect is produced by substituting bromide for chloride ions, as is shown in Fig. 1*A* and *B*. In this experiment the maximum tension of the contracture produced by 25 mM potassium is about 4 times larger with the toe muscle in bromide solutions than it is with the muscle in chloride solutions. This observation has been repeated in some twenty muscles in which bromide

substitution increased the maximum tension from 4 to 12 times. As in the case of the effect of bromide substitution on the muscle twitch, the onset is rapid. The maximum increase in the contracture tension is generally obtained during the 10–15 sec needed for the contracture to reach its maximum. However, after the muscle had remained in the bromide solution for 30 min or more, up to 5 min was required for the potentiation to disappear after replacement of the bromide with chloride solution.

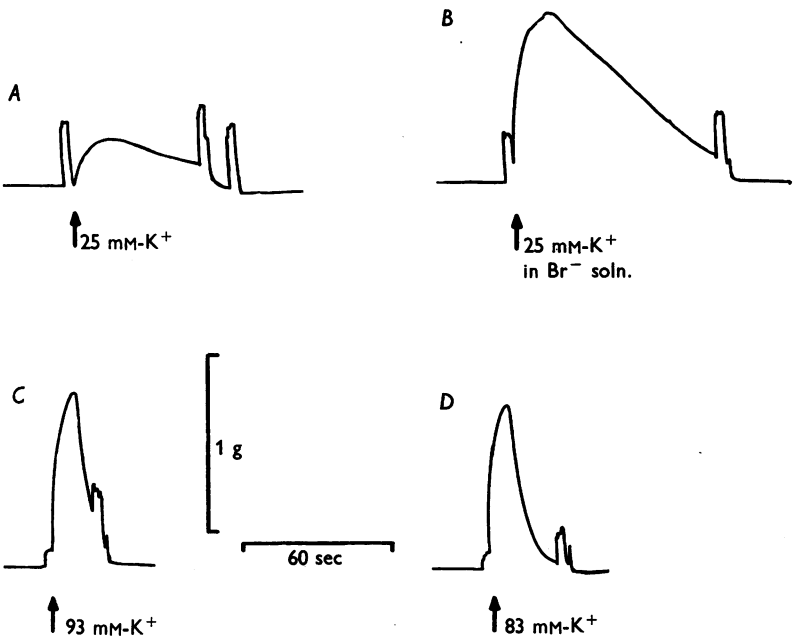

Fig. 1. Potassium-induced contractures in a single toe muscle of a frog. Responses *A*, *C*, and *D* obtained with the muscle in a chloride solution and *B* with the muscle in a bromide solution. The arrows indicate the points at which the test solutions were placed in the bath. The rectangular artifacts at the start and end of the contractures were caused by emptying the bath to change the solution.

Larger contracture tensions are obtained by increasing the potassium concentration above 25 mM. Thus it was possible to determine the potassium concentration in chloride solutions needed to cause a contracture equal to that due to 25 mM potassium in bromide solutions. This was done in the experiment shown in Fig. 1. In this case the maximum tension of the contracture produced by 25 mM potassium in bromide solution was between the maximum tensions produced by 83 and 93 mM potassium in chloride solution. Although contractures having similar maximum tensions can be obtained in this manner, it should be noted that the durations of the contractures produced by high potassium concentrations in chloride

(Fig. 1*C* and *D*) are considerably shorter than equal tension contractures induced by lower concentrations of potassium in bromide solutions (Fig. 1*B*).

The maximum tensions of contractures induced by various potassium concentrations in chloride and in bromide solutions are plotted in Fig. 2. The data obtained in two separate experiments are presented. The maximum tensions of 25 mM potassium-induced contractures with the muscles in bromide solutions were equal to the maximum tensions of contractures induced by 63–123 mM potassium with the same muscles in chloride

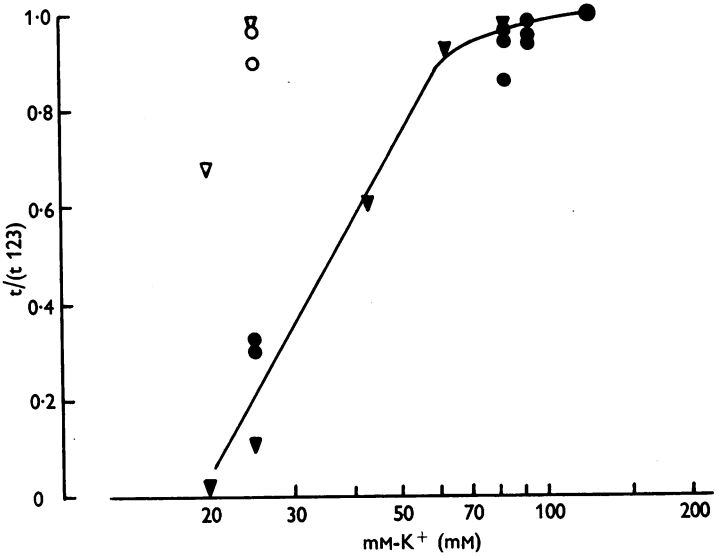

Fig. 2. Relation between the potassium concentration and the maximum tension of the contracture. Two experiments with different muscles, represented by different symbols. Open symbols, muscles in bromide solutions; solid symbols, muscles in chloride solutions. t/(t 123), maximum tension/maximum tension of 123 mM potassium-induced contracture. Semi-log. scale.

solutions. The relation between the potassium concentration and the maximum tension of the contractures produced in chloride solutions was similar in other muscles in which the effect of bromide was not determined. The only difference was that with some of these other muscles the curve was shifted to the right.

It was previously found that the maximum tensions of contractures produced by isotonic potassium chloride solutions in the small toe muscles used in these experiments were equal or nearly equal to the maximum tetanic tensions (Frank, 1960). It was found in the present study that bromide substitution had little or no effect on the maximum tension or the duration of contractures produced by 123 mM potassium solutions.

This seems to indicate that the events in excitation–contraction coupling are the same in the tetanus and in the maximum contracture.

When recordings were made at the time of substituting bromide solutions for chloride, a small contracture was observed. This contracture was never more than a quarter the size of contractures induced by 25 mM potassium.

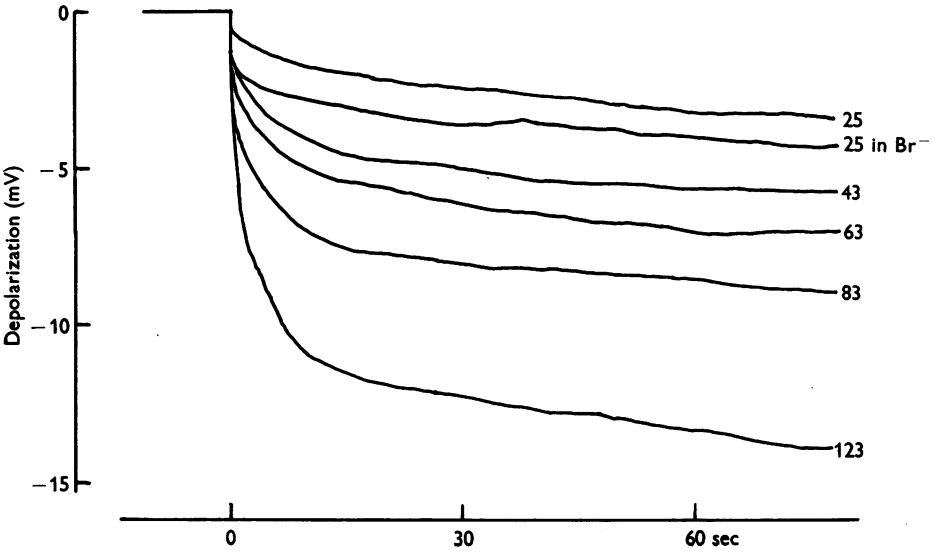

Fig. 3. Depolarizations produced by various potassium concentrations in a single toe muscle. Potassium concentrations given at the end of each record. Muscle in chloride solutions during all recordings except while recording the record marked 'in Br^-'. In this case the muscle was kept in a bromide solution for 5 min before and during the recording.

Effects of substituting bromide for chloride ions on potassium-induced depolarizations

Hutter & Padsha (1959) reported that substitution of nitrate for chloride ions increased the amplitude of the end-plate potential in a nerve–muscle preparation and of the electrotonic potentials in isolated frog muscle. These effects, plus the frequently reported increase in the size of the negative after-potential, made it desirable to study the effects of bromide ions on potassium-induced depolarizations.

When a bromide solution replaced the chloride solution bathing the part of a muscle in one of the compartments of the bath, that part of the muscle was depolarized about 25 % as much as by 25 mM potassium. This depolarization might well be responsible for the slight contracture which resulted from bromide substitution for chloride ions.

As can be seen in Fig. 3, the depolarization produced by 25 mM potassium

is slightly larger when the muscle is in a bromide solution. However, this slight increase seems inadequate to account for the large increase in the size of the contracture (Figs. 1 and 2). In order to produce contractures in chloride solutions having maximum tensions comparable to those produced by 25 mM potassium with the muscle in a bromide solution, it was necessary to use solutions having potassium concentrations of 63 mM or greater. The depolarization produced by 25 mM potassium with the muscle in a bromide solution was less than that produced by 43 mM potassium with the muscle in a chloride solution.

Effect of substituting bromide for chloride ions on the rate of contracture inhibition by calcium-free solutions

The above results are consistent with the hypothesis that the effects of anions on the submaximum contracture are not due to the electrical changes brought about by these non-physiological anions. The effect was next determined of bromide substitution on the rate at which the potassium-induced contracture was inhibited by placing the muscle in a calcium-free solution. It was found that bromide substitution appreciably slowed the rate of this inhibition.

The effect of bromide substitution was first tested with contractures induced by 25 mM potassium, and a decreased rate of inhibition was observed. However, two factors reduce the significance of this test. First, the contractures produced by 25 mM potassium are considerably larger when the muscle is kept in bromide solutions than when it is in a chloride solution. Secondly, an increase in the maximum tension of 25 mM potassium-induced contractures generally is observed if the response is tested shortly after exposing the muscle to a calcium-free solution (Frank, 1960). This increase is not observed in bromide solutions. Consequently, the inhibition curves obtained in the two types of solutions have distinctly different shapes. For these reasons the rest of the experiments were performed using 123 mM potassium solutions to induce contractures.

As has been shown above, the maximum tensions of contractures produced by isotonic potassium chloride and by isotonic potassium bromide were equal, or nearly so. However, the contractures were inhibited more slowly in calcium-free bromide solutions. The results of a typical experiment were plotted in Fig. 4. Experiments of this type were carried out with eleven different muscles. In each case a definite decrease in the rate of inhibition was found. Unfortunately, owing to instrument difficulties, quantitatively reliable results were obtained in only three of these experiments. The length of time in the calcium-free solutions required to produce a 50 % decrease in the maximum tensions of the contractures is shown in Table 1.

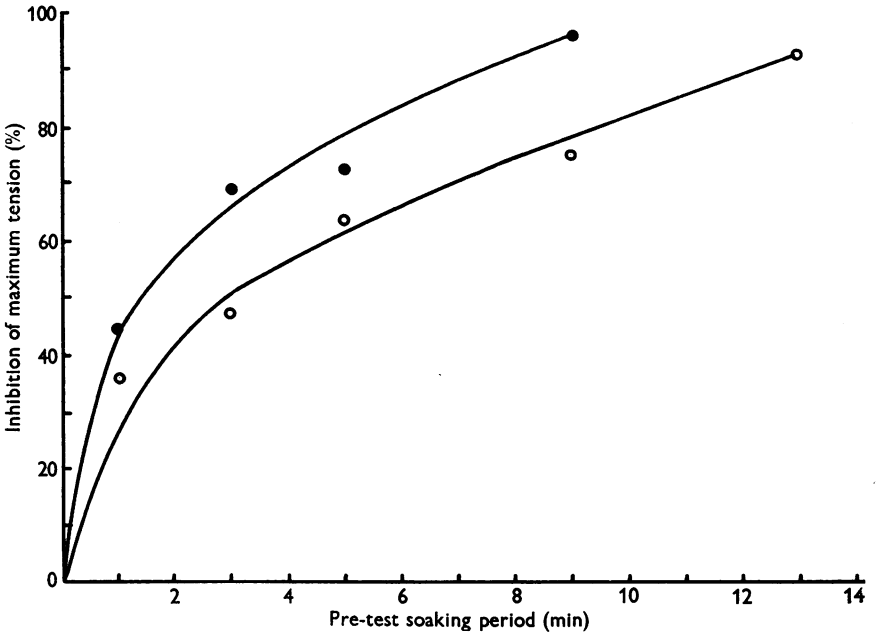

Fig. 4. Effect of substituting bromide for chloride solutions on the rate at which 123 mM potassium-induced contractures of a toe muscle were inhibited by placing the muscle in a calcium-free solution. Abscissa, time muscle was kept in a calcium-free solution before testing with potassium. ●, muscle in chloride solutions; ○, muscle in bromide solutions. Lines drawn by eye.

TABLE 1. Time in the calcium-free solution required to produce a 50 % decrease in the maximum tensions of 123 mM potassium-induced contractures

Muscle	In chloride (min)	In bromide (min)
1113	1·4	2·6
1209	2·0	7·0
1215	1·5	2·7

In three additional experiments only the time in a calcium-free solution required to eliminate completely the contracture was determined. In each of these cases the exposure time for complete elimination of the mechanical response was at least 50 % longer with the muscle in a bromide solution.

Effects of changes in extracellular calcium concentration on the maximum tensions of contractures produced by isotonic potassium chloride and isotonic potassium bromide

It was previously shown that the length of time for which a toe muscle has to be kept in a calcium-free solution in order completely to eliminate

the potassium-induced contracture is a measure of the time for equilibration between the calcium ion concentration of the extracellular spaces and that of the bathing solution (Frank, 1960). In these experiments equilibration was based on the frequently repeated observation that exposing a muscle to a solution with an altered calcium concentration for 2–3 times the equilibration period resulted in no further change in the amplitude or duration of the potassium-induced contracture than had occurred during the equilibration period itself. Thus by first determining the time a toe muscle had to be kept in a calcium-free solution to eliminate completely the potassium-induced contracture, and by using this interval to allow for equilibration, it was possible to determine the effects of changes in extracellular calcium concentration on the tensions of potassium-induced contractures. Since it took longer to eliminate the contractures with the muscles in bromide solutions, separate determinations of the elimination times in chloride and in bromide solutions were made on each muscle, and sufficient additional time was allowed for equilibration in bromide solutions after changing the calcium concentration of the bathing solution.

The relation found between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures is shown in Fig. 5. This type of relation was previously described for 25 mM potassium-induced contracture in chloride solutions (Frank, 1960). The curve obtained with 123 mM potassium-induced contractures does not show the initial potentiation as the calcium concentration is lowered as previously noted with 25 mM potassium-induced contractures.

In Fig. 5 the results are presented for three experiments in which the relation between extracellular calcium and contracture tension was determined with the muscles in chloride and in bromide solutions. It can be seen that substitution of bromide for chloride ions did not change the relation between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures. It was also observed that bromide substitution did not modify the duration of the contractures in this type of experiment.

Effect of substituting bromide for chloride ions on caffeine-induced contractures

It has been shown that essentially unmodified caffeine-induced contractures of skeletal muscle can be produced while the muscle is completely depolarized by potassium (Axelsson & Thesleff, 1958), or in the complete absence of extracellular calcium (Frank, 1960). It would seem that caffeine causes a mechanical response either by a process different from depolarization-induced contractures or by an action on the same excitation–contraction process but at a stage which follows depolarization and calcium

influx. Therefore it was of interest to see whether bromide substitution would modify caffeine-induced contractures and particularly whether it would increase the tensions of submaximal caffeine contractures.

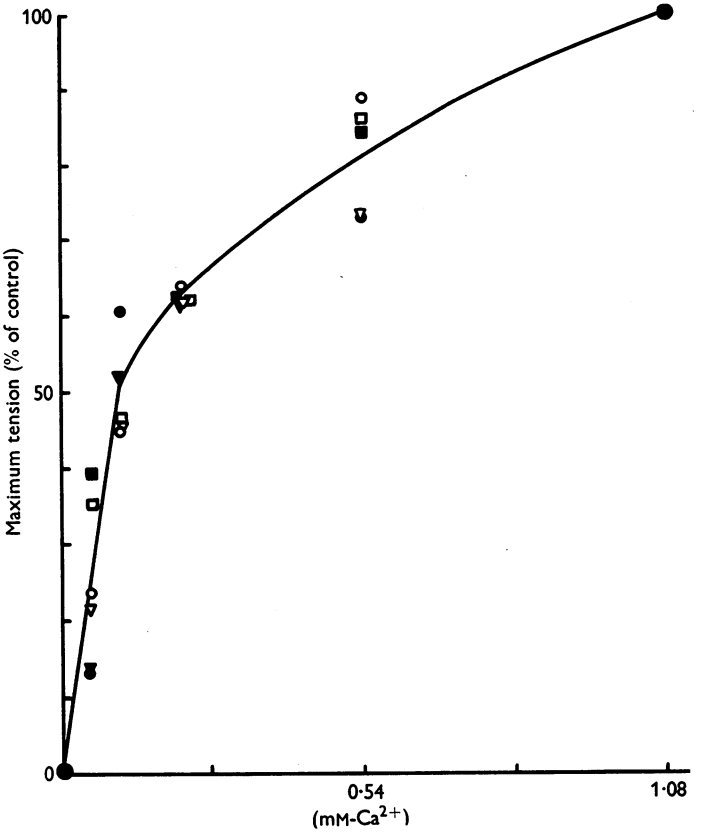

Fig. 5. Relation between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures of the toe muscle. Experiments with three different muscles represented by different symbols. The open symbols represent determinations made with the muscles in chloride solutions and the solid symbols determinations with the muscles in bromide solutions.

Caffeine contractures of one muscle produced in chloride and in bromide solutions are shown in Fig. 6. The maximum tension of the contracture produced by caffeine $5{\cdot}0 \times 10^{-4}$ g/ml. (Fig. 6*C*) was about the same as that produced by isotonic potassium chloride in the same muscle. In four experiments of this type the only change observed following bromide substitution was a slight but definite decrease in the maximum tensions of the caffeine-induced contractures.

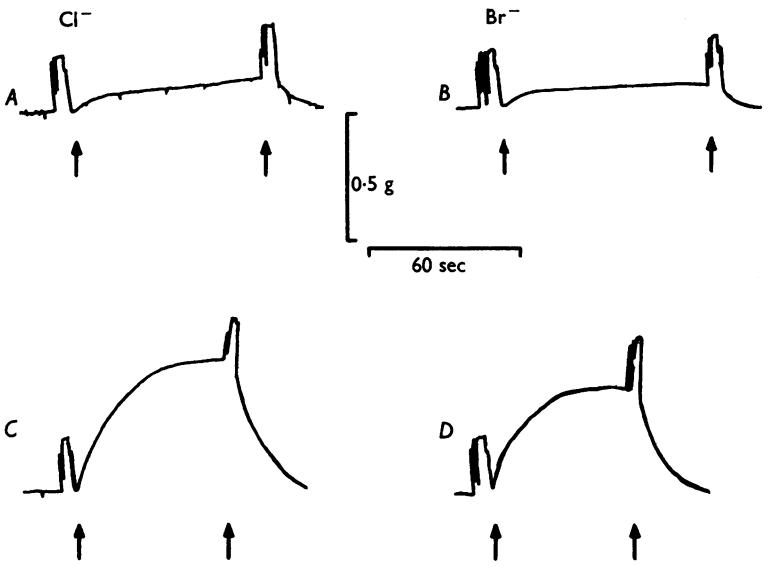

Fig. 6. Effect of substituting bromide for chloride solutions on the caffeine-induced contractures of a toe muscle. Test solutions contained caffeine $2{\cdot}5 \times 10^{-4}$ g/ml. in *A* and *B*, and $5{\cdot}0 \times 10^{-4}$ g/ml. in *C* and *D*. Arrows indicate the times when caffeine solutions were put into and removed from the bath. *A* and *C*, muscle in chloride solutions; *B* and *D*, muscle in bromide solutions.

DISCUSSION

The results of previous workers have shown that the increase in the size of maximal twitches of skeletal muscle caused by bromide ions and other anions below chloride in the lyotropic series is due to an increase in the duration of the active state of the contractile mechanism of the muscle cells, and further that the action of these ions is at the surface of the cells and not directly on the contractile elements within the cells (see p. 35). Therefore the effect of these anions on contraction results either from an increased depolarization during an action potential, or from an action on the processes which link the electrical and mechanical events, or from both.

At present excitation–contraction coupling can be presumed to occur in stages, starting with depolarization of the muscle fibre and ending with activation of the contractile mechanism. The substitution of bromide for chloride ions can increase the tension of twitches and of some types of submaximum contractures. However, the effects of bromide substitution on the depolarizations seem inadequate to account for the effects on the mechanical responses. Lubin (1957) recognized that the increase in the amplitude and duration of the negative after-potential of skeletal muscle fibres was not sufficient to account for the increase in the maximal twitch

which results from substitution of these anions for chloride. However, he suggested that the same change at the muscle fibre surface must be the cause of both effects.

In the previously reported studies from this laboratory on excitation–contraction coupling in skeletal muscle, the potassium-induced contracture has been used as a model for the events which occur during a contraction (Frank, 1958, 1960). This model also has been used in the present study. Therefore, it is of interest to compare the effects of substituting bromide for chloride ions on the two types of responses. It has been found that bromide substitution results in a marked increase in submaximum contractures induced by relatively low potassium concentrations but is without effect on maximum contractures induced by isotonic potassium chloride or potassium bromide solutions. This is analogous to the effects of bromide substitution on contractions in which the maximum twitch tension but not the maximum tetanic tension is increased. The rapid onset of the effect of bromide substitution on submaximum contractures indicates that, as in the muscle twitch, bromide ions act at the surface of the cells. Another similarity is the finding of a slight but obvious increase in the depolarization which accompanies each type of mechanical response in the presence of bromide ions. As is probably the case for the twitch, this slight increase is insufficient to account for the increase in the tension of the submaximum contracture.

Recently Hodgkin & Horowicz (1960) reported a potentiation of potassium-induced contractures in single skeletal muscle fibres by the substitution of nitrate or thiocyanite for chloride ions. As reported here they observed a slight increase in the potassium depolarization in the presence of nitrate ions. This change in depolarization was insufficient to account completely for the increase in contracture tension.

It is suggested here that the effects of substituting bromide for chloride ions on the mechanical responses of skeletal muscles are mainly due to an action of these anions on the calcium entry mechanism which links electrical and mechanical events in contraction. The evidence supporting the calcium entry mechanism of excitation–contraction coupling can be found elsewhere (see Frank, 1960, for references). The demonstration of a direct effect of bromide substitution for chloride ions on the rate at which the potassium-induced contracture is inhibited when the toe muscle is placed in a calcium-free solution supports the mechanism proposed here for the effects of bromide ions. Furthermore, the maximum possible tension of a potassium-induced contracture is determined by the extracellular calcium-ion concentration, and the inability of bromide ions to increase the tension of contractures made submaximum by reduction of the extracellular calcium concentration (Fig. 5) indicates that the effects of bromide

substitution on the potassium-induced contracture also are dependent upon some action of calcium ions.

It has been shown previously (Frank, 1960) that the rate at which the potassium-induced contracture of the toe muscle is inhibited when the muscle is placed in calcium-free solution is determined by the rate at which calcium ions are removed from the extracellular spaces of the muscle. Since the presence of bromide ions does not change the relation between the extracellular calcium concentration and the maximum tension of the 123 mM potassium-induced contracture, the decrease in the rate of inhibition must mean that the presence of bromide ions has in some way slowed down the loss of calcium ions from some site in the muscle. Since it is hard to visualize how bromide ions could interfere with the diffusion of calcium ions out of the extracellular spaces of the muscle, it appears probable that bromide ions act on the muscle fibre membrane to slow the loss of calcium associated with these membranes when the calcium concentration of the surrounding fluid is reduced.

Shanes (1958) has suggested that the effects of these anions which increase the muscle twitch could be explained by assuming that they increase the calcium concentration in the muscle fibre membrane. Since substitution of these anions for chloride does not increase the maximum tetanic tension it must be assumed that the maximum possible tension of the muscle is set by the state of the contractile mechanism of the cells and thus is not increased by the increased influx of calcium ions associated with each action potential during a tetanus. However, this explanation cannot account for the inability of bromide ions to increase the tension of contractures made submaximal by reduction of the extracellular calcium concentration (Fig. 5). All that can be said is that in some way bromide substitution favours the influx of calcium ions during a depolarization, but this effect probably is not due simply to an increase in membrane calcium concentration.

Bromide ions increase the size of the muscle twitch by increasing the duration of the active state of the contractile mechanism (Hill & Macpherson, 1954; Ritchie, 1954). Sandow (1955) presented some records indicating that potassium-induced contractures could be increased in duration by using nitrate in place of chloride ions. However, an interpretation of these records is complicated by the fact that relatively large strips of sartorius muscle (at least 1 mm across) were used and by the fact that the test solutions contained only 100 mM potassium. In the present study maximum contractures produced by 123 mM potassium were not increased either in amplitude or duration by bromide substitution, and even contractures induced by 25 mM potassium, which were greatly increased in amplitude, were not noticeably increased in duration. The present results

can best be interpreted as showing an increased effectiveness of relatively small depolarizations in producing a mechanical response in the presence of bromide ions. If bromide ions have the same mechanism of action in modifying muscle twitches and contractures, the increased duration of the active state during the twitch response in the presence of bromide ions would be due to an increased ability of the negative after-potential to maintain the active state. Further, if as suggested here the effects of bromide substitution are due to an action on the calcium entry mechanism, there should be an increased influx of calcium ions during a twitch produced with the muscle in a solution containing bromide ions compared to that produced in chloride-containing solutions. Indeed, Bianchi & Shanes (1959) have shown an increased influx of calcium ions per twitch when nitrate ions were substituted for chloride ions in the solution bathing the sartorius muscle of the frog.

SUMMARY

1. It was observed that substitution of bromide for chloride ions in solutions bathing the extensor longus digiti IV muscle of the frog increased from 4 to 12 times the maximum tensions of contractures induced by 25 mM potassium, but was without effect on maximum contractures induced by 123 mM potassium. The depolarization produced by 25 mM potassium also was slightly increased but this increase seemed insufficient to account for the marked potentiation of the mechanical response.

2. Substitution of bromide for chloride ions decreased the rate at which 25 and 123 mM potassium-induced contractures are inhibited when the toe muscle is placed in a calcium-free solution.

3. Bromide substitution did not increase the size of 123 mM induced contractures made submaximum by decreasing the extracellular calcium concentration, nor did it increase the tension of submaximum caffeine-induced contractures.

4. It is suggested that the increase in twitch tension and in the tension of submaximum potassium-induced contractures which occur when bromide is substituted for chloride is due to an increase in the excitation of the contractile mechanism of the muscle cells by small depolarizations. Further, it is suggested that this increase in the effect of small depolarizations is due to a relatively greater influx of calcium ions during depolarization in the presence of bromide ions.

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