Actions of Some Cations on the Electrical Properties and Mechanical Threshold of Frog Sartorius Muscle Fibers

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ABSTRACT With the use of a point voltage-clamp technique, the effects of Zn^{2+}, UO_2^{2+}, tetraethylammonium, and several other homologous quaternary ammonium ions on the electrical properties of the frog sartorius muscle and its mechanical threshold were studied. None of the agents separated the voltage thresholds for mechanical activation and delayed rectification. However, Zn^{2+}, UO_2^{2+}, and TEA, which are known to potentiate the twitch, caused some inhibition of the normal increase in potassium conductance during delayed rectification. Zn^{2+} and UO_2^{2+} also slowed the rate of development of the outward current. A strength-duration relation was studied for depolarization pulses capable of initiating contraction. With a depolarizing pulse of 2.5 msec the mechanical threshold is about -13 mv at about 20°C. UO_2^{2+}, 0.5 μM, which markedly reduced the outward current produced by such a short pulse, did not raise the mechanical threshold. All findings indicate that there is no direct causal relation between delayed rectification and mechanical activation.

INTRODUCTION

In the experiments to be discussed, the central question is whether there is a causal relation between the outward current flowing during delayed rectification of a muscle fiber and its mechanical activation. Some of the reasons for the study were the observations that under a voltage-clamped condition, the voltage threshold for contraction in a frog sartorius fiber was very close to that for delayed rectification, and that lyotropic anions, in lowering the former threshold, lowered the latter in strict parallel (Kao and Stanfield, 1968). Moreover, lyotropic anions (e.g., nitrate) which potentiated the twitch, also increased the outward current in delayed rectification. These observations appear consistent with a view (Freygang, 1965) that there might be some link between the outward current in delayed rectification and mechanical activation.

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There is, however, other evidence against a simple direct causal relation between the two. For instance, certain cations (tetraethylammonium, Zn^{2+}, UO_2^{2+}) can potentiate the twitch while prolonging the action potential. Since in the case of the action of tetraethylammonium ion on frog nerve, it is known that the prolonged action potential is due to the ability of the agent to inhibit delayed rectification (Hille, 1967; Koppenhöfer, 1967), it seems likely that the potentiated twitch in a muscle fiber with a prolonged action potential might be accompanied by a diminished outward current. Hagiwara and Watanabe (1955) first suggested such a possibility for TEA; Isaacson and Sandow (1963) made a similar suggestion for Zn^{2+}. In neither case, nor for UO_2^{2+}, is there direct evidence for diminished outward current. Since the voltage-clamp method used allowed us to observe currents associated with changes in membrane conductance, we have been able to obtain direct evidence along this line. We also made detailed studies of the actions of TEA, Zn^{2+}, and UO_2^{2+} on the electrical properties and the mechanical threshold of the frog sartorius fiber, and attempted to correlate our findings with the mechanically potentiating effects that these cations are known to have (see Sandow, 1965). In addition to TEA, we also studied the effects of a series of monoquaternary ammonium ions homologous with TEA: tetramethyl, tetrapropyl, tetrabutyl, and tetrapentyl. In the case of UO_2^{2+}, we further studied a "strength-duration" relation of depolarizing pulses on mechanical threshold. From all these experiments, we conclude that there cannot be any simple direct causal relation between the outward current in delayed rectification and mechanical activation.

METHODS

The material and methods used were the same as those previously described in detail (Kao and Stanfield, 1968).

Since an appreciation of the results given below depends on an understanding of the various methods used, a brief summary of these methods is given here. The chief method employed a current-passing microelectrode to control membrane voltage which was sensed by another microelectrode. A diagram of the setup can be found in Kao and Stanfield (1968, Fig. 1). It should be emphasized that since the applied current flows from a point source, it will be distributed along the muscle fiber in accordance with cable theory. In other words, the membrane voltage is not uniform for any length, but decays electrotonically from the current source (see also Heistracher and Hunt, 1969). The accuracy of the voltage control will depend, among other factors, on the proximity of the voltage-sensing electrode to the current electrode. In our experiments, the tips of the two electrodes were within 50 μ of each other, a distance somewhat less than the diameter of a fiber in most instances. Since the space constant of a fiber not undergoing regenerative increase of Na^+ conductance (because of the use of tetrodotoxin) is approximately 2 mm, the membrane potential at the voltage electrode is about 3 % lower than that at the current electrode. This estimate is based on

conventional cable theory; a more precise result could be obtained by considering the three dimensional potential field (see, for example, Adrian, Costantin, and Peachey, 1969).

In most of the experiments, the important aspect was the steady voltage attained. In a few experiments involving very short pulses, the transient of the voltage step was of some concern. Because of the limited amount of current that can be passed through a microelectrode (see also Adrian, Chandler, and Hodgkin, 1969), it was not possible to charge or discharge the various capacitances in the preparation instantaneously, particularly when large voltage steps were employed. Depending on the preparation, a step from -90 mv to -10 mv might take as long as 800 μsec. Most of the pulses used were several hundred milliseconds long, so that this slow transient was of no concern. The shortest pulses used were about 2–2.5 msec; so the final voltage step might last somewhat less than 2 msec. In these experiments (p. 635) the primary concern was a comparison of the strength-duration relation in normal and in UO_2^{2+}-treated fibers, rather than any absolute measurement of this relation; hence this source of error did not affect the conclusions.

The muscle preparation was observed at either a magnification of 60 or 120 times in polarized light. Under these magnifications, the sarcomeres were not distinguishable, and the mechanical threshold was taken as the voltage at which a visible movement or a change in birefringence of the impaled region was just detectable. These contractions no doubt involved most or all the myofibrils radially in a fiber, and were more substantial than contractions of only the superficial myofibrils described by Adrian, Costantin, and Peachey (1969). The mechanical threshold could usually be determined to within 1 mv, provided precaution was taken to avoid too frequent repetition (pp. 297 and 301 of Kao and Stanfield, 1968). Because of the spatial electrotonic decay of the voltage step from the impaled area, the observed contractions were limited in their longitudinal spread. At threshold, the visibly contracting area rarely exceeded a distance equivalent to one fiber diameter. With greater depolarizations, longer segments of the fiber contracted, but since there were no propagated action potentials in the TTX-treated preparations, there were no propagated contractions. Certain comments in this paper refer to changes in twitch tension produced by some agents; these comments are based on the results of other investigators in the literature and do not imply that we have any information on externally recordable tension in a point-clamped muscle fiber.

The threshold for delayed rectification was determined from plots of the voltage-current relation, and was taken as the voltage at which the slope of the curve increased by 10% or more (Kao and Stanfield, 1968). This increase in conductance is due primarily to an increase in potassium conductance, although on our evidence, we cannot exclude minor contributions from other ionic conductances (see p. 299, Kao and Stanfield, 1968). Since this threshold is determined from a plot, variations of 2 or 3 mv either way may be included. In the present experiments, slightly greater variation may be present in those situations in which a test agent had markedly reduced delayed rectification.

A phosphate-buffered Ringer solution was used in the experiments with quaternary ammonium compounds. In experiments with zinc and uranyl salts, a Ringer solution

containing no phosphate, but buffered with 5 mM tris (hydroxymethyl) aminomethane (Tris buffer), titrated to pH 7.2 with HCl, was used. This solution contained 115 mM Na^+ instead of 120 mM Na^+, and 126 mM Cl^- instead of 121 mM Cl^-, but the same concentration of other ions as in the phosphate-buffered Ringer. Zinc and uranyl were added to the solutions as acetates.

Tetrodotoxin (0.1 $\mu g/ml$), which does not affect the mechanical or delayed rectification thresholds (Kao and Stanfield, 1968), was used, when required, to abolish spikes.

All values shown in the tables are means and standard errors of means. Statistical comparisons for significance of difference were made on the basis of set comparisons by Student's t test.

RESULTS

Experiments with Quaternary Ammonium Compounds

Table I gives some properties of frog sartorius fibers in the presence of several quaternary ammonium compounds.

A. RESTING FIBERS

1. *Resting Potential* With the exception of the tetraethyl compounds, all the quaternary ammonium salts caused some significant depolarization. Tetramethylammonium (TMA) caused a slight depolarization ($P = 0.012$) when used at 58 mM concentration. The compounds with longer alkyl chains generally were found, in preliminary trials, to produce greater depolarizations as the chain length increased. Resting potentials as low as -30 mv could be produced by some compounds at concentrations of 5–10 mM. In order to avoid excessive depolarization, the concentrations of tetrapropyl (TPrA), tetrabutyl (TBA), and tetrapentyl (TPeA) compounds were reduced to the minimum level that would produce a clear reduction in the outward current. However, even in concentrations of 0.1–1.0 mM these compounds caused statistically significant depolarization (Table I, $P < 0.001$ in each case).

TEA and TBA were also used in solutions containing iodide and sulfate, respectively, in place of chloride. As iodide itself did not affect the resting potential, TEA iodide did not produce any depolarization. Although the mean resting potential in 1 mM TEA in sulfate was slightly lower than that in 1 mM TBA in chloride Ringer (Table I), this difference was not significant ($P > 0.3$).

2. *Effective Resistance* As in the previous experiments with some anions (Kao and Stanfield, 1968), all preparations in the present experiments were clamped at a holding potential of -90 mv. The effective resistance was taken from the slope dV/dI at a clamped potential of -100 mv. The effective resistance in 58 mM TEA chloride (Table I) is significantly higher ($P = 0.008$) than that in the standard phosphate-buffered Ringer solution. This finding is

TABLE I
EFFECTS OF QUATERNARY AMMONIUM IONS ON MECHANICAL THRESHOLD AND ELECTRICAL PROPERTIES OF FROG SARTORIUS MUSCLE FIBERS

	Concentration used	Resting potential	Effective resistance	Thresholds for		
				Spike	Contraction	Delayed rectification
	mM	*mv*	*MΩ*	*mv*	*mv*	*mv*
Isotonic solutions						
Normal phosphate-* buffered Ringer	115 NaCl	−90.8±1.0 (13)	0.26±0.03 (13)	−58.9±1.1 (10)	−48.8±0.8 (13)	−52.0±0.8 (13)
TMA chloride	58	−85.2±1.4 (6)‡	0.24±0.03 (6)‡	—	−51.0±1.2 (6)‡	−51.5±1.1 (6)‡
TEA chloride	58	−88.1±1.4 (7)	0.40±0.04 (7)	−59.0±1.2 (10)	−54.3±1.2 (7)	−53.1±0.9 (7)
TPrA chloride	1.0	−75.4±4.8 (5)	0.28±0.06 (5)	—	−49.2±1.1 (5)	−51.6±2.3 (9)
TBA chloride	1.0	−73.0±3.5 (9)	0.18±0.01 (9)	—	−51.7±1.1 (9)	−52.9±1.2 (9)
TPeA chloride	0.1	−77.9±1.7 (7)	0.29±0.02 (7)	—	−36.9±3.1 (7)	−40.7±3.0 (7)
NaI Ringer	58 NaI	−87.0±1.2 (10)	0.37±0.05 (10)	—	−64.6±0.4 (10)	−64.2±0.8 (10)
TEA iodide	58	−89.9±2.4 (8)	0.58±0.07 (8)	—	−67.4±1.1 (8)	−67.9±1.1 (8)
Sulfate Ringer*	40 mM Na₂SO₄	−90.6±1.0 (9)	0.44±0.05 (9)	—	−43.6±0.6 (9)	−44.7±0.4 (9)
TEA in sulfate	1.0	−68.8±2.3 (6)	0.37±0.03 (6)	—	−38.8±1.2 (6)	−39.7±0.6 (6)
Hypertonic solutions						
TEA chloride	58	−76.6±2.9 (9)	0.37±0.02 (9)	—	—	−53.4±1.2 (9)
TEA iodide	58	−87.4±2.8 (8)	0.56±0.06 (9)	—	—	−67.3±1.2 (8)

* Values in these rows are taken from Table 1 of Kao and Stanfield (1968) for comparison with other values.

‡ Means ± SE of mean followed by number of fibers used in parentheses. All fibers were pretreated with tetrodotoxin (0.1 µg/ml), and held at −90 mv. All experiments were performed at room temperature (about 20°C).

in agreement with that of Hagiwara and Watanabe (1955) and of Washio and Mashima (1963). In individual fibers, TEA chloride increased the membrane resistance to both inward and outward currents (e.g., fiber of Fig. 1); and potassium inward rectification appeared to be reduced.

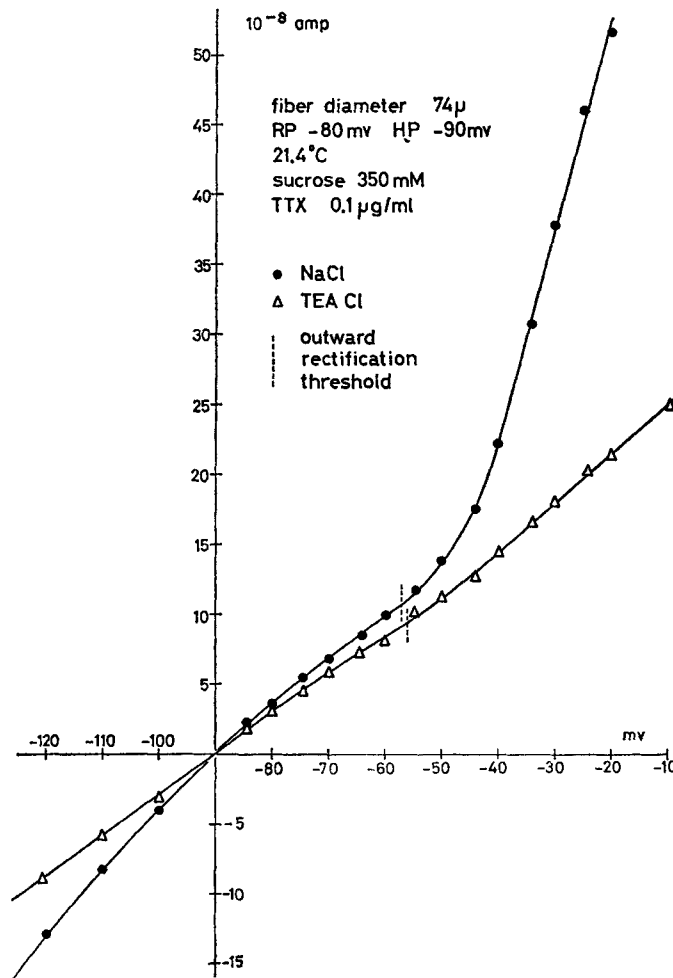The effective resistance in TEA iodide is also significantly higher than that

FIGURE 1. Voltage–total current relations in normal and TEA-treated fiber. Fiber diameter, 74 μ; resting potential -80 mv; holding potential -90 mv. 21.4°C. Tetrodotoxin (TTX) 0.1 μg/ml. This fiber was immersed consecutively in hypertonic normal (filled circles) and hypertonic TEA chloride (58 mM) Ringer solutions (open triangles). Recording was carried out between two microelectrodes to avoid the effect of junction potential on changing solutions. Abscissa, membrane potential in millivolts; ordinate, total current in 10^{-8} amp. Vertical dashed lines on the curves indicate thresholds for delayed rectification. This fiber has an unusually low threshold in normal Ringer solution (see Table 2 of Kao and Stanfield, 1968).

in NaI Ringer solution ($P = 0.03$). It is interesting to note that the effective resistance in TEA iodide corresponds with an increase in membrane resistance five times that in standard sodium chloride Ringer solution [$(0.58/0.26)^2$]. Since NaI increased the membrane resistance only twofold (see Table 1, Kao and Stanfield, 1968), most probably by lowering chloride conductance, the extra effect of TEA iodide must be attributed to some reduction of potassium conductance by TEA. This point will be supported by further evidence in later sections.

None of the other quarternary ammonium compounds significantly changed the effective resistance. The compounds higher in the series may be present in too low a concentration to increase the membrane resistance. The case of TBA should be mentioned particularly because Fatt and Katz (1953), working with crustacean muscle fibers, reported an increase in membrane resistance. Aside from a difference in the animal material, we also used a concentration which was less than one one-hundredth of the concentration they used, in order to avoid excessive depolarization of the frog muscle fibers.

B. ACTIVE FIBERS

1. *Spike Threshold*   TEA chloride (58 mM) did not significantly alter the threshold for inward sodium current even though the TEA Ringer contained only half the normal amount of sodium (Table I).

2. *Outward Currents*   TMA (58 mM) had little effect on the magnitude or the rate of development of the outward current. For fibers of similar diameter, the total outward current in TMA-treated fibers is comparable with that in untreated fibers. A distinct peak in the current trace, characteristic of delayed rectification, occurs in TMA-treated fibers, and is reached as rapidly as in untreated fibers. This is similar to the finding in the frog node of Ranvier (Hille, 1967).

All the other quaternary ammonium compounds reduced the outward current. Their general effects can be illustrated by the current records obtained in the presence of TEA salts (Fig. 2). The outstanding features are that: (a) the current magnitude is lower than that in untreated fibers at similar levels of depolarization, and (b) the hump in the current record due to delayed rectification is absent. The reduced current amplitude indicates a reduced conductance increase, which has also been verified by applying modulating pulses (hyperpolarizing by 10 mv) during a sustained depolarization. (A similar experiment in the presence of zinc is illustrated in Fig. 5.)

In a total current record obtained with a point voltage clamp, the absence of a distinct hump can only be taken as evidence that delayed rectification has been markedly reduced, but not necessarily completely suppressed. From a plot of the voltage–steady-state current (at about 100 msec) relation (Fig. 1),

outward rectification can still be seen. However, the question of whether this rectification has a delayed onset cannot be directly answered by the current records illustrated in Fig. 2. In a few rare instances in which the TEA action was not so marked because of a short period of treatment, a clear delay was present. This is the reason for assuming that the rectification found in plots represented by Fig. 1 is of a delayed nature. Another indication is that at a concentration of 1 mM, TPrA reduced the late total current, slowed the time
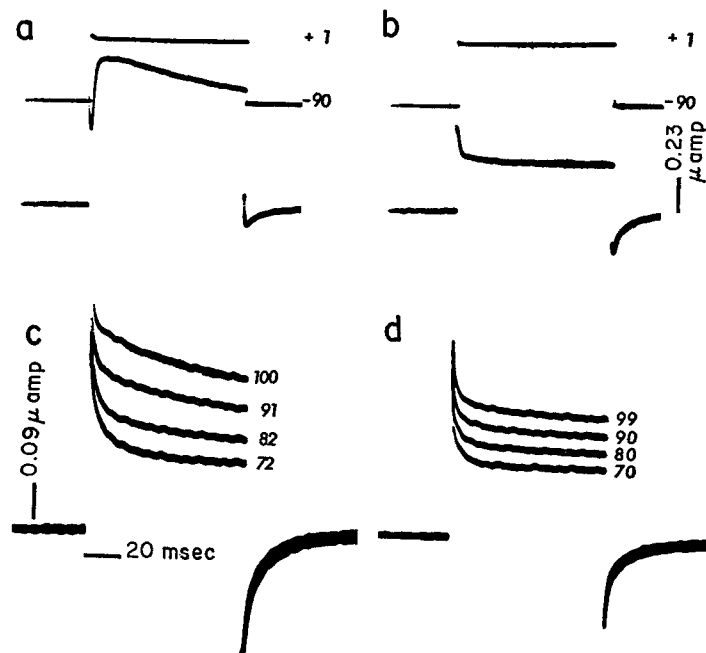

FIGURE 2.   Records of voltage and total current in normal, TEA chloride, and TEA iodide solutions. a and b are from same fiber as that shown in Fig. 1. Top trace, voltage, bottom trace, total current. a was taken with the fiber in normal Ringer, b with fiber in 58 mM TEA chloride. Numbers attached to voltage trace indicate level of clamped membrane potentials. c and d, total current records obtained by multiple exposure during step depolarizations. c, fiber in 58 mM TEA chloride Ringer. d, fiber in 58 mM TEA iodide Ringer. Number at right of each trace gives amplitude of depolarizing step in millivolts from holding potential of −90 mv. TTX, 0·1 μg/ml.

to peak of delayed rectification, but did not suppress the delayed rectification as markedly as is shown for TEA in Fig. 2. The last difference is probably caused by a much lower concentration of TPrA, but from the remaining hump in the current trace it may be surmised that some quaternary ammonium compounds can alter the kinetics of the change in potassium conductance as well as reduce the maximum conductance (see also Koppenhöfer, 1967). Lastly, in recent experiments using a three electrode voltage clamp (Adrian,

Chandler, and Hodgkin, 1966) where membrane current, rather than total electrode current is measured, it is possible to be certain that the outward rectification does have a delayed onset in the presence of 58 mM TEA (P. R. Stanfield, unpublished observations).

The difference in the current amplitudes in TEA chloride and TEA iodide (Fig. 2) can probably be attributed to the contribution of chloride conductance to the total conductance. When the delayed rectifying channel is open, the contribution of chloride conductance to the total conductance is not readily appreciated in a trace of total current. In TEA, however, the reduction of potassium conductance makes the relative contribution of chloride conductance greater, and the reduction of the latter by iodide may well be responsible for the smaller currents, even though the threshold for delayed rectification was lowered by TEA iodide. That this difference can be seen supports the conclusion that in frog sartorius, the potassium conductance of delayed rectification was reduced by TEA, as in other preparations (e.g., frog node of Ranvier, Hille, 1967; Koppenhöfer, 1967; squid giant axon, Armstrong and Binstock, 1965; Onchidium sub- and supraesophageal ganglion cells, Hagiwara and Saito, 1959; Spheroides supramedullary cells, Nakajima, 1966).

It is of some interest to note that while TPrA and TBA can act on the delayed rectifying channel of frog skeletal muscle at a concentration of only 1 mM, they are almost totally inactive on the frog node of Ranvier at the same concentration (Hille, 1967).

3. *Mechanical and Delayed Rectification Thresholds*   The actions of various quaternary ammonium compounds on these thresholds are shown in Table I. TMA did not significantly alter the mechanical or delayed rectification thresholds from those in Ringer soluton ($P = 0.09$ and 0.3, respectively). With the other compounds, the first general point which is evident is that while they all reduced the maximum potassium conductance, there is no clear trend in their effects on the mechanical and delayed rectification thresholds. Thus, TEA chloride significantly lowered the mechanical threshold ($P = 0.001$), as did TBA in chloride solution ($P = 0.04$), TPrA had no effect on it ($P > 0.3$), whereas TPeA markedly raised it ($P < 0.001$).

The effects of these compounds on the delayed rectification thresholds were generally in agreement with their effects on mechanical thresholds, except for the cases of TEA and TBA in chloride, which did not significantly lower the delayed rectification threshold. These apparent discrepancies can be explained by the greater difficulty in measuring the threshold; in fibers in which the conductance change was reduced, it was particularly difficult to be precise about this threshold (see also Kao and Stanfield, 1968). In no instance in any quaternary ammonium compound, however, was the observed mechanical

threshold different from the corresponding delayed rectification threshold by a statistically significant amount.

(a) *TEA iodide vs. TEA chloride*   As expected, TEA iodide produced a much greater lowering of the mechanical and delayed rectification thresholds than did TEA chloride; and the changes produced by TEA iodide were slightly greater than those found in NaI ($P = 0.02$ for mechanical threshold and 0.01 for delayed rectification threshold. For data on NaI, see Kao and Stanfield, 1968). These differences can be attributed to the combined actions of TEA and iodide.

(b) *TBA in sulfate vs. TBA in chloride*   Whereas 1.0 mM TBA chloride in standard chloride Ringer solution slightly lowered the mechanical threshold, and sulfate, replacing chloride, slightly raised it (Kao and Stanfield, 1968), 1.0 mM TBA chloride in sulfate Ringer solution had the unexpected effect of greatly raising the mechanical threshold (Table I). The delayed rectification threshold was similarly raised. These changes are in contrast to the additive effect of TEA and iodide. Because the sulfate salts used were apparently contaminated, muscle fibers immersed for long periods in a solution made with them tended to become electrically inexcitable; we now feel that the results shown in Table I for TBA chloride in sulfate Ringer may be due to injurious effects, and that the threshold values are not totally reliable. Although there has been no opportunity to repeat the TBA experiments, we include them because of the remarkable consistency with which the mechanical and delayed rectification thresholds are closely associated with each other.

4. *Hypertonic Solutions*   Because of the evidence already presented in the case of lytotropic anions (Kao and Stanfield, 1968) which showed that the increase of outward current at the mechanical threshold is not a movement artifact, we limited similar demonstrations here to the two TEA salts only. When freshly dissected muscles were immersed directly into a TEA chloride solution made hypertonic with 350 mM sucrose, there was always a marked depolarization of the fibers. The probable explanation of this phenomenon is as follows. In hypertonic Ringer solution the internal volume of a fiber becomes smaller, and KCl tends to become more concentrated. Such an increase in internal KCl makes E_{Cl} less negative and E_K more negative. If in the presence of TEA the resting potential becomes more dependent on E_{Cl}, because of a reduction in potassium conductance, a depolarization can be expected. TEA would further accentuate this by slowing the escape of the KCl because of the lowered P_K. As might be expected with this hypothesis, the depolarization could be lessened if the muscles were first immersed in hypertonic NaCl Ringer for 30 min before being transferred to a hypertonic TEA chloride solution, but some depolarization still occurred (Table I). On the other hand,

in hypertonic TEA iodide solution, in which both potassium and chloride conductances were reduced, there was no depolarization.

The effective resistances in hypertonic TEA chloride and in hypertonic TEA iodide (Table I) were similar to those in the corresponding isotonic solutions ($P > 0.3$ in each case). The delayed rectification thresholds in the hypertonic solutions were also similar to those in the isotonic solution ($P > 0.3$ in each case), extending a previous conclusion that the increase in outward current at the mechanical threshold is real.

Experiments with Zinc and Uranyl Ions

A. TRIS-BUFFERED RINGER   As described in the Methods section, Zn^{2+} and UO_2^{2+} were applied in a Tris-buffered Ringer to avoid the formation of insoluble phosphate compounds. For a basis of comparison, it was necessary first to determine the mechanical threshold and some electrical properties of muscle fibers in the standard Tris-buffered Ringer. The results are shown in Table II. Although the resting potential, effective resistance, and thresholds for spike and delayed rectification are not significantly altered from those in phosphate-buffered Ringer (see Table I), the mechanical threshold is slightly but significantly lower ($P = 0.04$).

B. zn^{2+} AND UO_2^{2+}

1. *Resting Potential and Effective Resistance*   The procedure used in these experiments was to equilibrate the isolated muscles in the various test solutions for 30 min before impalement, a period adequate for the development of the full effect of Zn^{2+} (Isaacson and Sandow, 1963; Sandow and Isaacson, 1966).

From Table II, it may be seen that neither Zn^{2+} nor UO_2^{2+} had any significant effect on the resting potential. But Zn^{2+} (0.05 mM) did significantly increase the effective resistance ($P = 0.04$), whereas UO_2^{2+} (0.5 μM) did not. These observations are consistent with those of Mashima and Washio (1964) and of Hutter and Warner (1967).

2. *Spike Threshold* Zn^{2+} (0.05 mM) was found to raise the spike threshold significantly ($P = 0.030$) by about 3 mv. UO_2^{2+} (0.5 μ), did not have this effect.

3. *Outward Currents*   Both Zn^{2+} and UO_2^{2+} reduced the magnitude of the outward current at all levels of depolarization. This effect is best illustrated by comparing currents in the same fiber in superimposed current tracings (Fig. 3) and in a plot of voltage–total current relations at about 100 msec plotted from such records (Fig. 4). It may be seen that although the threshold for delayed rectification is not altered (Table II), the conductance change associated with delayed rectification is reduced by Zn^{2+}.

TABLE II

EFFECTS OF ZINC AND URANYL IONS ON
MECHANICAL THRESHOLD AND ELECTRICAL
PROPERTIES OF FROG SARTORIUS FIBERS

	Concentration used	Resting potential	Effective resistance	Thresholds for		
				Spike	Contraction	Delayed rectification
	μM	mv	$M\Omega$	mv	mv	mv
Normal Tris-buffered Ringer	—	-86.4 ± 1.0 (9)*	0.22 ± 0.02 (9)*	-59.0 ± 0.9 (8)	-52.4 ± 1.0 (8)*	-54.8 ± 0.9 (8)*
Zinc (isotonic)	50	-85.2 ± 1.3 (9)	0.29 ± 0.03 (8)	-56.3 ± 0.7 (9)	-51.2 ± 1.0 (9)	-53.4 ± 0.8 (9)
Uranyl (isotonic)	0.5	-87.0 ± 1.2 (10)	0.30 ± 0.04 (10)	-60.6 ± 1.4 (10)	-52.8 ± 1.3 (10)	-55.4 ± 0.9 (10)
Zinc (hypertonic)	50	-85.5 ± 1.8 (12)	0.36 ± 0.04 (12)	—	—	-53.6 ± 1.6 (12)
Uranyl (hypertonic)	0.5	-87.0 ± 2.1 (6)	0.39 ± 0.02 (6)	—	—	$-56.2\pm.12$ (6)

* Means $\pm$ SE of mean, followed by number of fibers used in parentheses. All fibers were pretreated with tetrodotoxin (0.1 $\mu g/ml$), and held at -90 mv. All experiments were performed at room temperature (about 20°C).

Another way of showing the effect of Zn^{2+} or UO_2^{2+} on the membrane conductance is shown in Fig. 5, in which modulating pulses (hyperpolarizing 10 mv) were superimposed on a long depolarizing pulse. As might be expected, the current associated with the modulating pulses follows a temporal relation parallel with that of the outward current due to the depolarizing pulse. Thus, when the outward current is largest, so is the modulating current, and when the outward current declines with time, so does the modulating current. Some appreciation of the change in membrane conductance may be
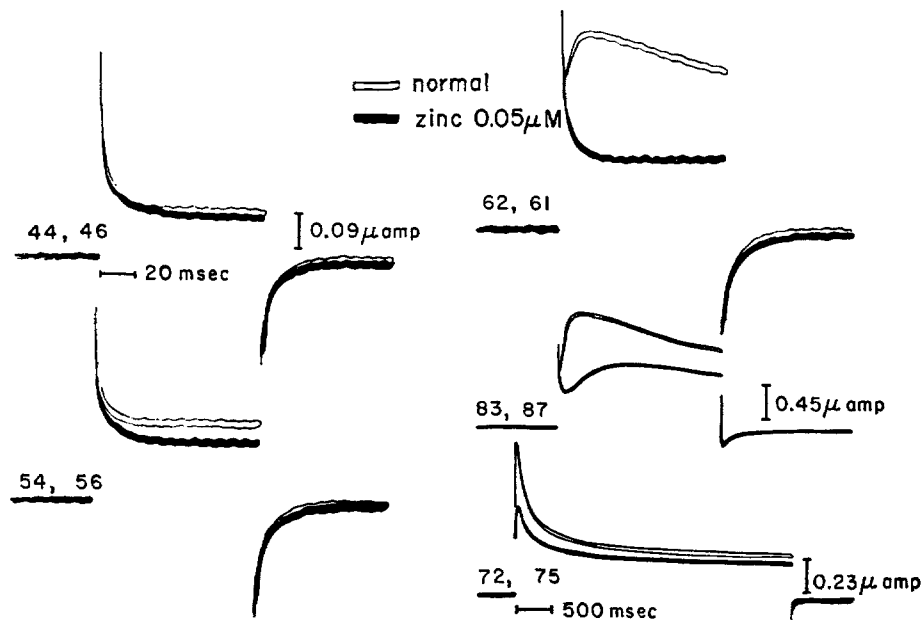
FIGURE 3.   Effect of Zn^{2+} on outward current. Superimposed tracings of total current records from a fiber immersed consecutively in normal hypertonic Tris-buffered Ringer and in hypertonic 0.05 mM zinc Ringer. Number at left of each pair of tracings indicates amplitude of depolarizing pulse in millivolts from holding potential of −90 mv, in the order normal, zinc. Fiber diameter, 53 μ; resting potential, −88 mv; temperature, 20.7°C. TTX, 0.1 μg/ml.

gained by comparing the amplitude of the modulating current in the resting fiber with that during the depolarization. In untreated fibers, the ratio between the modulating current at the height of the outward current and that at rest is 5.5, whereas in fibers treated with 0.05 mM Zn^{2+}, this ratio is 2.5. As the currents shown are total electrode currents, and as the membrane conductance is not linear when the delayed rectifying channel is open, it is not possible to estimate more precisely the relative membrane currents in either case. However, in a limited way, these observations can be taken to mean that Zn^{2+} reduced the membrane conductance during delayed rectification, as it did in the resting fiber (see p. 630).

In addition to reducing the current amplitude, Zn^{2+} and UO_2^{2+} also characteristically slowed the rate of development of the outward current. This slowing is evident at all levels of depolarization that are sufficient to elicit delayed rectification (Fig. 3). Although the lack of a uniform membrane potential in a point clamp precludes any precise interpretation (see also Kao and Stanfield, 1968), some appreciation of this slowing may be seen from the following measurements. At a membrane potential of -10 mv, the mean
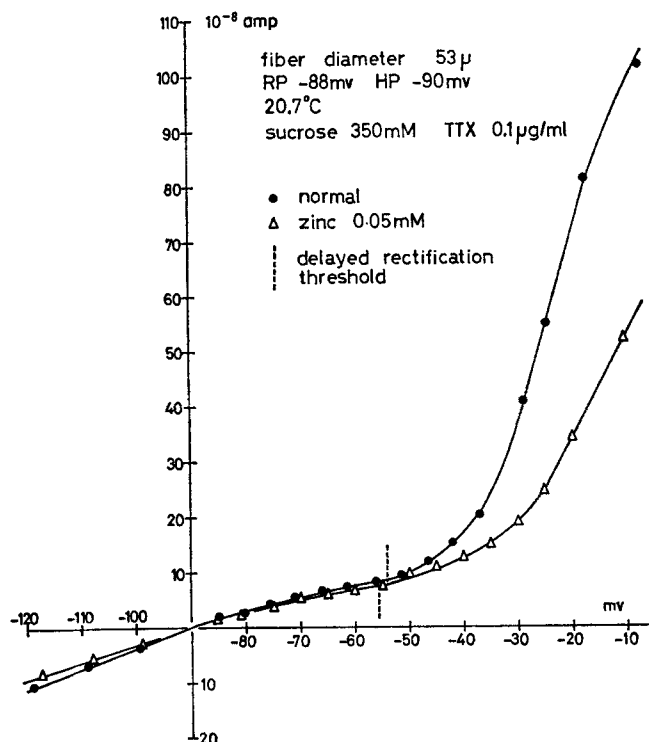
FIGURE 4. Voltage–total current relations in normal and in Zn^{2+}-treated fiber. Same fiber as in Fig. 3. Abscissa, membrane potential in millivolts; ordinate, total current in 10^{-8} amp. Vertical dashed lines, thresholds for delayed rectification.

time to peak of the outward current in standard Tris-buffered Ringer is 4.7 ± 0.1 msec (mean $\pm$ SEM of nine fibers), that in Zn^{2+} Ringer is 28.1 ± 2.2 msec (seven fibers), and that in UO_2^{2+} Ringer is 39.2 ± 7.0 msec (nine fibers). The differences between fibers in standard Tris-buffered Ringer and fibers treated with Zn^{2+} and UO_2^{2+} are significant ($P < 0.001$ in each case).

The conductance increase associated with delayed rectification of the frog sartorius muscle fiber characteristically falls off with time during a sustained depolarization (Nakajima, Iwasaki, and Obata, 1962; Adrian et al., 1966). At a membrane potential of -10 mv, the potassium inactivation has a half-time

of 177 $\pm$ 14 msec (mean $\pm$ SEM of six fibers) in standard Tris-buffered Ringer. In Zn^{2+} Ringer, the half-time is 322 $\pm$ 29 msec (eight fibers); in UO_2^{2+} Ringer, it is 245 $\pm$ 57 msec (five fibers). The difference between fibers in control and in Zn^{2+} solutions is significant (P = 0.002), whereas that between fibers in control and in UO_2^{2+} solutions is not (P = 0.26).

4. *Mechanical and Delayed Rectification Thresholds*   Neither Zn^{2+} (0.05 mM) nor UO_2^{2+} (0.5 μM) altered either the mechanical threshold (P > 0.3 in each case) or the delayed rectification threshold (P = 0.3 for Zn^{2+} and > 0.3 for
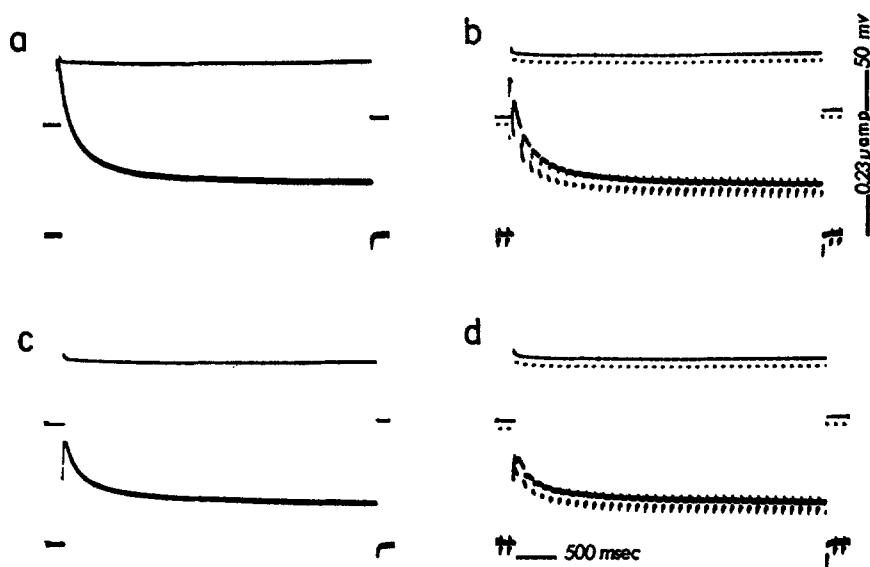

FIGURE 5.   Records of voltage and current in normal and in Zn^{2+}-treated fiber. a and b in normal Ringer; c and d in 0.05 mM Zn^{2+} Ringer. Top trace, voltage; bottom trace, current. a and c, traces without modulating pulses; b and d, modulating pulses of 10 mv amplitude, 10 msec duration were applied at a frequency of 10 sec^{-1}. In each instance b followed a and d followed c in 1 min. This interval was too short for full recovery from K inactivation, as can be seen from the slightly lower maximum outward current.

UO_2^{2+}). The findings on the mechanical threshold are in agreement with earlier observations by others using potassium depolarization for studying mechanical threshold (Sandow, Taylor, and Preiser, 1965; Edman and Grieve, 1966). The absence of any significant effect of Zn^{2+} (0.05 mM) on the mechanical and delayed rectification thresholds may be contrasted with its action (p. 630) in raising the spike threshold.

5. *Hypertonic Solutions*   As shown in Table II, the resting potentials and effective resistances of fibers immersed in hypertonic solutions containing Zn^{2+} or UO_2^{2+} are not significantly different from those in the corresponding isotonic solutions (P values ranging from 0.09 to >0.3). The threshold for

delayed rectification is also essentially the same as in isotonic solutions ($P > 0.3$ in each case), confirming a previous conclusion that the increase of outward current at the mechanical threshold is not a movement artifact (Kao and Stanfield, 1968).

6. *Strength-Duration Relation for Mechanical Activation and the Effect of UO_2^{2+} upon It*   Sandow et al. (1965) proposed a concept of the mechanically effective period for the action potential, assuming that the mechanical threshold is the same whether contraction is elicited by means of a high potassium solution or
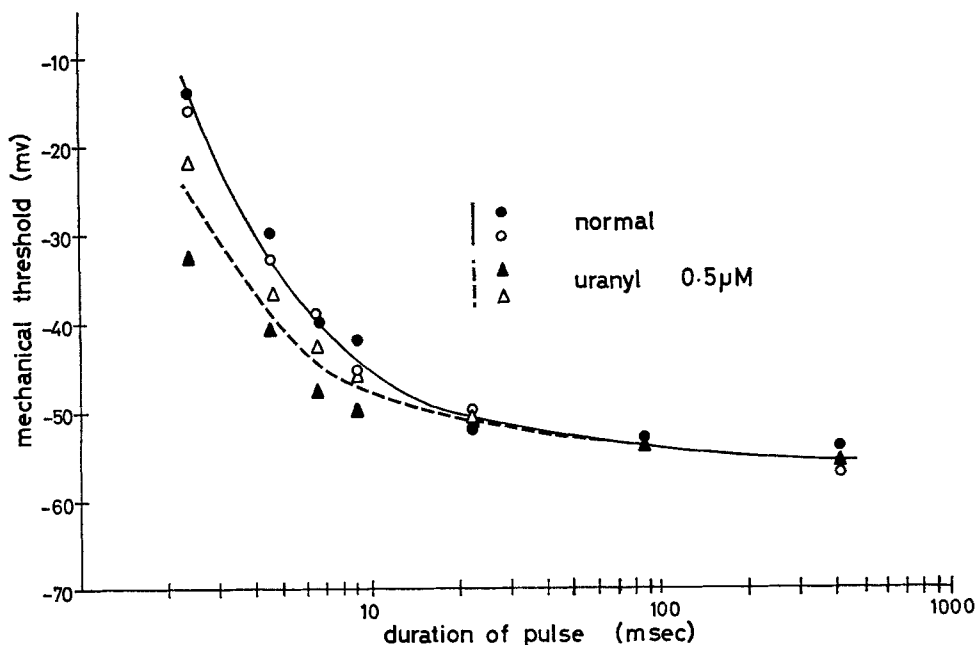

FIGURE 6. Strength-duration relations in normal and in UO_2^{2+}-treated fibers. Two separate fibers. Room temperature, 20–22°C. TTX, 0.1 μg/ml. Note that pulse duration is plotted in log scale.

a depolarizing pulse of relatively brief duration. With the point voltage-clamp technique, we have been able to investigate the relation between the threshold and pulse duration. Our experiments show that a strength-duration relation exists for the depolarizing pulse just necessary to elicit visible contraction (Fig. 6). Thus, with pulses of about 50 msec duration, the mechanical threshold is about −49 mv. With longer pulses (up to 580 msec) the mechanical threshold is only slightly lower. With pulses shorter than 50 msec, however, the mechanical threshold rises rapidly. When the pulse duration is 2.5 msec, the mechanical threshold is −13 mv. Because of the difficulties in seeing minute and fleeting local contractions, we did not determine the mechanical

threshold of pulses shorter than 2.5 msec. These findings are generally similar to those of Adrian, Chandler, and Hodgkin (1969), and differ from them only because of the different temperatures.

In the presence of 0.5 μM UO_2^{2+}, the mechanical threshold for pulses longer than 40 msec is the same as that in untreated fibers (Fig. 6). But for shorter pulses, the mechanical threshold appears to be slightly lowered by UO_2^{2+}. Because our observations were limited, we cannot be certain whether the slight lowering is a genuine effect of UO_2^{2+}. However, as the results show a consistent relationship, even though pulses of different durations were applied in a random sequence, we can state that UO_2^{2+} did not increase the mechanical threshold at short pulses. The significance of this point will be discussed (p. 638).

DISCUSSION

Cations and Electrical Properties

TEA AND INWARD RECTIFICATION   Our results suggest that TEA might block the inwardly rectifying ("anomalous") channels as well as the outwardly rectifying ("delayed") potassium channels. Three lines of evidence are available on this: (a) The large increase in membrane resistance on hyperpolarization found in TEA iodide solution suggested that TEA iodide affected more than the chloride conductance, and therefore, presumably also reduced the potassium conductance. (b) The depolarization found in hypertonic TEA chloride solutions could readily be explained by a greater dependence of the resting potential on E_{Cl} in the presence of TEA. (c) In the same fiber, the current-voltage relation in TEA Ringer is more nearly linear at membrane potentials negative to the threshold for delayed rectification than is the relation in normal Ringer. The conclusion that TEA affects the inward rectification has been confirmed and extended by Stanfield (1969).

Zn^{2+} AND SPIKE THRESHOLD   Our results show that Zn^{2+} (0.05 mM) raised the spike threshold significantly without affecting either the mechanical threshold or the delayed rectification threshold. When they first found a lowering of the mechanical threshold by lyotropic anions, Hodgkin and Horowicz (1960) suggested that the anions could have adsorbed onto the surface membrane, thereby altering the surface potential and the potential gradient through the membrane. Kao and Stanfield (1968) adopted this explanation and used it to explain the lowering of the threshold for delayed rectification as well as the mechanical threshold by the anions. However, they found that the anions did not affect the spike threshold, and suggested that the anions might have been excluded from around the sodium channels because of a local concentration of negative charges around these channels. Such a distribution of negative charges should have an opposite effect on cations

which ought to be preferentially adsorbed around the sodium channels. The present observations with Zn^{2+} are consistent with this view, as are the observations with Ca^{2+} (Costantin, 1968). This view apparently applies only to small cations, for large cations, such as TEA and UO_2^{2+}, do not have the same effect on spike threshold, possibly because of some steric hinderance.

PROLONGATION OF THE ACTION POTENTIAL BY TEA, ZN^{2+}, AND UO_2^{2+} All these ions are known to prolong the action potential by slowing the repolarization phase (Hagiwara and Watanabe, 1955; Washio and Mashima, 1963; Sandow, 1965; Sandow et al., 1965). Our results show that the slowed repolarization is to be attributed to a reduction in the intensity of the outward current flowing through the membrane (Figs. 1–5), and, in the case of Zn^{2+} and UO_2^{2+}, also to a slowing of the rate of development of this current (Fig. 3).

OUTWARD CURRENT   There are several points of parallelism between mechanical activation and the flow of outward current in delayed rectification. The onset of the two processes appears to occur hand in hand: (*a*) the voltage thresholds at which both processes begin lie very close together (see Kao and Stanfield, 1968, and the present results), and (*b*) under a variety of experimental conditions the two thresholds are lowered or raised in strict parallel (Kao and Stanfield, 1968; Costantin, 1968, and the present results). There is a parallelism between the ability of lyotropic anions to increase the magnitude of the outward current during delayed rectification (Kao and Stanfield, 1968) and their ability to potentiate the twitch (see Sandow, 1965).

Such parallelism, however, cannot be accepted as evidence for a causal relation between the outward current in delayed rectification and mechanical activation, either with respect to the onset of the two processes, or with respect to any influence of the current magnitude on tension. The latter possibility is readily countered by the present observations that TEA, Zn^{2+}, and UO_2^{2+}, which are known to potentiate the twitch, actually reduce the magnitude of the outward current in delayed rectification.

Evidence against the onset of the outward current as a cause of mechanical activation can be found in two recent papers. Heistracher and Hunt (1969) depolarized a snake muscle in 95 mM K_2SO_4, but clamped the fiber at -100 mv. Contractions occurred when the membrane potential was made less negative as in normal fibers, but the delayed current was inward rather than outward as in normal fibers. More recently, using a three electrode voltage-clamp method in which membrane current rather than total electrode current was observed, Adrian, Chandler, and Hodgkin (1969) found that with a rather short pulse visible contractions could be produced with a threshold depolarization which produced little outward current. They also showed that the strength-duration relations for a conductance increase in delayed rectification and for mechanical activation have different time characteristics.

STRENGTH-DURATION RELATION AND EFFECT OF UO_2^{2+} Our evidence against a causal relation between the onset of delayed rectification and mechanical activation can be found in the effects of UO_2^{2+} on the strength-duration relation for mechanical activation. If mechanical activation were dependent on a current which switches on with a delay, then the strength-duration relation should show at least in part a reflection of this delay. Since UO_2^{2+} slowed the delayed current, and also greatly reduced the magnitude of the outward current, it would be expected that the mechanical threshold for short pulses would be raised markedly. Therefore, our observation that UO_2^{2+} did not raise the mechanical threshold with short pulses is clearly incompatible with any suggestions that the outward current is a cause of the mechanical activation.

In conclusion, the close association of the mechanical threshold with the threshold for delayed rectification may be due to their unrelated, though markedly similar, dependence on the voltage gradient through the membrane.

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