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PERCHLORATE-INDUCED ALTERATIONS IN ELECTRICAL AND MECHANICAL PARAMETERS OF FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. The effect of the perchlorate anion (ClO_4^-) on the potential dependence of mechanical and electrical parameters was investigated in skeletal muscle fibres of the frog.

2. Two main methods were employed: twitches and K contractures were induced in isolated fibres from the semitendinosus or iliofibularis muscle, and point voltage clamp was applied in sartorius and short toe muscle fibres.

3. Twitch height was unaffected below 10^{-4} M-ClO_4^-, it usually increased several-fold in the concentration range of 10^{-3} to 10^{-2} M-ClO_4^- and continued to rise slowly between 10^{-2} and 10^{-1} M-ClO_4^-.

4. ClO_4^- caused a parallel shift of the activation curve, which relates peak force to membrane potential, towards more negative potentials by up to 40 mV (70 mM-ClO_4^-). The shift in force activation was not accompanied by a corresponding shift in the potential dependence of force inactivation.

5. In the presence of ClO_4^-, maximum force development upon depolarization to −60 or −50 mV could be maintained for several minutes, suggesting that spontaneous relaxation after full depolarization is due to a potential-dependent inactivation process, and not to an exhaustion of Ca^{2+} release.

6. ClO_4^- shifted the threshold for the initiation of the action potential only slightly towards more negative potentials (~10 mV at 70 mM-ClO_4^-). Little or no shift was observed in the lower concentration range (< 10 mM) where the threshold of force activation was shifted by about 20 mV.

7. ClO_4^- slightly depressed the activation of the delayed rectifier without causing any distinct change in its threshold potential.

8. Electrophoretic injection of ClO_4^- (internal ClO_4^- concentration ($[ClO_4^-]_i$) ~ 1 mM) induced similar effects to those following external application of this anion, i.e. a shift of force activation towards more negative potentials.

9. Of several other anions tested, only dichromate, which resembles ClO_4^- in its tetrahedal structure, similarly caused force activation after repolarization.

10. We conclude that at low concentrations (< 10 mM) ClO_4^- rather specifically improves excitation–contraction coupling by direct interference with the gating mechanism which activates Ca release from the sarcoplasmic reticulum. At higher concentrations, it may also influence potential-dependent membrane processes by adsorption to the outer surface of the membrane.

INTRODUCTION

In 1973, Foulks, Miller & Perry showed that perchlorate (ClO_4^-) in the external solution caused depolarized and mechanically refractory frog skeletal muscles to develop force upon repolarization to membrane potentials more negative than −40 mV. As an explanation they suggested that 'the effects of perchlorate include disproportionate shifts in the relation between log $[K]_o$ and K-contracture tension, and between log $[K]_o$ and relaxation rate'. ($[K]_o$ represents the external K concentration.) We confirmed and extended the authors' results (Foulks *et al.* 1973; Foulks & Perry, 1979) and arrived at similar conclusions. In our investigation of the ClO_4^- effect we measured the potential dependence of mechanical and electrical parameters. The results show that low concentrations of ClO_4^- shift mainly the threshold of force activation to more negative potentials, probably by direct interference with membrane-bound sensors which control Ca release from the sarcoplasmic reticulum (s.r.). Investigations of the action of ClO_4^- on intramembrane charge movements, which are supposed to occur in the wall of the transverse tubular system and to trigger Ca^{2+} release from the s.r., supported this assumption (Fuxreiter, Gottschalk, Kovács & Lüttgau, 1983; Lüttgau, Kovács, Gottschalk & Fuxreiter, 1983).

A preliminary account of some of our experiments was published recently (Gomolla, Gottschalk & Lüttgau, 1982).

METHODS

Preparation

Muscle fibres of the frog (*Rana temporaria*) were used throughout. For the first series of experiments we isolated single fibres or small bundles from semitendinosus and iliofibularis muscles and measured isometric force (RCA transducer 5734) induced by electrical stimulation or by a quick increase in the external K^+ concentration (for details see Lüttgau, 1965). In the second series we used small bundles of fibres (five to twenty fibres) dissected from the M. lumbricalis digiti IV of the hind limb. One tendon was connected to a transducer (Endevco, type 8107/2); the other end was fixed with needles to the bottom of the experimental chamber. The mechanical activity of *one* fibre was investigated by applying depolarizing voltage-clamp steps from various holding potentials. Voltage-clamp control was achieved in a conventional way (Costantin, 1968; Fink & Wettwer, 1978) with two internal micro-electrodes, one (filled with 2 M-K citrate) for injecting current and the other (filled with 3 M-KCl) for potential control.

The fibres in this preparation were about 1·5 mm long and 70 μm thick. According to Caputo & Fernandez de Bolaños (1979) they possess a length constant of about 2 mm, which allows a rather uniform depolarization even under point-voltage-clamp conditions. In our experiments we always replaced external Cl^- by an impermeant anion and blocked the delayed rectifier with tetraethylammonium (see 'Solutions'). This was done to improve the uniformity of the membrane potential, in particular to preserve this uniformity during voltage-clamp steps within the potential range where the K conductance is normally activated. The steep potential dependence of force activation shown in Fig. 6 indirectly shows the good uniformity achieved. For mechanical flexibility the shafts of the micro-electrodes were thinly coated with rubber cement which became stiff after a few minutes. The electrode glass was then gently broken about 2–3 mm above the tip. The flexibility obtained in this way was adequate to keep the electrode tips inside the fibres even during vigorous contractions. However, leakage currents usually increased with the number of contractures, ultimately limiting the useful life span of the fibres. Under most favourable conditions, five to twelve reproducible contractures could be obtained from one fibre. Several records obtained under different conditions are shown in Fig. 1. Occasionally, fibre movements caused some disturbance in the current record (Fig. 1*B*) which could mostly be eliminated by gently pushing the electrodes deeper

into the fibres. The fibre in Fig. 1*B* also shows an atypical underswing after the contracture. This and similar artifacts were often due to rearrangements of fibres in the whole bundle during the first contracture or to activities of other fibres. The former could be prevented by dissecting bundles with only one layer of parallel fibres. However, even then it was difficult to maintain the same resting force for longer periods of time or to follow up long-lasting force activities. This preparation, introduced into physiological research by Caputo & Fernandez de Bolaños (1979), is thus especially suitable for measuring brief contractures under reasonable voltage-clamp conditions in intact muscle fibres.

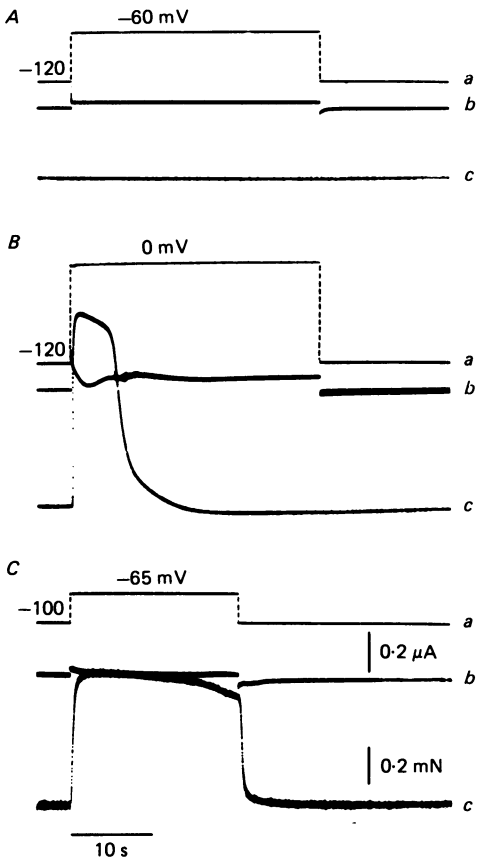

Fig. 1. Voltage clamp in short toe muscle fibres. *A*, *B* same fibre, TEA sulphate solution (solution F); temperature, 6·7 °C; *C* another fibre in solution F with 7 mM-ClO_4^-; temperature, 5·7 °C; *a* potential, *b* current, *c* force.

Solutions

Standard Ringer solution (solution A, Adrian, 1956; in mM): NaCl, 115·0; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85. Muscles and single fibres were dissected and stored in this solution before the experiment.

Solutions used in experiments with single isolated fibres (*solutions* B–D). Tris Ringer (solution B): standard Ringer (solution A) in which NaCl and the phosphate buffers were replaced by Tris Cl. Tris ClO_4^- Ringer (solution C): Tris Ringer (solution B) plus 8 mM-Tris ClO_4^- and 5 mM-MOPS (3-(*N*-morpholino)propanesulphonic acid) as buffer. The contracture solutions with an elevated K concentration corresponded to those given by Hodgkin & Horowicz (1959). Solution D, for a complete depolarization of the fibre, contained in mM: K_2SO_4, 95·0; $CaSO_4$, 8·0 (free $Ca^{2+} \sim 4$ mM);

Na_2HPO_4, 1·08 and NaH_2PO_4, 0·43. Solutions to establish membrane potentials between −50 and −15 mV (corresponding to 20 and 75 mM-K, respectively) had a constant $[K]_o \times [Cl]_o$ (external K and Cl concentrations) product of 300 mM^2 and contained SO_4^{2-} as the impermeant anion, sucrose to maintain osmolarity and Tris Cl or MOPS as buffers. ClO_4^-, $Cr_2O_8^{2-}$ and further anions were added together with $Tris^+$ under a corresponding reduction in $sulphate^{2-}$.

Solutions used in voltage-clamp experiments (solutions E–G). Toe muscle preparations were transferred into the experimental chamber filled with standard Ringer (solution A). Subsequently this solution was replaced by a sulphate Ringer solution (solution E, Hodgkin & Horowicz, 1959) of the following composition (in mM): Na_2SO_4, 38·75; K_2SO_4, 1·25; Na_2HPO_4, 1·08; NaH_2PO_4, 0·43; sucrose, 113·0; $CaSO_4$, 8·0 ($Ca^{2+} \sim 4$ mM). Tetrodotoxin (TTX; 5×10^{-7} g/ml) was added to this and the following solutions to suppress action potentials. Fibres usually developed force in this solution (depolarization induced by Cl^- removal) which slowly declined within 5–10 min. After relaxation, solution F containing tetraethyl ammonium (TEA) sulphate was applied (in mM): TEA sulphate, 40·0; K_2SO_4, 1·25; $CaSO_4$, 8·0 ($Ca^{2+} \sim 4$ mM); MOPS, 5; sucrose, 113. This solution often induced a second transient increase in force. Finally solution G was applied. It consisted of solution F, but with sucrose partially replaced by an osmotically equivalent amount of $NaClO_4$ or Tris ClO_4. The pH of all solutions was 7·0.

Temperature

Single fibre experiments were done at room temperature and voltage-clamp experiments at temperatures between 2 and 22 °C.

Statistics

All numerical results shown in this paper are given as mean and S.E. of the mean. Comparisons of differences were made by Student's paired *t* test.

RESULTS

The results are divided into three parts. In the first part we describe experiments with isolated fibres from semitendinosus and iliofibularis muscles. The investigations took place in a type of chamber first used by Hodgkin & Horowicz (1960*a*) which allows a quick exchange of solutions. Therefore, we used this method to measure the speed of action of ClO_4^- and to establish entire dose–response relations with one fibre. The relatively simple procedure was also used to screen anions which are chemically or structurally related to ClO_4^-, and to confirm earlier measurements of other authors.

In the second part we employed the more laborious voltage-clamp method. Because it allows nearly instantaneous voltage shifts over a wide potential range we preferred this method for measuring the potential dependence of force activation and inactivation before and after ClO_4^- application.

The third part deals with the effect of ClO_4^- on the potential dependence of the Na conductance and the delayed rectifier. These measurements were performed on the sartorius muscle as a classical preparation for electrophysiological experiments.

Experiments with isolated single fibres

Repolarization induced activation of force. The striking observation by Foulks *et al.* (1973) that fully depolarized and mechanically refractory muscle fibres develop force upon repolarization aroused our interest in ClO_4^- as a tool for the study of excitation–contraction coupling. Entering this new field, we first repeated some of these authors' most impressive experiments using single isolated muscle fibres. In most cases the following procedure was adopted: Ringer solution was first replaced by Tris Ringer (solution B) to suppress the initiation of action potentials and

twitches. After 2 min this solution was exchanged for Tris Ringer with ClO_4^- (solution C, usually with 8 mM-ClO_4^-) and only 20 s later, complete depolarization was achieved with a solution containing 190 mM-K (solution D, again with the same concentration of ClO_4^-). The depolarization induced a phasic contracture of normal appearance (Fig. 2*C*). After relaxation the fibres were repolarized to about –50 mV

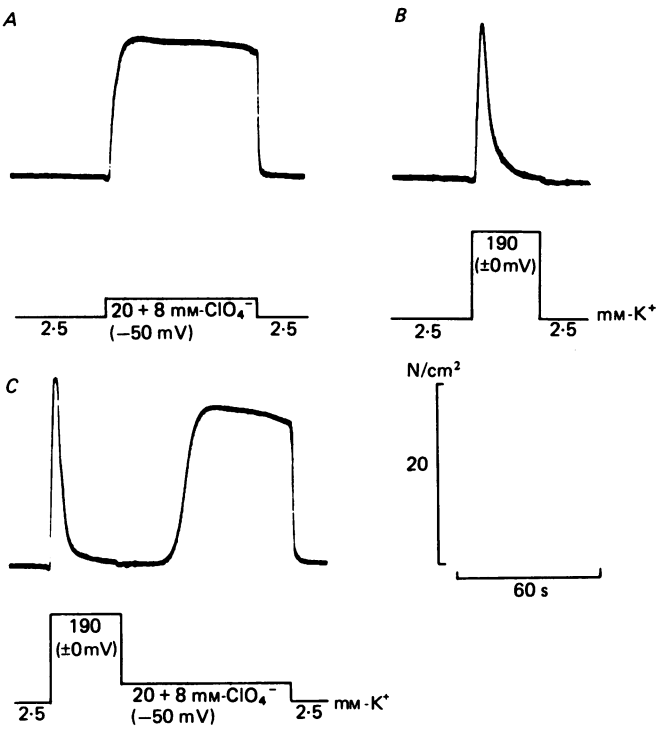

Fig. 2. Activation of force in the presence of ClO_4^-. *A* and *C*, depolarization- and repolarization-induced activation of force. 8 mM-ClO_4^- was applied 20 s prior to the first depolarization and present in all subsequently applied solutions besides that which in *C* finally repolarized the fibre to the resting value. *B*, control without ClO_4^-. *A* and *B*, contractures from the same isolated semitendinosus fibre, *C* another fibre. Action potentials were prevented by the exchange of Na with Tris 2 min prior to depolarization; temperature, 20–22 °C.

with a solution containing 20 mM-K at a constant $[K]_o \times [Cl]_o$ product, again with ClO_4^-. In most cases repolarization induced a second rise in force which usually started after a delay of about 20 s and reached up to 80 % of the maximum value. Force could be maintained for a long period of time (see below). In the experiment of Fig. 2*C* quick relaxation was ultimately achieved by repolarizing the fibre in standard Ringer. A similar fast relaxation could also be obtained by a full depolarization with a K-rich solution. Under both conditions relaxation also occurred in the presence of ClO_4^-.

This ClO_4^- effect, which was first described by Foulks *et al.* (1973), to some extent resembles the repolarization-induced reactivation of the Na conductance caused in nerve fibres by a scorpion venom (Calahan, 1975). However, that effect could only

be obtained after a preceding depolarization, of which ClO_4^- acts independently. This is shown in Fig. 2*A*. In the presence of ClO_4^- a direct depolarization to −50 mV caused an immediate increase of force to a steady level which approached the maximum (shown in Fig. 2*B* without ClO_4^-). Under normal conditions no or at best minor and transient contractures can be induced at −50 mV. The repolarization-induced force became smaller when the temperature was lowered (cf. Foulks *et al.* 1973) and it disappeared when the preceding depolarization was prolonged. In some fibres it could not be induced at all. Because of this low reproducibility we did not attempt a voltage-clamp analysis of this phenomenon.

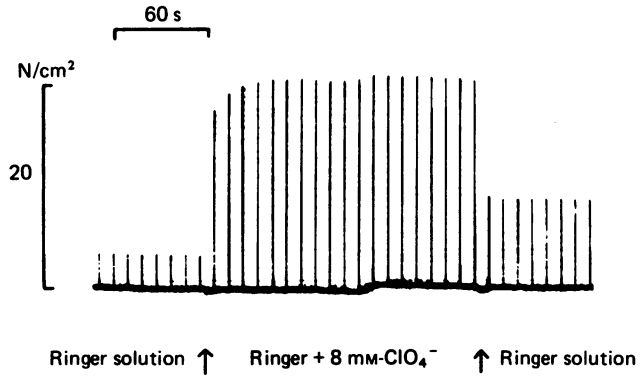

Fig. 3. Speed of action of ClO_4^-. Ringer solution (solution A) plus 8 mM-ClO_4^- was flushed through the experimental chamber for about 3 min (indicated by arrows) while twitches were induced continuously at 0·1/s. Isolated fibre from the M. semitendinosus; temperature, 20–22 °C.

Speed of action. ClO_4^- caused a quick and distinct increase in twitch height. Since this form of mechanical activity can easily be induced and evaluated (cf. Hodgkin & Horowicz, 1960*b*) we used maximum twitch force to measure the speed of action. In the experiment shown in Fig. 3 an isolated fibre was stimulated every 10 s. At the time indicated by the first arrow Ringer solution (solution A) was replaced by a solution of the same composition plus 8 mM-ClO_4^- (The steady flow replaced the chamber volume nearly every second). The first twitch following solution exchange was nearly six times larger than normal. Twitch height increased further and reached a steady level of about seven times the original value within 20–30 s. Upon removal of ClO_4^- it immediately fell to about two to three times the value in Ringer prior to the application of ClO_4^-. Further decline was slow. The ClO_4^- effect was reversible even at 100 mM-ClO_4^- if the application lasted only a few minutes. We conclude from this and similar experiments that ClO_4^- acts fast. The equilibration within 20–30 s, with a rise time of only a few seconds, may reflect the time needed for diffusion of ClO_4^- into the T system, its distribution in the membranes of the transverse tubular system (Hodgkin & Horowicz, 1960*b*) and the establishment of a new intracellular equilibrium between Ca pools and Ca buffers. The slow recovery is less easily explained. It may partly arise from a diffusion of ClO_4^- into the intracellular space during the time of exposure.

Dose–response relation. The relation between twitch height and the external ClO_4^-

concentration is shown in Fig. 4. Two isolated fibres were employed, one showing a large, the other a less distinct increase in force. We adopted the following procedure: the fibres were continuously stimulated at 0·1/s throughout the experiment. After a steady state had been reached, standard Ringer (solution A) was replaced by Ringer in which part of NaCl had been replaced by $NaClO_4$. After 2 min this solution was again replaced by standard Ringer for 5 min before the next test solution with a higher ClO_4^- concentration was applied, again for 2 min. Twitch height was evaluated after 1 min when a steady state had been reached. In one of the two fibres the first increase in force became visible at 10^{-4} M, it increased steeply between 0·5 and 5×10^{-3} M and approached saturation beyond 10^{-2} M.

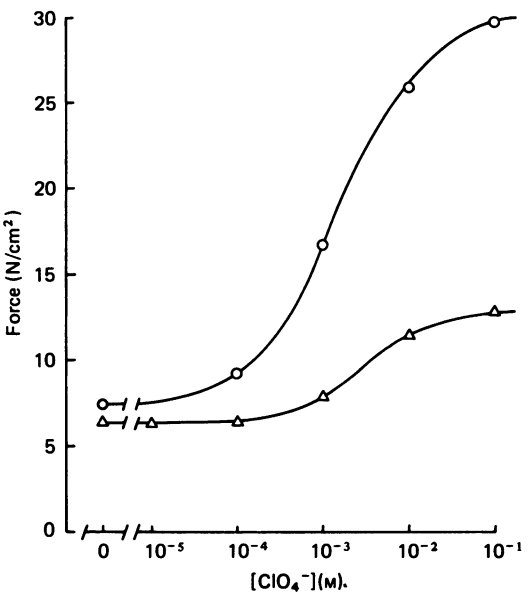

Fig. 4. Dose–response relation. The increase in twitch height in dependence of the external ClO_4^- concentration (abscissa, logarithmic scale). Two experiments with isolated fibres from the M. iliofibularis; temperature, 22 °C.

Interpretation of these curves is difficult since twitch height depends on different factors, such as duration of the action potential, surface charge density or intramembrane charge movement (see 'Discussion'). However, this method makes it possible to measure the entire dose–response relation with one fibre and to detect the first sign of ClO_4^- action.

The effect of chemically and structurally related anions. Foulks & Perry (1979) showed that repolarization-induced contractures could also be observed after the application of caffeine and other so-called potentiators of contraction. However, the effects were smaller, not typical and poorly reproducible. We have tested further anions with properties similar to ClO_4^-. These screening tests were done with small bundles of five to ten muscle fibres from the semitendinosus. Substances with a high oxidation potential were toxic (e.g. 0·05–8 mM-MnO_4^-) or caused some increase in twitch height (1 mM-H_2O_2). A repolarization-induced force, however, was not obtained. Three

anions structurally related to ClO_4^- were tested. Silicate (10 mM-Na_4SiO_4, tetrahedal structure like ClO_4^- but with four negative charges) showed no effect but acted deleteriously when applied for longer periods of time. Thiosulphate (20 mM-$S_2O_3^{2-}$, tetrahedal structure, one oxygen atom replaced by sulphur, two negative charges) reduced twitch height. Chromate (10 mM-CrO_4^{2-}, tetrahedal structure, two negative charges) increased twitch height and caused a contracture upon depolarization to −50 mV (normal threshold) which reached 50% of the maximum. Repolarization-induced contractures were not obtained. Interestingly, dichromate (10 mM-$Cr_2O_4^{2-}$,

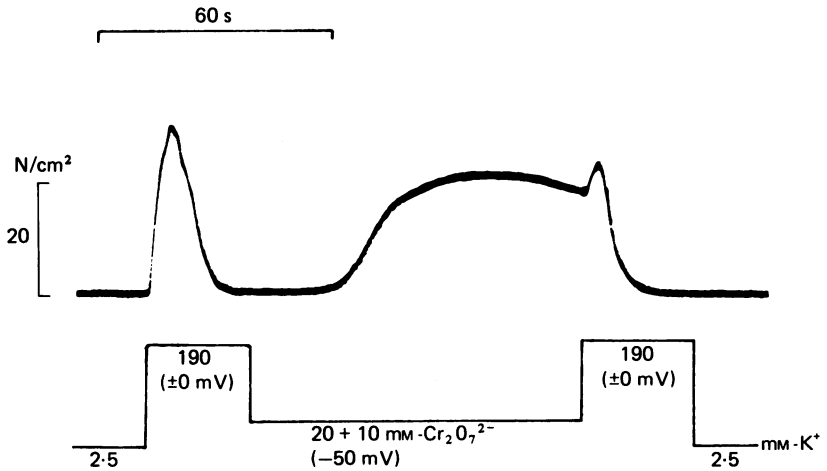

Fig. 5. Repolarization-induced force development in the presence of 10 mM-$Cr_2O_7^{2-}$ with a bundle of eight semitendinosus fibres; 10 mM-$Cr_2O_7^{2-}$ was applied 20 s prior to the first depolarization and present in all subsequently applied solutions; temperature, 20–22 °C.

two tetraheda joined by a common oxygen atom, each with a single charge) was still more effective than chromate. A depolarization to −50 mV induced 80% of maximum force, and after a full depolarization followed by force inactivation a repolarization to −50 mV induced an activation of force almost as distinct as that obtained with the same concentration of ClO_4^- (Fig. 5).

Voltage-clamp experiments with short toe muscle fibres

The ClO_4^- experiments described above and those reported earlier by Foulks *et al.* (1973) show that this anion enables the development of force at potentials more negative than the usual threshold potential (−50 mV). The long-lasting contractures (Fig. 2) suggest in addition that fast inactivation of force is retarded or completely absent in this potential range. Below, these preliminary observations are confirmed by a quantitative study of the potential dependence of both force activation and inactivation in the presence of ClO_4^-.

Activation of force. Fibres were bathed in TEA sulphate Ringer (solution F) in which the corresponding amounts of $NaClO_4$ replaced osmotically equivalent amounts of sucrose (solution G) (see 'Methods'). The fibres were kept for at least 5 min at holding potentials more negative than the expected threshold for activation of contraction (−110 to −140 mV) before force was induced by depolarizing steps of 30 s duration.

Without ClO_4^-, force first appeared at steps to −50 mV and reached a maximum between −40 and −45 mV (Fig. 6). This approximately agrees with earlier measurements on the same preparation by Caputo & Fernandez de Bolaños (1979) whose curve with data from five different fibres is slightly less steep. ClO_4^- caused a parallel shift of the activation curve to more negative potentials by up to 40 mV. A further evaluation of these measurements is shown in Fig. 7. Here the potential at which the force reached half of its maximum value is plotted against the ClO_4^- concentration. The curve is steep in the lower concentration range, it becomes flatter beyond

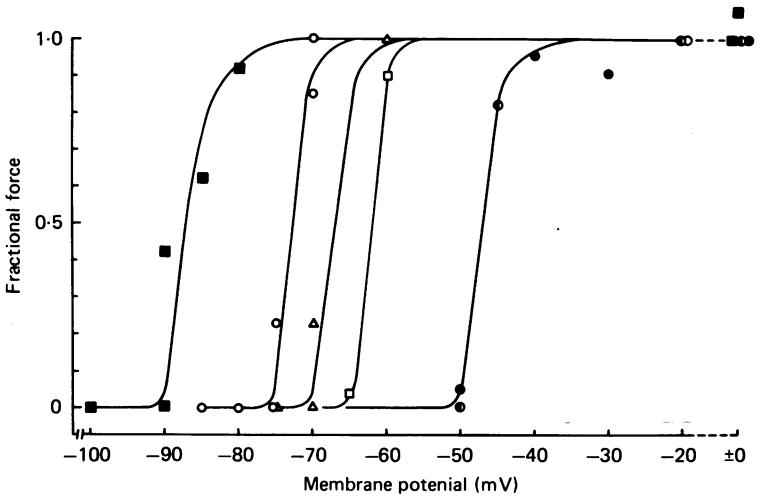

Fig. 6. Dependence of force activation on membrane potential. The fibres were kept for at least 5 min at a holding potential of −110 mV (−140 mV at 70 mM-ClO_4^-) before force was induced by depolarizing steps to membrane potentials given at the abscissa. Ordinate, fractional force. Each symbol represents one fibre; temperature, 5·8–6·7 °C. Concentration of ClO_4^- (mM): ◐, ●, 0; □, 1; △, 10; ○, 20; ■, 70.

10 mM-ClO_4^- and continues to rise slowly up to 70 mM-ClO_4^-. (Also included in this Figure is the shift in the threshold potential for the action potential which will be discussed in more detail in a subsequent section.) From the threshold near −90 mV at 70 mM-ClO_4^- one would expect spontaneous contractures in isolated semitendinosus fibres at ClO_4^- concentrations above 70 mM. This was not observed. In toe muscle preparations, however, spontaneous contractures frequently occurred in ClO_4^- solutions. These probably developed in damaged and slowly depolarizing fibres and impeded the identification of force transients from the voltage-clamped fibre under investigation, in particular during long-lasting activities.

Injection of ClO_4^-. In order to get some information about the site of action of ClO_4^- we applied this anion internally with the help of a third glass micro-electrode filled with 3 M-$NaClO_4$. The current passed through this electrode, for electrophoretic injection of ClO_4^-, lasted for one to several seconds and reached up to 20 nA. Under the assumptions that (1) the fibres were 1·8 mm long and 70 μm thick, that (2) half of the current was carried by ClO_4^- (most probably an over-estimation) and that (3) the anions were distributed uniformly throughout the fibre volume, we arrived at

intracellular ClO_4^- concentrations between 0·4 and 1·8 mM. The bath contained solution F at a temperature of 7 °C. After the injection procedure voltage-clamp steps of 45 s duration were applied from −100 to −60, −50 or ±0 mV. In one fibre with a calculated internal ClO_4^- concentration of about 0·5 mM, we observed no force at −60 mV, nearly maximal and maintained force at −50 mV and a normal transient force development at ±0 mV. In three further experiments we also obtained

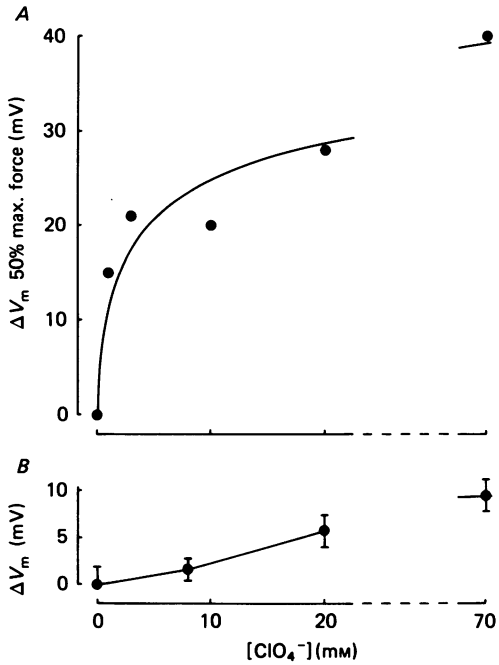

Fig. 7. Dose–response relation. Shift to more negative potentials of force activation (*A*) and threshold of the action potential (*B*) in dependence of external ClO_4^- (abscissa). Ordinate: *A*, ΔV_m (membrane potential) at 50% max. force ($\Delta V_m = 0$ mV at $V_m = -47$ mV); temperature, 6–6·5 °C, toe muscle. *B*, ΔV_m of action potential threshold ($\Delta V_m = 0$ mV at $V_m = -51$ mV), sartorius muscle; temperature, 12–13 °C.

long-lasting contractures at −60 or −50 mV reaching 30–60% of the maximum strength. These preliminary experiments seem to suggest that no differences exist between the internal or external application of ClO_4^-. In both cases non-relaxing contractures could be induced at voltages more negative than the normal threshold potential.

Inactivation of force. The potential dependence of force inactivation was usually tested with two subsequent potential steps. Initially the membrane was depolarized to a conditioning potential between −60 and −20 mV until a new steady state in inactivation was reached. Subsequently the remaining attainable force was elicited by a full depolarization (e.g. Lüttgau & Oetliker, 1968). This procedure is difficult to perform in the presence of ClO_4^- since fibres do not relax spontaneously during the conditioning procedure so that fatigue processes might affect the results. However, the characteristics of the ClO_4^- effect allow an alternative approach which is shown in Fig. 8. The fibre was depolarized for 20 s from a holding potential of

−100 mV to potential levels as shown. With 7 mM-ClO_4^- in the external solution the threshold of force activation was reached at about −73 mV. At −50 mV, relaxation, regarded as a sign of inactivation, did not occur. However, it became obvious at −25 mV, and upon full depolarization the developing phasic contracture could no longer be distinguished from a normal contracture at this potential.

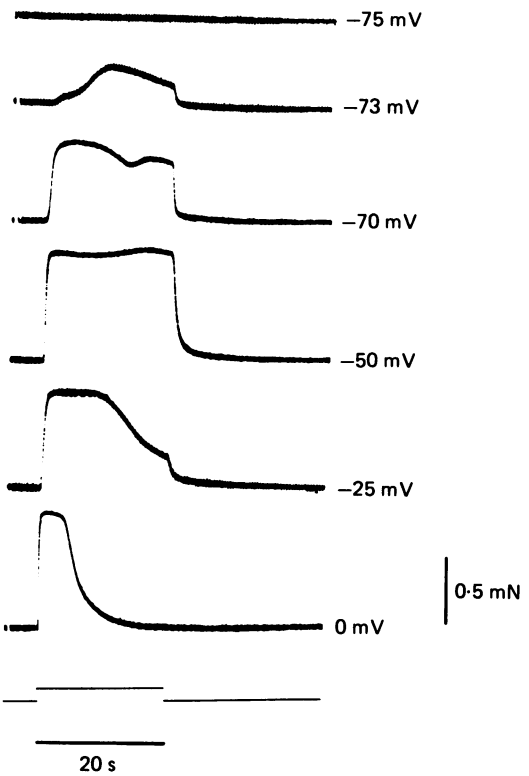

Fig. 8. The effect of 7 mM-ClO_4^- upon force during a depolarizing voltage step of 20 s duration to potential levels given at the right of each contracture. Holding potential −100 mV; temperature, 6 °C.

The complicated time course of the contractures at −73 and −70 mV (Fig. 8), which suggests two phases of activation, was often, although not always, observed at rather negative potentials. They resemble those found after the application of low concentrations of caffeine (Lüttgau & Oetliker, 1968) and may be the expression of regenerative processes (cf. Lüttgau & Spiecker, 1979).

The inactivation experiment was repeated in four additional fibres from different preparations with similar results. In these measurements the depolarizing voltage step lasted for 30 s (in the presence of 10 or 20 mM-ClO_4^-). For steps to −55 and −50 mV the activated force did not decrease throughout the depolarization period. Steps to −20 and ±0 mV led to a complete relaxation following maximal force development while contractures relaxed only partially during steps to −40 and −30 mV. At the latter values a constant force had not been reached before the end

of the pulse suggesting that the fibres were not yet in a steady state. These experiments were performed at 6 °C. In one experiment at 2 °C we observed a 50 % inactivation taking place during a step to −50 mV while force was maintained at −60 mV (10 mM-ClO_4^-). It is known that a drastic decrease in temperature shifts the inactivation curve towards more negative potentials (Caputo, 1972). This effect might explain the partial relaxation at −50 mV which was not found at higher temperatures.

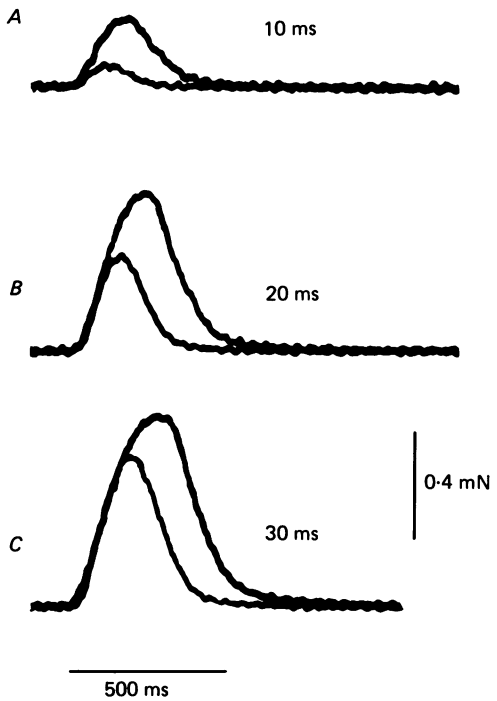

Fig. 9. Activation of force with short depolarizing pulses. The fibre was depolarized from a holding potential of −85 mV to −15 mV before and after the application of 20 mM-ClO_4^-. Pulse duration *A*, 10; *B*, 20 and *C*, 30 ms. The larger force in the superimposed contractures was at each duration obtained in the presence of ClO_4^-. The small oscillations are mechanical artifacts; temperature, 6 °C.

The experiments show that in a potential range more negative than −50 mV, in which force activation occurs only in the presence of ClO_4^-, inactivation of force is either absent or follows a very slow time course. Between −50 and ±0 mV inactivation kinetics appears to be little affected by ClO_4^-. The potential dependence of the steady-state inactivation curve was probably not altered dramatically although the results do not exclude minor shifts in either direction.

Short depolarizing pulses. In Fig. 9 we applied short depolarizing pulses from a holding potential of −85 mV to −15 mV first without ClO_4^- and second a few minutes after the application of 20 mM-ClO_4^-. The superimposed contractures show that ClO_4^- increased the rising time and consequently the maximum of force. It had no effect upon the rate of rise. Similar effects were obtained when force was induced by propagated action potentials (D. Berwe, unpublished observation).

Long-lasting contractures. The experiments described in the preceding sections suggest that in a potential range between −90 and −50 mV ClO_4^- brings about an activation of force which is not followed by a spontaneous relaxation. However, depolarizations lasted only for 20–30 s. In further experiments with 10–20 mM-ClO_4^- and potential steps from −120 to −60 or −55 mV, we extended the depolarization period until fibres had relaxed from the induced force development. The main results from ten experiments can be summarized as follows: maximum force could be

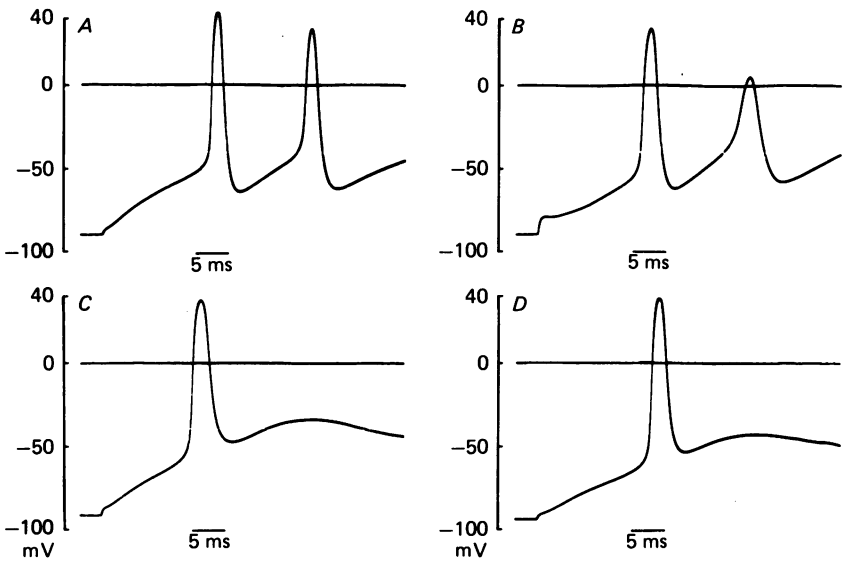

Fig. 10. The effect of ClO_4^- upon the action potential. Different sartorius preparations. All solutions contained 350 mM-sucrose to prevent twitches; temperature, 12·5 °C. *A*, control, threshold −47 mV; *B*, 8 mM-ClO_4^-, threshold −52 mV; *C*, 20 mM-ClO_4^-, threshold −55 mV; *D*, 70 mM-ClO_4^-, threshold −59 mV.

maintained for at least one to several minutes. Then force declined towards zero within 10–20 min. In several fibres the slow decline was suddenly interrupted by a quick relaxation. A definite explanation of the decline in force cannot be given. When fully depolarized at the end of the long-lasting contracture some fibres developed nearly maximal force. This could be explained by assuming that those Ca^{2+} releasing sites which were active at −50 mV slowly became inactivated and that further sites were activated upon full depolarization. Other fibres, however, developed only minor contractures upon full depolarization so that slow relaxation was probably due to an exhaustion of Ca^{2+} release or energy reserves.

The effect of ClO_4^- on electrophysiological parameters of sartorius muscle fibres

The so-called chaotropic anion ClO_4^- adsorbs to artificial phospholipid membranes and induces a negative electrostatic potential at the membrane surface (McLaughlin, Bruder, Chen & Moser, 1975). Such an adsorption to the outer side of muscular membranes should shift the voltage dependence of membrane processes to more negative potentials without altering the membrane potential measured with internal micro-electrodes. If this accounts for the observed shift in force activation, other

membrane processes should also be affected. To test the adsorption hypothesis we have measured the threshold potential for activation of Na and K channels. These experiments were performed with micro-electrodes in muscle fibres from the sartorius.

Resting potential. In Ringer solution (solution A) a mean resting potential of $-86{\cdot}4 \pm 1{\cdot}03$ mV ($n = 8$) was measured. It shifted to $-90{\cdot}0 \pm 0{\cdot}47$ mV ($n = 16$; $P < 0{\cdot}01$) following addition of 20 mM-$NaClO_4$ to the Ringer solution and returned to $-87{\cdot}8 \pm 0{\cdot}49$ mV ($n = 16$; $P < 0{\cdot}01$) within a few minutes after re-application of normal Ringer.

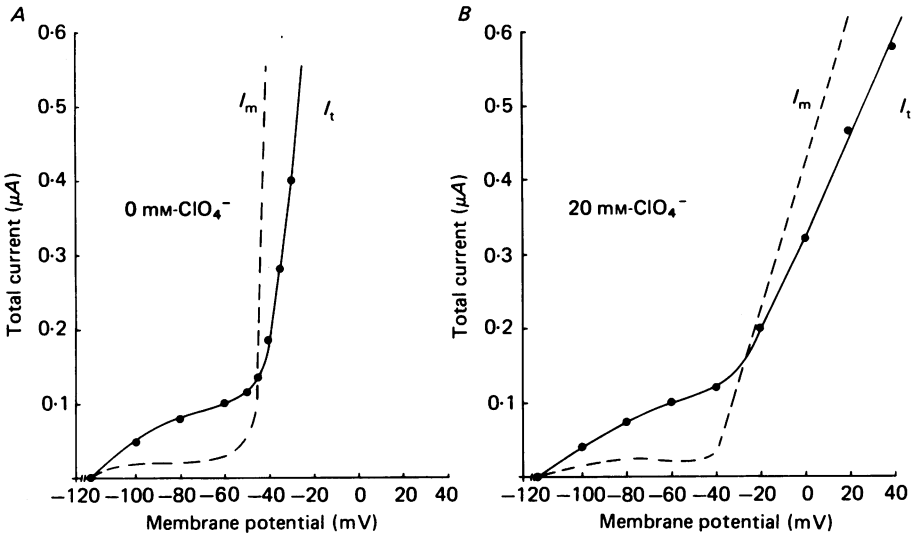

Fig. 11. Current–voltage relationship in sulphate Ringer without (*A*) and with 20 mM-ClO_4^- (*B*). The total clamp current (I_t) read at the end of a 200 ms pulse was plotted on the ordinate against the corresponding membrane potential on the abscissa. From the measured curves membrane current density (I_m; arbitrary units) was derived using Cole's theorem (dashed line). Holding potential −120 mV; temperature, 3 °C.

Action potential. Since our point-voltage-clamp method did not resolve fast ionic currents we used the threshold for the initiation of an action potential as a measure for the potential dependence of Na current activation. The solutions contained 350 mM-sucrose to prevent twitching, and NaCl in standard Ringer (solution A) was iso-osmotically replaced by $NaClO_4$. The membrane was depolarized by rectangular constant current pulses of 100 ms duration passed through a citrate electrode while the action potential was measured with a KCl electrode impaled close to the current electrode. The current was increased until an action potential occurred within a latency period of 13–16 ms. The threshold potential was defined as the intersection of the tangent along the electrotonic potential during the current pulse with that along the upstroke of the action potential. In Fig. 10 representative action potentials at different ClO_4^- concentrations can be seen and Fig. 7 shows the threshold potential shift plotted against the ClO_4^- concentration. Even at high concentrations ClO_4^- caused only minor alterations in the shape of the action potential, in particular a prolongation in spike duration and a decrease in overshoot. The threshold potential

shifted from $-51{\cdot}4 \pm 1{\cdot}9$ mV ($n = 10$) under normal conditions to $-53{\cdot}4 \pm 1{\cdot}5$ mV (8 mM), $-56{\cdot}7 \pm 1{\cdot}8$ mV (20 mM) and $-60{\cdot}9 \pm 1{\cdot}2$ mV (70 mM). Whereas the difference in potential was insignificant between 0 and 8 mM ($P < 0{\cdot}5$) it became significant at higher ClO_4^- concentrations (20 mM: $P < 0{\cdot}02$; 70 mM: $P < 0{\cdot}01$). The data correspond reasonably well with those of Dani, Sánchez & Hille (1983) in voltage-clamp experiments with the cut-fibre method. After full replacement of external Cl^- by ClO_4^- they observed a shift of the voltage dependence of opening of sodium channels by about 13 mV to more negative potentials.

K conductance (delayed rectifier). The activation of the delayed K^+ conductance was measured in fibres from the sartorius muscle under point-voltage-clamp conditions. Ringer solution was replaced by sulphate Ringer (solution E) plus 350 mM-sucrose to suppress twitches. In solutions containing ClO_4^-, Na_2SO_4 was partially replaced by $NaClO_4$ keeping the Na concentration constant. Fig. 11 shows representative current–voltage relations without and with 20 mM-ClO_4^-.

In the presence of ClO_4^- the curve is less steep in the potential range in which the delayed rectifier is activated. This effect became more and more pronounced with increasing ClO_4^- concentrations. However, a distinct shift of the steep part of the current–voltage relation to more negative potentials comparable to that of force activation was not found (twelve complete curves; 0–70 mM-ClO_4^-). In some experiments a slight inflexion of the curve, similar to that described by Heistracher & Hunt (1969) for the action of SCN^- in short snake muscle fibres, was seen in a potential range 5–10 mV more negative than the linear extrapolation of the steep part of the current–voltage relation. Because of this additional complication and the partial block of the delayed rectifier, we did not attempt a statistical treatment.

DISCUSSION

ClO_4^- anions shift the activation curve which relates peak force to the membrane potential towards more negative potentials by up to 40 mV. This shift is not accompanied by a corresponding change in the potential dependence of force inactivation. Consequently a potential range exists between -50 and -90 mV where force can be activated without subsequent inactivation and where reactivation can occur after a preceding depolarization-induced inactivation. In this interpretation of 'repolarization-induced force activation', which rests upon the activation–inactivation model developed by Hodgkin & Horowicz (1960*a*), we agree with previous authors (Foulks *et al.* 1973). On the other hand the phenomenon is probably difficult to explain with Frank's alternative model of excitation–contraction coupling according to which a *depolarization*-induced release of Ca^{2+} from the inner side of the tubular membrane initiates Ca^{2+} release from the s.r. (cf. Frank, 1982).

The action of ClO_4^- allows the direct observation of the restoration time course of mechanically refractory muscle fibres (Fig. 2*C*). Following some delay, which may only partly be due to a slow repolarization of the membrane, restoration of force occurred relatively fast. Maximum speed of force restoration was only slightly less than that of force activation (Fig. 2*A*). However, insufficient reproducibility of the phenomenon in voltage-clamp experiments restrained us from further exploiting this interesting new experimental approach to investigate inactivation kinetics. ClO_4^- also

makes it possible to maintain maximum force for several minutes by depolarizing fibres to −60 mV. This result demonstrates convincingly that spontaneous relaxation, occurring within a few seconds after force activation by full depolarization of untreated fibres, is due to a membrane-bound inactivation process rather than to exhaustion of energy reserves or a depletion of Ca^{2+} stores. It also suggests that Ca^{2+} turnover must be reduced rigorously during long-lasting muscular activities.

In 1973, Schneider & Chandler suggested that Ca^{2+} release from the s.r. is controlled in some way by intramembrane charge movements in the wall of the transverse tubular system. These charge movements are modified by ClO_4^-. In preliminary reports Fuxreiter *et al.* (1983) and Lüttgau *et al.* (1983) recently showed that the voltage dependence of intramembrane charge movement is steeper in the presence of 8 mM-ClO_4^-, shifting the mid-point voltage $\bar{V}$ by about 25 mV to more negative potentials. The amount of charge transferred remained unaltered. In addition, the anion caused a characteristic prolongation of the declining phase of the 'off' response. We regard these alterations in charge movement kinetics as the principal cause for the shift in the potential dependence of force activation. However, the present results do not yet allow a quantitative approach. In addition we are still altogether in the dark about a detailed molecular mechanism of the described effects. If some idea about the specific action of ClO_4^- emerges it may become feasible to distinguish between the two competing models about the spread of activation from the T-system to the s.r., i.e. the charge movement concept (Schneider & Chandler, 1973), which we applied to interpret our results, or the hypothesis (Mathias, Levis & Eisenberg, 1980) that suggests the existence of activatable pores between the two membranal systems.

ClO_4^- can apparently reach its site of action from either side of the membrane. It acts as though obstructing charge movement from the active to the resting position. From the experiments with related anions it appears possible that the tetrahedal structure is of some importance. The structural basis of the ClO_4^- effect was also investigated by Foulks & Perry (1979) and Foulks & Morishita (1980). In their publications they tested the role of divalent cations and further substances as well as that of temperature in this context and arrived at the conclusion that the phenomenon may result from the effects of ClO_4^- on hydrophobic forces which determine the conformation of macromolecular compounds of the membrane.

Whatever the molecular mechanism may prove to be, ClO_4^- must be regarded as a useful tool for investigating excitation–contraction coupling for at least two reasons: (1) it rather specifically shifts the force activation curve towards more negative potentials and thus allows long-lasting contractures and a direct investigation of the kinetics of force restoration. (2) It separates the potential range of force activation from that of force inactivation and that of the activation of Na and K channels. This implies that in the presence of ClO_4^- charge movements associated with force activation are not contaminated by gating currents responsible for other potential-controlled membrane processes.

It is known that the so-called chaotropic anions (for further details see Hanstein, 1979) ClO_4^- and SCN^- adsorb to artificial phospholipid membranes, creating a negative electrostatic potential at their surface (McLaughlin *et al.* 1975). The effect of low ClO_4^- concentrations (< 10 mM) described here probably can not be explained

by adsorption because the anion acts in the same way if it is applied internally and it rather specifically shifts only the potential dependence of force activation. Since adsorption effects become manifest at high concentrations (>50 mM) they might provide an explanation for the shift in the action potential threshold and the continuous slow shift in force activation with increasing ClO_4^- concentrations. The evidence for this assumption, however, is not very convincing. A definite potential shift was observed in action potential threshold and force activation. Concerning the thresholds of force inactivation and the activation of the delayed rectifier our experiments allow only the cautious statement that a small shift in potential dependence cannot be excluded. In addition the threshold of the action potential can also be influenced by secondary reactions, for example by the observed partial inhibition of the delayed rectifier or the shift of the voltage dependence of Na channel inactivation to more negative potentials (Dani *et al.* 1983). Further complications arise if earlier measurements by Kao & Stanfield (1968) are considered. These authors showed that comparable concentrations (58 mM) of the chaotropic anion SCN^-, caused no shift in the action potential threshold but large shifts in the thresholds for contraction (19 mV) and delayed rectification (17 mV). This suggests that the two anions act quite differently and that apparently no uniform interpretation of the action of chaotropic anions upon biological membranes is possible.

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