

Effects of Low-Chloride Solutions On Action Potentials of Sheep Cardiac Purkinje Fibers

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ABSTRACT The rapid repolarization during phase 1 of the action potential of sheep cardiac Purkinje fibers has been attributed to a time- and voltage-dependent chloride current. In part, this conclusion was based on experiments that showed a substantial slowing of phase 1 when larger, presumably impermeant, anions were substituted for chloride in Tyrode's solution. We have re-examined the electrical effects of low-chloride solutions. We recorded action potentials of sheep cardiac Purkinje fibers in normal Tyrode's solution and in low-chloride solutions made by substituting sodium propionate, acetylglycinate, methylsulfate, or methanesulfonate for the NaCl of Tyrode's solution. Total calcium was adjusted to keep calcium ion activity of test solutions equal to that of control solutions. Propionate gave qualitatively variable results in preliminary experiments; it was not tested further. Low-chloride solutions made with the other anions gave much more consistent results: phase 1 and the notch that often occurs between phases 1 and 2 were usually unaffected, and the action potential duration usually increased. The only apparent change in the resting potential was a transient 3–6 mV depolarization when low-chloride solution was first admitted to the chamber, and a symmetrical transient hyperpolarization when chloride was returned to normal. If a time- and voltage-dependent chloride current exists in sheep cardiac Purkinje fibers, our results suggest that it plays little role in generating phase 1 of the action potential.

INTRODUCTION

For more than two decades, the sheep cardiac Purkinje fiber has been a popular preparation for electrophysiological studies of heart muscle. Detailed analysis of the ionic basis of electrical activity in this preparation became possible when Deck et al. (1964) developed a method of voltage clamping short segments of Purkinje fiber. As this technique has been applied, an extraordinarily complicated picture of the ionic basis of the electrical activity has emerged, and it now seems that at least five time-dependent currents interact to produce the action potential (McAllister et al., 1975). This complexity is disturbing, but experimental evidence exists for each of these currents, and any attempt to reconsider present ideas must begin with a re-examination of the evidence.

Two of the time-dependent currents have been of particular interest to us. The "slow inward" current, or I_{sl} , seems at least partly due to the movement of calcium ions (Vitek and Trautwein, 1971) and it appears to be closely linked to

contraction of the Purkinje fiber (Gibbons and Fozzard, 1975) and ventricular muscle (Trautwein et al., 1975). In the Purkinje fiber, however, analysis of I_{si} is hampered by a large transient outward current that arises when the preparation is clamped to voltages more positive than -20 mV. This transient outward current has been called the "chloride" current or I_{Cl} (Dudel et al., 1967), the "positive dynamic" current (Peper and Trautwein, 1968), and most recently " I_{qr} " (McAllister et al., 1975). We shall refer to it either as the transient outward current or as I_{qr} , because these terms imply nothing about the ion or ions responsible for it.

The history of I_{qr} is interesting. Carmeliet (1961) first suggested that chloride movements play a substantial role in generating the Purkinje fiber action potential. When Carmeliet replaced chloride by acetylglycinate, he found a transient increase in the rate of spontaneously active preparations, prolongation of the plateau of the action potential with occasional standstill at the plateau voltage, a slowing of repolarization immediately after the upstroke, and an elevation of the plateau that was most marked if the preparation was stimulated rapidly. Hutter and Noble (1961) reported similar changes of spontaneous frequency and action potential duration when chloride was replaced by methyl-sulfate or pyroglutamate.

Under voltage clamp conditions, Deck et al. (1964) noted a large initial outward current when the preparation was clamped to inside positive potentials. Dudel et al. (1967) investigated the possibility that this current was due to chloride movement; in most of their experiments maleate or gluconate was used to replace chloride. The initial current was greatly reduced in chloride-free solution and Dudel et al. concluded that it was due to the inward movement of chloride ions. Dudel et al. pointed out that this "chloride current" would cause rapid repolarization immediately after the upstroke of the action potential (i.e. during phase I of the action potential), and they published action potential records showing a dramatic reduction of the rate of phase I repolarization in low-chloride solution. (For their action potential experiments, Dudel et al. only reduced chloride to 25 mM because the preparations tended to depolarize in chloride-free solution.)

The presently accepted role of I_{qr} in generating the action potential is essentially that proposed by Dudel et al. (1967). I_{qr} is a relatively large and transient outward current with a threshold at approximately -20 mV, and it is thought to be due to the inward movement of chloride ions. I_{qr} appears to be largely responsible for the initial rapid phase I repolarization after the spike of the action potential, and the deactivation of this current, together with the onset of I_{si} , is thought to generate the notch that often appears between the spike and the plateau of the Purkinje fiber action potential (Noble and Tsien, 1969; McAllister et al., 1975). The I_{qr} system is slow to recover from inactivation (Hauswirth et al., 1972; Fozzard and Hiraoka, 1973), and the slow repriming rate accounts for the observation that the notch is often smaller or absent during stimulation at moderate or high rates (McAllister et al., 1975). During a voltage clamp depolarization, I_{qr} and I_{si} are thought to overlap in time at voltages positive to -20 mV (Vitek and Trautwein, 1971), and it is this overlap that makes complete analysis of I_{si} difficult (Gibbons and Fozzard, 1975).

In order to make the development of present ideas about I_{qr} seem this straightforward, however, some disturbing features of the history of this current must be omitted. Peper and Trautwein (1968) noted that I_{qr} and I_{si} (they called them the positive and negative dynamic currents) had many characteristics in common. They recovered along similar time courses, both were reduced when chloride was replaced by propionate, and both were increased when chloride was replaced by nitrate. It should be pointed out that inward current tails were taken as indications of an inward current system in their experiments. The similar behavior of inward and outward currents led Peper and Trautwein to conclude, for a time at least, that both were due to the same current system. Another problem is that while I_{qr} is reported to be reduced in the presence of some chloride substitutes, there has been no convincing demonstration that I_{si} can be seen in a low-chloride or chloride-free solution. To explain these problems, Trautwein (1973) suggested that chloride reduction alters the membrane, thus affecting the permeability of several ions. That may be so, but if I_{qr} is a chloride current, and if chloride replacement cannot be used to eliminate it, progress in understanding Purkinje fiber electrical activity is going to be slowed. We therefore thought that other possible explanations should be explored before this suggestion is accepted.

There are a number of possible problems in chloride replacement studies that seem to have received little attention in earlier experiments on Purkinje fibers. Each of the anions that has been used to replace chloride reduces the calcium ion activity of Tyrode's solution (see Appendix). It is probable, then, that the changes in electrical activity noted in low-chloride solutions have been due to a combination of effects caused by chloride reduction and effects caused by reduced calcium ion activity.

Another possible problem is changes of junction potentials in the recording system as chloride is reduced. These should have been detected in action potential experiments, but they might not have been noticed in voltage-clamp experiments because of the feedback control of voltage. If this were to occur, the actual membrane potential would change while the recorded voltage would stay constant.

Finally, the sodium chloride of the bathing solution was often replaced by the sodium salt of a rather weak organic acid (e.g., propionate). In the undissociated form, many weak acids readily enter skeletal muscle cells (Horowitz and Caputo, 1968; Woodbury and Miles, 1973), and conductance changes may then occur as a result of decreasing intracellular pH (Woodbury and Miles, 1973).

Any of these problems could have led to incorrect conclusions about the effects of chloride on Purkinje fiber electrical activity. Furthermore, if a large body of data were obtained with various chloride substitutes, it might well appear that chloride reduction altered the properties of the membrane so that several conductance systems were changed. With these thoughts in mind, we decided to re-examine the effects of low chloride on the action potential. In order to survey the effects of several anions, we concentrated on those aspects of earlier action potential experiments that have had a strong influence on present ideas about the ionic nature of I_{qr} .

As a practical matter, it would have been difficult or impossible to document the extent to which the problems we suggested have influenced the results and conclusions of earlier work. Instead, we tried to design experiments that minimized the possible influence of changes in calcium ion activity and junction potentials, and we tested anions (propionate, acetylglycinate, methylsulfate, and methanesulfonate) of acids with a wide range of pK values.

MATERIALS AND METHODS

Solutions

Normal Tyrode's solution contained (mM): NaCl, 137; KCl, 5.4; MgCl₂, 1.05; NaHCO₃, 13.5; NaH₂PO₄, 2.4; dextrose, 11.1; CaCl₂, either 1.8 or 2.7. Reagent grade chemicals (obtained from either Mallinckrodt Chemical Co., St. Louis, Mo., or Baker Chemical Co., Phillipsburg, Pa.) and glass-redistilled water were used.

To make low-chloride Tyrode's solutions, the NaCl of the normal solution was replaced by an equimolar amount of sodium propionate, sodium acetylglycinate, sodium methylsulfate, or sodium methanesulfonate. The sources and preparation of these salts were as indicated in the Appendix. Normal and low-chloride Tyrode's solutions were saturated with 95% O₂-5% CO₂ at 36-37°C; the pH of the solutions was 7.3-7.4.

In most of these experiments, the total calcium concentration in the low-chloride solution was increased so that the calcium ion activity was the same in the normal and low-chloride solutions. The necessary total calcium concentration in the low-chloride solution was determined by multiplying the total calcium concentration in the normal solution (1.8 or 2.7 mM) by the following factors: for propionate or acetylglycinate solutions, 1.5; for methylsulfate solution, 1.2; for methanesulfonate solution, 1.0. These factors were based on calcium electrode studies of these solutions (Appendix, Table I).

The experiments were performed at 36-37°C; during an experiment, the temperature was kept constant within $\pm 0.2^\circ\text{C}$. Solutions were changed by use of a valve assembly like that described by Gibbons and Fozzard (1971). Solution flow rates were slower than in the 1971 study, to avoid dislodging the electrode, so several seconds were required to completely change solutions.

Membrane Potential Recording

Intracellular potentials were recorded with micropipette electrodes filled with 3 M KCl (resistance 6-8 M Ω). Conventional techniques were used, with one important exception. The transmembrane potential is usually measured as the difference between an intracellular microelectrode and an extracellular microelectrode placed close to the surface of the preparation. Carmeliet (1961) and Dudel et al. (1967) used this technique in their chloride replacement studies. We used it in several experiments, but discontinued it because, when both electrodes were in the bath solution, 0- ± 3 mV developed between the two electrodes as Tyrode's solution was replaced by low-chloride solution. This suggested that the electrodes were not behaving as saturated KCl bridges, and that they were sometimes developing unequal junction potentials in low-chloride solutions. When we recorded between each microelectrode and a saturated KCl-agar bridge (Hodgkin and Horowicz, 1959), a voltage of 3-7 mV developed as the solutions were changed, as noted by Hutter and Noble (1961). Since only one electrode would be in the external solution during an experiment, a 3-7 mV error would be introduced into the voltage measurements with our electrodes. We therefore adopted the technique described by Woodbury and Miles (1973) and used a flowing KCl-calomel electrode as the external reference electrode. To avoid significant contamination of the bath solution by KCl, a

porous plug electrode with a low flow rate (Fisher 13-639-56, Fisher Scientific Co., Pittsburgh, Pa.) was chosen and the electrode was positioned downstream from the preparation.

In principle, this should be the best arrangement, and the voltage recorded should be close to the true membrane potential. Frequently, however, we withdrew the voltage electrode from the cell (into Tyrode's solution) at the end of an experiment and found that the trace did not return to the zero that we had set at the beginning of the experiment. When the difference was more than just a few millivolts, there was usually good reason to suspect drift even as the experiment was in progress. When this happened, we could not be certain which of the electrodes was responsible. We accepted data on the configuration of the action potential and on changes in resting membrane potential occurring over periods of a few minutes from preparations where drift occurred during the experiment. However, statements about longer-term voltage changes, such as comparisons of resting potentials in normal and low-chloride solutions, are based on experiments in which electrode withdrawal at the end of the experiment gave a zero potential within 3 mV of that set at the beginning of the experiment.

We usually stimulated the preparation by means of a second intracellular microelectrode. In a few experiments, we used an external electrode placed close to the surface of the preparation. One stimulus was delivered every 15 s (0.067 s^{-1}) throughout most experiments. The slow rate was used because I_{qr} reprimed slowly (Hauswirth et al., 1972; Fozzard and Hiraoka, 1973), and we wished to see the maximum effect of any changes in this current. Later, when it appeared that we were getting results different from those Dudel et al. (1967) had obtained, we began to include periodic trains of stimuli delivered at 0.67 s^{-1} to provide a more direct comparison with their data.

RESULTS

Propionate

Propionate has been used as a chloride substitute in voltage clamp experiments (Dudel et al., 1967; Fozzard and Hiraoka, 1973), so its effects on the action potential are of interest. Fig. 1 shows the results we obtained from three preparations. The records labeled A1 show the first exposure of one preparation to a propionate Tyrode's solution. The controls were obtained in Tyrode's solution containing 2.7 mM calcium, and 4 mM calcium was used in the propionate solution to maintain a constant calcium ion activity. After 3 min in propionate Tyrode's solution, the rate of phase 1 repolarization was considerably slower than the control rate. The action potential duration was longer than the control after 1 min in the propionate solution, but after 3 min it was shorter than the control, and in the steady state (9 min), it was dramatically shorter. When the preparation was washed with Tyrode's solution, the action potential configuration returned to normal. A second exposure to the propionate solution gave essentially the same result as that illustrated in records A1.

In the second experiment (B1, Fig. 1), the control Tyrode's solution contained 1.8 mM calcium and we did not correct to maintain constant calcium ion activity as we changed solutions. As in experiment A1, phase 1 repolarization became slower (see the 8-min record). However, after a transient shortening (B1, 1-min record), the action potential became longer than the control (B1, 8-min record). After 9 min in propionate solution the preparation remained at the plateau voltage after a stimulus and did not repolarize until chloride

solution was readmitted to the chamber (B1, 10-min record). After washing, a second exposure to the propionate solution gave the same result. A third preparation gave results different from either of the first two. Data from this preparation are shown in Fig. 1 for the second (records C2) and third (records C3) exposures to propionate Tyrode's solution. After the preparation equilibrated in Tyrode's solution with 1.8 mM calcium, the solution was changed to propionate Tyrode's solution with the total calcium adjusted to keep the

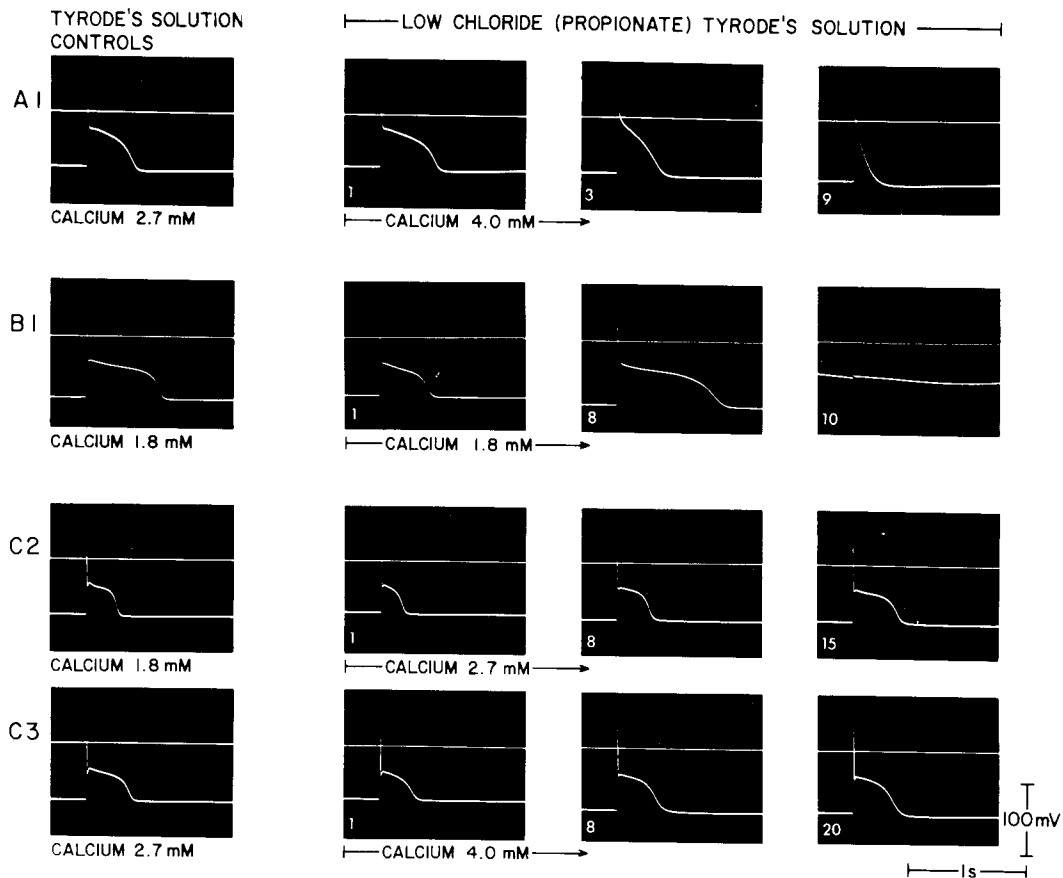


FIGURE 1. Action potentials in propionate Tyrode's solution. Results are shown from experiments on three preparations. The letters A, B, and C to the left of the control records indicate different preparations that were exposed to propionate; the numbers after the letters indicate whether the illustration shows the first, second, or third exposure to the low-chloride solution. A similar letter and number scheme is used in the following figures showing the effects of other chloride substitutes. Numbers in the lower left of experimental records give the number of minutes in low-chloride solution, and the horizontal lines across the records are drawn at 0 mV. The control records were taken just before the change to propionate solution. All action potentials were obtained at a stimulation frequency of 0.067 s^{-1} . Phase 1 of the action potentials from preparations B and C was retouched for clarity.

calcium ion activity constant. The control action potential had a rather rapid phase I repolarization rate and a conspicuous notch between the spike and the plateau. After a transient shortening in the propionate solution (C2, 1-min record), the action potential became slightly longer than the control (C2, 15-min record). The notch was still present after 15 min in propionate Tyrode's solution, and we could detect no change in the rate of phase I repolarization.

The preparation was washed with Tyrode's solution containing 2.7 mM calcium and exposed to propionate Tyrode's solution (4 mM calcium) under the same conditions that gave the result in the experiment labeled A1. In fact, propionate solution left over from experiment A1 was used in C3, and the Tyrode's solution inlet was disconnected to be certain that Tyrode's solution was not entering the chamber. Again, as shown in the records labeled C3, the action potential had a prominent notch even after 20 min in the low-chloride solution and there was no apparent slowing of phase I repolarization.

This seemed an extraordinary amount of variability for three consecutive experiments with the same chloride substitute. There are differences between the action potentials of different Purkinje fibers, as one can see by examining the control action potentials in Fig. 1, but it was difficult to see how there could be this much variability in the response to propionate if the only effect of replacing chloride by propionate were a reduction in chloride conductance. It did not seem likely that the amount of calcium was the cause, since C3 was so different from A1. Considering the difficulty of determining why the variability occurred, we decided to abandon further tests with propionate.

Acetylglycinate

Acetylglycinate gave more reproducible results than propionate, but the effects of replacing chloride by this anion were still not as consistent from preparation to preparation as we would have liked. To achieve a reasonable level of confidence in the results, we performed experiments on 13 Purkinje fibers from seven sheep. In all experiments, the control Tyrode's solution contained 1.8 mM total calcium. Acetylglycinate Tyrode's solutions contained either 1.8 mM calcium (constant calcium concentration) or, except for one experiment noted below, 2.7 mM (constant calcium ion activity).

The action potential duration always increased in acetylglycinate Tyrode's solution (see, for example, Fig. 2). The duration of some action potentials increased only a few percent, while others became several times as long as the controls when sodium chloride was replaced by sodium acetylglycinate. In an attempt to see if the magnitude of the increase in duration was influenced by changes in the calcium ion activity, we exposed preparations to acetylglycinate solution in which the activity of calcium was kept constant by increasing the total calcium concentration. After a steady state was reached, the preparations were washed in normal Tyrode's solution and then exposed to acetylglycinate Tyrode's solution in which the total calcium concentration was the same as that in the control Tyrode's solution. This procedure gave rather unsatisfactory results, because a second exposure to acetylglycinate often caused a change that was quantitatively different from that seen during the first exposure, even when the same solution was used both times. We can say with confidence that

the duration increased regardless of whether or not we kept the calcium ion activity constant in the acetylglycinate solution. However, we cannot say whether adjusting the total calcium concentration of the acetylglycinate solution affected the magnitude of the increase. This question was examined again during the experiments in which methylsulfate was used as a chloride substitute.

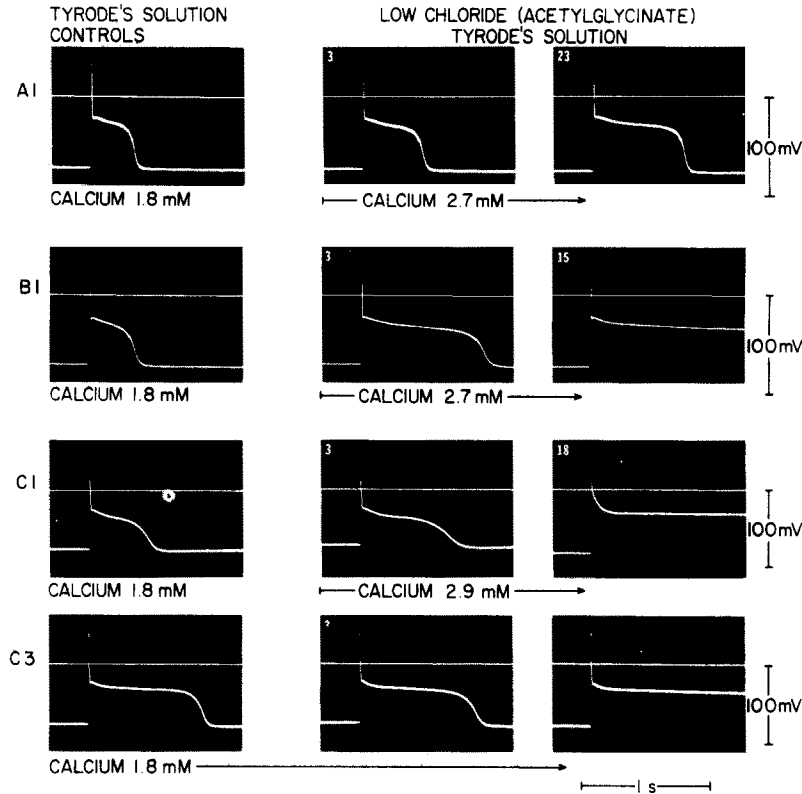


FIGURE 2. Action potentials in acetylglycinate Tyrode's solution. Action potential records are shown from three preparations. Labeling is as in Fig. 1 except that numbers in the upper left corner of each experimental record give the number of minutes in the low-chloride solution. The stimulation frequency was 0.067 s^{-1} for all records. Phase 1 of most action potentials was retouched.

At the sweep speed used in Fig. 2, we could see no change in the rate of phase 1 repolarization of 10 preparations as acetylglycinate replaced chloride. This was true whether or not the calcium ion activity was kept constant as the solution was changed. If there was a notch separating the spike from the plateau of the action potential, as there frequently was at the stimulation rate of 0.067 s^{-1} , the notch usually remained throughout the perfusion with acetylglycinate Tyrode's solution. The records labeled A1 and B1 in Fig. 2 show results from 2 of the 10 preparations that behaved in this way.

The behavior of the other three preparations was different. During the first

exposure to acetylglycinate Tyrode's solution, the rate of repolarization during phase 1 slowed substantially in these preparations, essentially as reported by Hiraoka and Hiraoka (1975). However, in the course of trying to determine the importance of maintaining constant calcium ion activity, each of these preparations was exposed three times to acetylglycinate Tyrode's solution. With each successive exposure, the effect of acetylglycinate solution on phase 1 was less. During the third exposure, we could no longer see any effect on phase 1 in two of these preparations; there was still a slight slowing of phase 1 of the third preparation during the third exposure to acetylglycinate Tyrode's solution. The results from the preparation that gave the most dramatic response to the first acetylglycinate exposure are shown in Fig. 2 (fiber C). These data were obtained at a time when only a rough estimate of the effect of acetylglycinate replacement on calcium ion activity was available, so 2.9 mM calcium was used in the acetylglycinate solution to be sure that the calcium ion activity did not fall. The preparation was exposed to this solution until a steady state was reached (18 min, see C1, Fig. 2). It was then washed for 27 min in normal Tyrode's solution and re-exposed to the same acetylglycinate solution. The second exposure (not illustrated in Fig. 2) caused a much smaller change in the rate of phase 1 repolarization. Acetylglycinate solution with a constant total calcium concentration was used for the third exposure to low chloride (C3, Figure 2), because the purpose of the experiment was to examine the effect of changing the calcium ion activity. There was no detectable change in phase 1 during the third exposure to chloride. Because there was a substantial difference between the first and the second exposures to the same solution, we believe that the disappearance of the effect on phase 1 was due either to elapsed time or to the repeated washing and re-exposure to acetylglycinate solution, and that the lack of any effect on phase 1 during the third exposure did not result from our having used a different total calcium concentration. Three attempts to confirm this conclusion by repeatedly exposing such a preparation to the same solution failed, because none of the preparations showed a change in phase 1 even during the first exposure to acetylglycinate Tyrode's solution.

For the purpose of reconsidering previous evidence about I_{qr} , the critical observation above is that low chloride solution did not appear to change the rate of phase 1 repolarization of most preparations. The sweep speed used in Fig. 2 should have allowed us to detect a slowing of phase 1 as dramatic as that reported by Dudel et al. (1967) and Hiraoka and Hiraoka (1975), but a small change might not have been apparent. We therefore recorded the upstroke and phase 1 at a high sweep speed in three of the preparations in which no change in phase 1 was apparent at normal sweep speeds. Fig. 3 shows control and steady-state records from two of these preparations. One set of records, labeled A1, is from the preparation labeled A in Fig. 2. In each of the three preparations, it was clear that acetylglycinate Tyrode's solution did not significantly alter the time course of phase 1 repolarization.

These results are substantially different from the results of low-chloride solutions reported by Dudel et al. (1967) and Hiraoka and Hiraoka (1975), and they do not confirm Carmeliet's (1961, Fig. 13) report that phase 1 is slower in acetylglycinate solution. However, these data do not necessarily conflict with

the other effects that Carmeliet noted. He stated that the plateau of the action potential was elevated in low-chloride (acetylglycinate or pyroglutamate) solutions, and that this effect was most marked at high stimulation rates. To see if we could demonstrate the same changes that Carmeliet saw at a higher rate of stimulation, we tested two of the above preparations at a stimulation rate of 0.67 s^{-1} . Control and steady-state records from these preparations are shown in Fig. 4. At this stimulation rate, the plateau of each preparation was slightly elevated in acetylglycinate solution. Fiber D of Fig. 4, in particular, qualitatively resembles Carmeliet's (1961, Fig. 12) illustration of the effect of acetylglycinate solution. Low-chloride solution caused phase 1 to end at a slightly less negative voltage, but as at the lower stimulation rate, phase 1 repolarization was not detectably slower than the control.

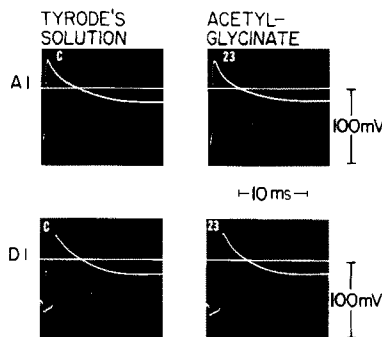


FIGURE 3. Fast sweep-speed recordings of phase 1 in acetylglycinate Tyrode's solution. The upstroke, all of phase 1, and the beginning of phase 2 are shown. Control records (those with C in the upper left corner) were taken just before the change to low chloride; steady-state records were each taken after 23 min in the experimental solution. The records labeled A are from the same experiment labeled A in Fig. 2. The stimulation frequency was 0.067 s^{-1} . The calcium concentration in the control solution was 1.8 mM, while that in the acetylglycinate solution was 2.7 mM.

Methylsulfate

The effects of replacing the sodium chloride of Tyrode's solution by sodium methylsulfate were examined in experiments on six preparations. The control Tyrode's solution always contained 1.8 mM calcium. Methylsulfate Tyrode's solution contained either 2.2 mM calcium (constant calcium ion activity) or 1.8 mM calcium (constant calcium concentration). Preparations were exposed to methylsulfate Tyrode's solution with constant calcium ion activity until the action potential stimulated at 0.067 s^{-1} no longer changed detectably with time. To determine the importance of maintaining a constant calcium ion activity in the experimental solution, several were then exposed to methylsulfate Tyrode's solution containing 1.8 mM calcium.

When the bathing solution was changed from Tyrode's solution to methylsulfate Tyrode's solution (2.2 mM calcium), action potentials obtained at 0.067 s^{-1} gradually lengthened. The action potential duration peaked after 2-4 min in

methylsulfate solution and then stayed constant or declined slowly to a steady state with additional incubation in methylsulfate Tyrode's solution. After 20–40 min in methylsulfate solution, the steady-state action potential duration was longer than the control in four preparations (up to 170% control), equal to the control in a fifth preparation, and shorter than the control in a sixth preparation. Fig. 5 shows representative results from three experiments.

In three experiments, after equilibration in methylsulfate Tyrode's solution containing 2.2 mM calcium, the solution bathing the muscle was changed to methylsulfate solution containing 1.8 mM calcium. In each experiment, the action potential became shorter as the calcium ion activity in methylsulfate Tyrode's solution decreased. The effect of changing the calcium ion activity in

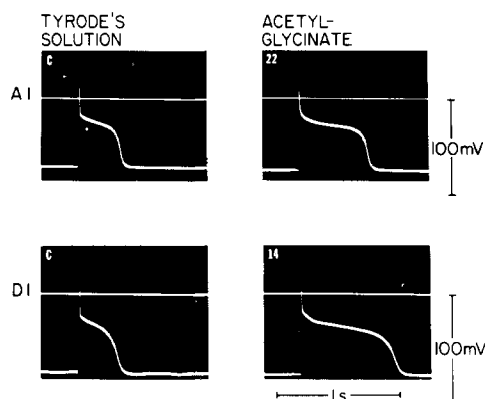


FIGURE 4. Effect of acetylglucinate at a higher stimulation rate. Records were obtained at a stimulation rate of 0.67 s^{-1} in normal and acetylglucinate Tyrode's solutions, from the same preparations shown in Fig. 3. The control records (those marked C) were taken shortly before the change to low-chloride solution; steady-state records were obtained during a period of stimulation at 0.67 s^{-1} 22 min (records A1) and 14 min (records D1) after the solution change. The calcium concentration in the control solution was 1.8 mM, and the concentration in the acetylglucinate solution was 2.7 mM. Phase 1 was retouched in all records.

the solution is shown for preparation B in Fig. 5. After 15–60 min in methylsulfate solution, action potential durations returned to values near the controls within 10–20 min after normal Tyrode's solution was readmitted to the chamber.

At the sweep speeds used in Fig. 5, we could see no slowing of phase 1 in any of the six preparations as sodium methylsulfate replaced sodium chloride. At a stimulation rate of 0.067 s^{-1} , there was a notch between the spike and the plateau of all six preparations, and the notch persisted in all preparations throughout exposure to methylsulfate solutions. Action potentials were recorded at a high sweep speed from two preparations to allow a more precise determination of the effect of chloride replacement on phase 1. Records from both experiments are shown in Fig. 6. In both experiments the maximum rate of repolarization, which occurred 1–2 ms after the peak of the action potential, was greater in the methylsulfate Tyrode's solution than it had been when

measured earlier in normal Tyrode's solution. The rate of repolarization measured 4 ms after the peak of the action potential, when I_{qr} would presumably have a greater effect on the rate of repolarization, was essentially the same as control values in both preparations after more than 20 min in methylsulfate solution. The increases in the maximum rate of repolarization may have been due to elapsed time, since the maximum repolarization rate increased slowly in

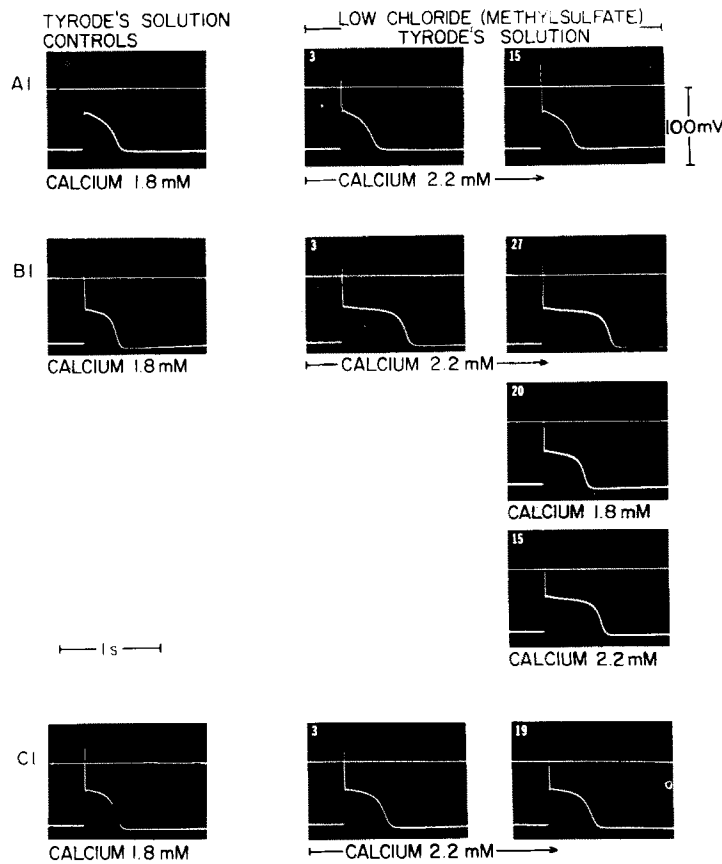


FIGURE 5. Action potentials in methylsulfate Tyrode's solution. The action potentials are from experiments on three preparations. The numbers in the upper left of the records indicate the number of minutes since the last solution change. In the case of the records from fibers A and C, the numbers give the time in low-chloride solution. For fiber B, the times on the records in the far right column indicate the following: for the record in 2.2 mM calcium, 27 min since the initial change to methylsulfate solution; for the record below it, 20 additional min in methylsulfate solution with a total calcium concentration of 1.8 mM; for the final record from fiber B; 15 min after returning to methylsulfate Tyrode's solution with a total calcium concentration of 2.2 mM (for a total of 62 min in low-chloride solution). The stimulation rate was 0.067 s^{-1} in these experiments and phase 1 of most action potentials was retouched.

the methylsulfate solution and the increased maximum rate persisted when the preparations were washed with normal Tyrode's solution.

Five of the above preparations were stimulated for brief periods at 0.67 s^{-1} . Results from three of these preparations are shown in Fig. 7. At the higher stimulation rate, we could see no consistent difference between action potentials recorded in methylsulfate solution and the control action potentials recorded in normal Tyrode's solution.

Methanesulfonate

The sodium chloride of Tyrode's solution was replaced by sodium methanesulfonate in experiments on seven preparations. All solutions used in this series of experiments contained 1.8 mM calcium. The duration of action potentials

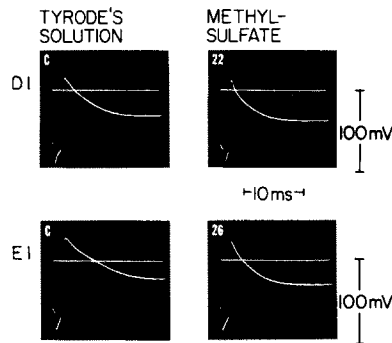


FIGURE 6. Fast sweep-speed recordings in methylsulfate Tyrode's solution. The upstroke of the action potential, all of phase 1, and the beginning of phase 2 are shown. Control (marked C) and steady-state (22 and 26 min) records are shown from experiments on two preparations (D and E) of the methylsulfate series. The stimulation frequency was 0.067 s^{-1} ; the calcium concentration was 1.8 mM in the control Tyrode's solution and 2.2 mM in the methylsulfate solution.

stimulated at 0.067 s^{-1} increased in all seven preparations when normal Tyrode's solution was replaced by methanesulfonate Tyrode's solution. In four preparations, the duration increased throughout the perfusion with methanesulfonate solution, although the rate of increase was quite slow after 15 min in methanesulfonate Tyrode's solution. Action potential durations of the other preparations peaked after 2–10 min in methanesulfonate solution, and then declined slowly to a steady state. In each of the experiments, the duration after 20–40 min in methanesulfonate solution was longer than the control duration, with the amount of the increase ranging from a few percent greater than control to about twice the duration of the control action potentials. Representative results from three preparations are shown in Fig. 8. Upon washing with normal Tyrode's solution, action potential durations returned to values close to the controls within 10 min.

Action potential records were taken at fast sweep speeds from five preparations. The maximum rate of repolarization, occurring shortly after the peak of the action potential, and the rate of repolarization 4 ms after the peak were

measured in normal Tyrode's solution and after equilibration in methanesulfonate solution. There was no consistent effect of chloride replacement on either measurement; rates increased slightly in some preparations and decreased slightly in others when chloride was replaced by methanesulfonate. For the group of five fibers, the measurements made in methanesulfonate solution were not significantly different from the control measurements ($P > 0.5$ by paired t -test). Steady-state control and experimental records from four fibers,

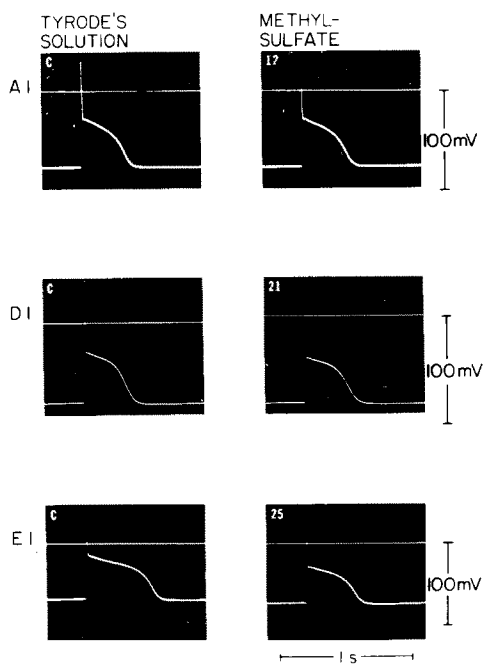


FIGURE 7. Effect of methylsulfate at a higher stimulation rate. The action potentials shown were obtained from three preparations during periods of stimulation at 0.67 s^{-1} before (records marked C) and 12, 21, or 25 min after the start of perfusion with methylsulfate Tyrode's solution. The calcium concentration was 1.8 mM in the control Tyrode's solution, and 2.2 mM in the methylsulfate solution. Phase 1 was retouched in all records. Other results from fibers A and D were also shown in Figs. 5 and 6, respectively.

showing all of phase 1 and the beginning of phase 2, are reproduced in Fig. 9.

All seven preparations were stimulated for brief periods at 0.67 s^{-1} in the course of these experiments. Action potentials recorded from three preparations at this stimulation rate are shown in Fig. 10. The results resembled those obtained at 0.067 s^{-1} , in that the only consistent effect of chloride replacement was that the action potentials were longer in the methanesulfonate solution.

Resting Potential

Detailed measurements of the resting membrane potential were made in acetylglucinate, methylsulfate, and methanesulfonate experiments in which

long-term drift was low (see Materials and Methods). The transmembrane voltage just before a stimulus, i.e. after nearly 15 s of inactivity, was taken as the resting potential. When the sodium salt of acetylglycinate, methylsulfate, or methanesulfonate was substituted for NaCl, there was a transient 3–6 mV depolarization. Peak depolarization occurred 15–60 seconds after the change of solution, and the resting potential then returned to the control level, usually within 3 min. A transient hyperpolarization with a similar magnitude and time course occurred when the control Tyrode's solution was readmitted to the

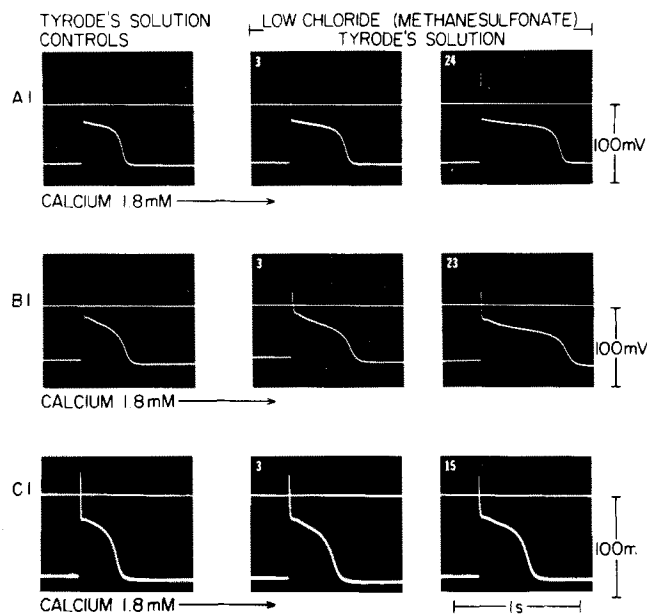


FIGURE 8. Action potentials in methanesulfonate Tyrode's solution. Action potentials from three preparations are shown. The small numbers in the upper left of the records again indicate time in low-chloride solution. The stimulation rate was 0.067 s^{-1} for all action potentials. Phase 1 was retouched.

chamber. These changes were apparent even in the preparations where long-term drift was greater than 3 mV.

After the transient depolarization in the above low-chloride solutions, the resting potential of most preparations stayed steady at a level very close to the control voltage. If any slow changes occurred, they were smaller than 3 mV, and therefore could not reliably be distinguished from slow drifts in the recording system.

Carmeliet (1961) noted that low-chloride solutions sometimes caused arrest at the plateau voltage. This apparently was a much more serious problem for Dudel et al. (1967). In our experiments, the only preparations that came to a standstill on the plateau were some in which the calcium ion activity was allowed to decrease as another anion replaced chloride (see B1, Fig. 1).

DISCUSSION

Electrical Effects of Reducing Chloride

The rationale behind replacing chloride by larger anions is that the membrane will be less permeable to the larger anions. Propionate, methylsulfate, and methanesulfonate have very low conductances in frog skeletal muscle (Woodbury and Miles, 1973), and Anderson and Foulks (1973) concluded that methyl-

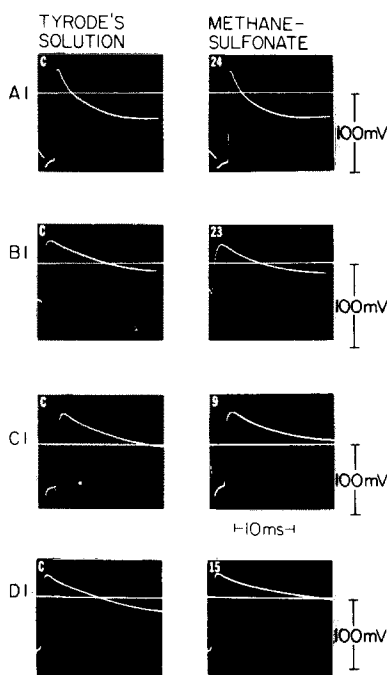


FIGURE 9. Fast sweep-speed recordings in methanesulfonate Tyrode's solution. The upstroke, all of phase 1, and the beginning of phase 2 are shown for four fibers. Action potentials of three of these fibers (A-C) were shown at a normal sweep speed in Fig. 8. Control records are marked C; steady-state records were obtained after the specified number of minutes in low-chloride solution. The stimulation rate was 0.067 s^{-1} , and the calcium concentration was 1.8 mM, for all records.

sulfate is an impermeant anion for frog ventricle. There is no direct evidence showing that these anions do not enter Purkinje fibers, but it seems reasonable to assume, as others have, that the anions we used are much less permeant than chloride. Currents carried by chloride should therefore be reduced when one of these larger anions is substituted for chloride, and alterations in the action potential may make it possible to deduce the normal role of chloride as a charge carrier. In practice, this may not be easy to do, even if one assumes that the larger anions are completely impermeant and that the only direct effect of chloride reduction is on chloride currents. There is substantial variability in the action potentials of Purkinje fibers, but perhaps more important, the voltage is not controlled and the voltage changes that occur when chloride is removed

may induce secondary effects in nonchloride currents. However, taking into account some of the most consistent voltage clamp observations, we think the action potential data reported here raise serious questions about the ionic basis of I_{qr} .

We have no doubt that a transient outward current (I_{qr}) appears during voltage clamp depolarizations. This was seen in the earliest voltage clamp records (Deck et al., 1964) and the presence of this current has been confirmed

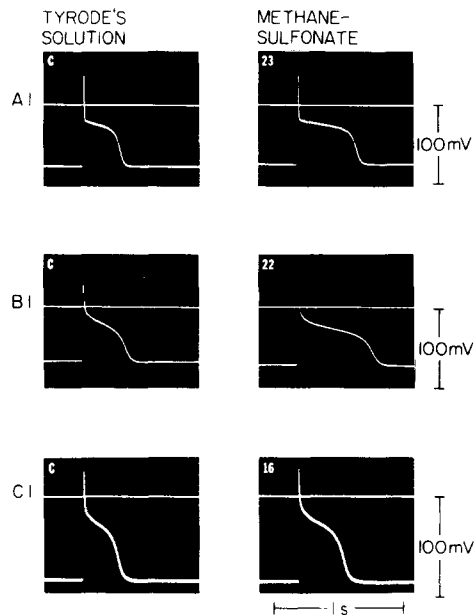


FIGURE 10. Effect of methanesulfonate at a higher stimulation rate. The action potentials shown were obtained during periods of 0.67 s^{-1} stimulation before (records marked C) and during perfusion with methanesulfonate Tyrode's solution. The records are from the same preparations labeled A-C in Figs. 8 and 9. The calcium concentration was 1.8 mM throughout all experiments in methanesulfonate. Phase 1 of the action potentials was retouched.

many times. Such an outward current could account for the initial rapid phase 1 repolarization after the spike of the Purkinje fiber action potential, as Dudel et al. (1967) suggested, and it could, along with other currents, cause the notch that often appears between the spike and the plateau (McAllister et al., 1975). McAllister et al. had to make many assumptions about early currents in their efforts to reconstruct the Purkinje fiber action potential, but their work represents the best estimate of what should happen if I_{qr} is substantially reduced. There should be a dramatic slowing of phase 1 and the notch should disappear (McAllister et al., 1975, Fig. 6).

Discounting our results with propionate as not allowing any meaningful interpretation, we are left with experiments on 26 Purkinje fibers using acetylglycinate, methylsulfate, and methanesulfonate as substitutes for chloride. In the majority of these experiments no slowing of phase 1 was apparent. Only

in three experiments (in acetylglycinate) did we see anything similar to the slowing of phase 1 reported by Dudel et al. (1967, Fig. 8), and in two of these fibers the effect did not persist through repeated exposures to the low-chloride solution. Furthermore, when there was a conspicuous notch between the spike and the plateau of the action potential in normal solution, it was usually present in low-chloride solutions as well. Thus, while most of the action potentials recorded in 8% chloride were longer than normal, they otherwise resembled normal action potentials.

The lack of a change in phase 1 repolarization in low-chloride solutions suggests that, under our experimental conditions at least, phase 1 repolarization is not caused by a time-dependent chloride current. This is a deliberately cautious statement because there are, as we mentioned above, limitations on what one can conclude about ionic currents from action potential experiments. In particular, we would emphasize that there may be a time-dependent chloride current in Purkinje fibers—our experiments do not rule out that possibility—and it could contribute to the transient outward current (I_{qr}) seen in voltage clamp experiments without having the proper voltage or time dependence to affect the action potential. But if there is such a current, its contribution to I_{qr} must be fairly small. If we are correct, the ionic basis of most of the transient outward current is still unknown.

From the earliest experiments on chloride reduction, Carmeliet (1961) and Hutter and Noble (1961) concluded that there is a “background” (i.e. time-independent) chloride conductance in Purkinje fibers. The changes that we saw are consistent with this conclusion. With such a background conductance, there should be a transient outward movement of chloride upon changing to a low chloride solution, and a transient inward movement on returning to normal solution. As Hutter and Noble (1961) pointed out, these chloride movements could cause brief depolarizations and hyperpolarizations like the resting potential changes reported here. A time-independent chloride conductance should also contribute to repolarization (Hutter and Noble, 1961), and replacing the external chloride with an impermeant anion might well lengthen the action potential.

Does Chloride Reduction Cause Nonspecific Conductance Changes?

In the Introduction, we cited Trautwein's (1973, p. 801) conclusion that chloride reduction alters the membrane and changes the conductances of several ions. We believe our experiments allow us to assess whether this is cause for serious concern. Except for their increased durations, action potentials recorded in methanesulfate or methanesulfonate Tyrode's solution closely resembled the control recordings. Because the configuration of the action potential changed little in these solutions, which contained only 8% of the normal chloride concentration, there is little reason to argue that serious nonspecific changes attended the chloride reduction. This would also be true of most of the experiments in which acetylglycinate replaced chloride.

Although we think it unlikely that chloride reduction, per se, causes nonspecific conductance changes, there are a variety of reasons for which nonspecific changes might occur in individual experiments or with particular substitutes.

We alluded to some of these problem areas in the Introduction; the results of the experiments give some clues as to how important they may be as sources of error. The most obvious problem is that the level of ionized calcium may change as other anions are substituted for chloride. The calcium ion activity did not seem to make a qualitative difference in our experiments, but we deliberately chose to examine substitutes that had relatively small effects on calcium ion activity. This question will be discussed in more detail when we compare our results to those of others.

Junction potential changes or, more properly, changes in the tip potentials of electrodes, certainly were a problem that required more than casual attention. If we had not used a flowing KCl-calomel electrode as an indifferent electrode, we would have concluded that low-chloride solutions lead to a 3–7 mV depolarization of the resting membrane potential. In a voltage clamp experiment, the feedback circuit would act to compensate for the apparent depolarization, passing sufficient hyperpolarizing current to keep constant the recorded voltage. The error could have serious consequences because one could easily be led to compare a current record obtained at one voltage in normal chloride with one obtained at a more negative voltage in low-chloride solution.

Finally, we pointed out that replacing NaCl by the salts of weak acids could also cause nonspecific conductance changes because, as Woodbury and Miles (1973) pointed out, many weak acids can cross membranes in the undissociated form. They may then dissociate inside cells and titrate cell buffers in the acid direction. The pK of propionic acid is 4.87 (Kortüm et al., 1961), so at a pH of 7.2 approximately 0.5% of the total propionate would be present as propionic acid. Acetylglycinate is better in this respect with a pK of 3.67 (Kortüm et al., 1961), which would correspond to about 0.03% undissociated acid at pH 7.2. With a pK of -1.2 (Albert and Serjeant, 1971), methanesulfonic acid should be completely dissociated at physiological pH. The pK of methylsulfate is not available in the literature, but a very crude calculation based on the pH of a water solution of the City Chemical Corp. sodium methylsulfate would place the pK between those of acetylglycinate and methanesulfonate.

Roos (1975) found that substantial amounts of lactic acid (pH 3.7) entered rat diaphragm. Acetylglycinate may therefore be on the borderline of acceptability, if one wishes to minimize the possibility that significant amounts of undissociated acid enter the cell. Conceivably, fiber-to-fiber variability in the response to propionate and the lesser degree of variability that we found with acetylglycinate could be related to the ability of different fibers to maintain a reasonably constant intracellular pH when faced with a proton load. This is speculation, of course, but in the absence of better criteria for selecting chloride substitutes, it seems reasonable to prefer the salts of strong acids.

Comparison with Previous Work

Our results are substantially different from those of Dudel et al. (1967, Fig. 8), for these workers obtained a very marked slowing of phase 1 repolarization when 75% of the chloride in Tyrode's solution was replaced by maleate. In the Appendix we indicate that maleate probably was an unfortunate choice as an anion to replace chloride because of its effects on calcium ion activity and ionic

strength. Since we did not test maleate, we cannot be certain that these effects would explain the different results, but it is probable that the calcium ion activity in the maleate and gluconate solutions used by Dudel et al. was substantially less than that in the control solution. In our experiments, the small change in calcium ion activity that occurred when methylsulfate was substituted for chloride without calcium adjustment clearly affected the action potential duration, so it is possible that the much larger change in calcium ion activity caused by maleate or gluconate could have serious effects. In this regard, it may be significant that Carmeliet (1961) and Dudel et al. (1967) found that preparations tended to arrest at the plateau voltage in low-chloride solutions. When we kept the calcium ion activity constant, that arrest never occurred. Further evidence of the importance of maintaining a constant calcium ion activity comes from the work of Peper and Trautwein (1968). They found that the positive dynamic current (I_{qr}) was substantially smaller in calcium-free solution.

Our data do not conflict with the results obtained by Hutter and Noble (1961), nor do they conflict with many of Carmeliet's (1961) results. Carmeliet reported that chloride reduction elevated the plateau of the action potential and made its duration longer, and he stated that the plateau elevation was more marked at higher frequencies. We found this several times at a frequency of 0.67 s^{-1} , and it might have been a more consistent observation if we had tested still higher rates. Given that I_{qr} recovers from inactivation relatively slowly (Hauswirth et al., 1972; Fozzard and Hiraoka, 1973), stimulation at a very slow rate (e.g., 0.067 s^{-1}) should provide the most sensitive test of whether I_{qr} is reduced by low-chloride solutions, while changes in the early part of the plateau caused by reduced background chloride current might be seen more readily at higher rates of stimulation. While most of our results seem compatible with Carmeliet's observations and conclusions, we could not confirm his report that phase 1 is slower in low-chloride solutions (1961, Fig. 13).

Our results also agree with a comment made by Noble and Tsien (1969). Although they did not show any records, they mentioned that action potentials with notches could be recorded in Tyrode's solution in which all but 5 mM of the chloride had been replaced by methylsulfate. Under voltage clamp conditions, they noted that a sizable transient outward current could be seen in low-chloride solution.

The most recent report of the effects of chloride reduction on the Purkinje fiber action potential was by Hiraoka and Hiraoka (1975). Using acetylglycinate and propionate as chloride substitutes, they found substantial reductions in the rate of phase 1 repolarization in low chloride. They used propionate more often than acetylglycinate, and propionate was used in each of their published experiments (Masayasu Hiraoka, personal communication). Our admittedly limited experience with propionate led us to conclude that it gave less reliable – or at least less consistent – results than the other anions. However, Hiraoka and Hiraoka obtained similar results with acetylglycinate, so there is a genuine difference between their results and ours that needs to be resolved. We can offer no explanation for the difference, except to reiterate our concern about

the effects of reduced calcium ion activity and the possibility that it may be a mistake to use the salts of weak acids.

If chloride movement is not the principal source of I_{qr} , it is appropriate to ask what ion or ions could be responsible. From the total current record in a voltage clamp experiment, one cannot distinguish between inward movement of negative ions and outward movement of positive ions. Outward potassium movement is therefore a logical candidate as the source of most of I_{qr} . This is only a suggestion on our part, of course, for our experiments do not provide any evidence as to what other ions might be involved. However, Haldimann (1963) stimulated sheep Purkinje fibers at 0.5 s^{-1} in Tyrode's solution similar to ours, and obtained similar control action potentials. The addition of 20 mM tetraethylammonium chloride dramatically slowed phase 1 repolarization and abolished the notch between the spike and the plateau of the action potential. Since tetraethylammonium ions reduce outward potassium currents in several excitable tissues (see, for example, Hille, 1967), Haldimann's observation would be consistent with the suggestion that I_{qr} is a potassium current. Voltage clamp analysis may help settle this question. If I_{qr} is largely the result of potassium movement, it will be particularly interesting to see what the relation is between I_{qr} and other plateau currents that are thought to be carried, wholly or in part, by potassium.

APPENDIX

Calcium Ion Activity In Low-Chloride Tyrode's Solutions

In analyses of chloride permeability, other anions often are substituted for chloride in physiological solutions. The amount of foreign anion introduced is usually large relative to the calcium concentration, and some anions might substantially reduce the calcium ion activity of solutions. Because membrane conductance systems are sensitive to calcium ion activity, this could be a serious source of error in studies of the electrical effects of chloride reduction (Boistel and Fatt, 1958; Hutter and Noble, 1960; Christoffersen and Sibsted, 1975).

Principles and Technique

We used Orion 92-20 and 93-20 calcium-selective electrodes (Orion Research, Inc., Cambridge, Mass.) to compare the calcium ion activity of low-chloride Tyrode's solutions to that of normal Tyrode's solutions. A flowing KCl-calomel reference electrode (Fisher 13-639-52, Fisher Scientific Co., Pittsburgh, Pa.) was used with the 92-20 electrode; an Orion 90-01 single junction reference electrode was used with the 93-20 electrode. The application of ion-selective electrodes to problems of this type is not yet routine, so it is necessary to specify the assumptions we made and the errors that might arise.

For an ideal Nernst electrode, the voltage difference between the ion-specific electrode and a suitable reference electrode is given by:

$$E = E_0 + \frac{2.3 RT}{z_1 F} \log \{i\}, \quad (1)$$

where R , T , and F have the usual meanings with $2.3 (RT/F)$ equal to 59 mV at 25°C , $\{i\}$ is the activity of the ion i to which the electrode responds, z_1 is the charge of the ion, and E_0 is a standard potential that depends in part on the choice of reference electrode.

For our purposes, it is more convenient to write Eq. (1) in the following form:

$$E = E_0 + S \log \{i\}, \quad (2)$$

where S replaces $2.3 (RT/z_iF)$. If ion i is a bivalent cation, the theoretical value of the "slope factor" S is 29.5 mV at 25°C.

E_0 in Eq. (1) and (2) depends on the choice of reference electrodes, as noted; it may also change slightly over long periods of time. We avoided these concerns by arranging experiments so that the parameters of interest were voltage differences obtained during tests of standards and unknowns, all of which were tested over a 1- or 2-h period. If E_u and E_s represent the voltages in an unknown and a standard solution, obtained with an electrode that behaves according to Eq. (2), then the calcium ion activity in the unknown solution, relative to that in the standard, is given by:

$$\frac{\{Ca^{++}\}_u}{\{Ca^{++}\}_s} = 10^{[(E_u - E_s)/S]}. \quad (3)$$

We used Eq. (3) to compare the calcium ion activity in substituted Tyrode's solution to that in normal Tyrode's solution. Tyrode's solution is not a standard solution in the usual sense, because the activity of calcium in Tyrode's solution is also unknown. Our approach therefore gives only the ratio of calcium ion activities. The derivation of additional information from the data would have required further assumptions, without adding materially to our conclusions.

Voltages were read to ± 0.2 mV, with a Beckman model 76A pH meter (Beckman Instruments, Inc., Fullerton, Calif.), or to ± 0.1 mV with an Orion model 801A millivolt-pH meter (Orion Research). For a bivalent ion electrode, a precision of ± 0.2 mV corresponds to approximately $\pm 1.6\%$ precision in determining ion activity.

Certain nonrandom errors are possible in these experiments. With either of the reference electrodes used, the liquid junction potential at the reference electrode is likely to change as one changes from Tyrode's solution to a solution containing one of the chloride substitutes. Using the Henderson equation (Bates, 1964, p. 40) and the value of the limiting ionic conductance for propionate given by Frankenthal (1963), we estimated that the potential at the junction between 4 M KCl and the test solution should increase approximately 1 mV as we changed from a Tyrode's solution containing 137 mM NaCl to one containing 137 mM sodium propionate. A 1-mV increase in the liquid junction potential on changing to the low-chloride solution would result in a ratio calculated by Eq. (3) that is $\sim 8\%$ lower than the true ratio. We were unable to find values for the limiting ionic conductances of the other ions tested and there is some uncertainty in the liquid junction potential calculation, so the data are presented without correction for changes in liquid junction potential.

Some error will also arise because the Orion electrode is not perfectly selective for calcium ion. Ross (1967) gave an empirical equation for the response of an ion-selective electrode in the presence of interfering ions. We used this equation and the selectivity constants that the manufacturer gives for the electrode to estimate the interference from other ions in Tyrode's solution. Only the Mg^{++} and Na^+ should produce significant interference. At the lowest calcium concentration tested (0.9 mM), the total error caused by these ions should be approximately +0.9 mV. The error decreases as the calcium concentration is increased and at 5.4 mM calcium the combined error should be about +0.3 mV. The effect of the competing ions, then, is to reduce slightly the slope of the curve relating electrode voltage to the logarithm of calcium ion activity. We followed the usual practice of using the experimental slope, rather than the theoretical slope, in calculations using Eq. (3). Where there were two complete sets of data in normal-chloride Tyrode's solution and in low-chloride Tyrode's solution, we fitted the two sets individ-

usually and used the mean of the two slopes for calculations. The two slopes were usually within 1–2 mV of each other. An uncertainty of 1 mV in determining S corresponds to an uncertainty of 0.015 in determining ratios by Eq. (3).

Solutions

In addition to CaCl_2 , Tyrode's solution contained (mM): NaCl, 137; KCl, 5.4; MgCl_2 , 1.05; NaHCO_3 , 13.5; NaH_2PO_4 , 2.4; dextrose, 11.1. Tyrode's solution without added calcium was prepared from stock solutions of the various salts, and Tyrode's solution with 5.4 mM CaCl_2 was prepared from the same stocks and a CaCl_2 stock solution made from freshly opened $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The 0 and 5.4 mM calcium solutions were then mixed to prepare Tyrode's solutions with calcium concentrations between 0.9 and 5.4 mM. Reagent grade chemicals (Mallinckrodt Chemical Co., St. Louis, Mo., or Baker Chemical Co., Phillisburg, Pa.) and glass-redistilled water were used.

Low-chloride solutions were prepared by replacing the 137 mM NaCl of normal Tyrode's solution with one of the following: 137 mM sodium acetylglycinate (prepared by NaOH neutralization of acetylglycine from Sigma Chemical Co., St. Louis, Mo.); 137 mM sodium gluconate (obtained from ICN-K&K, Plainfield, N. J.); 137 mM sodium methanesulfonate (prepared by NaOH neutralization of methanesulfonic acid from Eastman Organic Chemicals, Rochester, N. Y.); 137 mM sodium methylsulfate ("electronic grade" from City Chemical Corp., New York).

A note of warning may help others who wish to use methylsulfate. According to Mutschler and Lindmar (Appendix to Lüllman, 1961), sodium methylsulfate produces a clear, neutral solution. Stock solutions (274 mM) of the electronic grade chemical from City Chemical Corp. were clear, with a pH of 7.9–8.0. However, five of six lots from other suppliers (Eastman Organic Chemicals and ICN-K&K) gave solutions that were strongly acid or contained substantial amounts of undissolved material, or both. The sixth lot of chemical (from ICN-K&K) contained a significant amount of contaminant calcium. Some of the variability might be related to how the chemical was stored by suppliers; City Chemical advised us that it should be refrigerated to ensure stability.

Normal and low-chloride Tyrode's solutions were saturated with 95% O_2 -5% CO_2 at room temperature (21–24°C) before calcium determinations. The pH of solutions was spot checked, usually with 2.7 mM calcium solutions. At room temperature, pH was 6.9–7.2. To estimate the overall effect of temperature, some experiments were done with the solutions warmed to 36–38°C.

Results and Conclusions

The data comparing ionized calcium in Tyrode's solution containing chloride substitutes to that in normal Tyrode's solutions are summarized in Table I. Gluconate and maleate

TABLE I
RATIOS OF CALCIUM ION ACTIVITY IN TYRODE'S SOLUTION
CONTAINING CHLORIDE SUBSTITUTES TO THE CALCIUM ION ACTIVITY
IN NORMAL TYRODE'S SOLUTION

Chloride substitute	Added calcium (mM)						n
	0.9	1.8	2.7	3.6	4.5	5.4	
Gluconate	0.24 ± 0.02	0.20 ± 0.03	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	3
Maleate	0.32 ± 0.04	0.27 ± 0.01	0.26 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.04	3
Propionate	0.65 ± 0.02	0.63 ± 0.02	0.65 ± 0.04	0.64 ± 0.06	0.68 ± 0.10	0.69 ± 0.17	3
Acetylglycinate	0.66 ± 0.04	0.63 ± 0.04	0.67 ± 0.02	0.66 ± 0.03	0.68 ± 0.03	0.66 ± 0.04	3
Methylsulfate	0.83 ± 0.05	0.82 ± 0.03	0.84 ± 0.03	0.83 ± 0.04	0.81 ± 0.04	0.80 ± 0.03	3
Methanesulfonate	0.88 ± 0.10	0.87 ± 0.09	0.86 ± 0.01	0.88 ± 0.06	0.92 ± 0.10	0.91 ± 0.10	5

Mean values ± SD for tests of n solutions at each added calcium concentration.

drastically reduced calcium ion activity, while acetylglycinate and propionate had smaller, but still substantial, effects. Methylsulfate caused only a modest decrease in calcium ion activity. These general statements would hold regardless of whether the data were corrected for junction potential changes as indicated above. Methanesulfonate, on the other hand, had only a small effect on the ratio computed by Eq. (3). If a residual junction potential correction were applied to the data for this anion, it would probably be reasonable to conclude that substitution of methanesulfonate for chloride had no significant effect on calcium ion activity.

In a limited series of experiments, voltage measurements were made in normal and low-chloride Tyrode's solutions warmed to 36–38°C. Because of the problem of keeping large numbers of solutions warm, the normal and low-chloride solutions were compared only at a total calcium of 2.7 mM. Methylsulfate, propionate, acetylglycinate, maleate, and gluconate were tested in this way. At the higher temperature, voltage readings were 3.5–5 mV higher than measurements made of the same solutions at room temperature. However, at a total calcium of 2.7 mM, the difference between the voltages obtained in substituted and normal Tyrode's solution ($[E_u - E_s]$ in Eq. [3]) did not appear to change because of the increased temperature. The experiments at the higher temperature were not repeated enough times to allow statistical comparisons of the data but, for the substitutes tested at 36–38°C, the voltage differences were within the range of values obtained at room temperature and the ratios of calcium ion activities were comparable to those reported in Table I. A more definitive determination of the effect of temperature would have required thermostating and jacketing of the entire test setup (Moore, 1969). For the degree of accuracy necessary for most biological experiments, it seemed reasonable to conclude that the ratios reported in Table I would change little at 36–38°C.

Of the anions we tested, gluconate and maleate would seem to be very poor choices as chloride substitutes because of their drastic effects on calcium activity. There is an additional problem when maleate is used: because it is bivalent, either the sodium concentration or the ionic strength must be changed if it is to be used as a chloride substitute.

If the effect of an anion on calcium ion activity were the only important consideration in chloride replacement studies, methanesulfonate would be a better choice than the other anions we tested. It would seem reasonable to use this anion without calcium adjustment. Others may be used, however, providing the total calcium concentration is raised so that the calcium ion activity is kept constant. Our data (Table I) or those of Christoffersen and Skibsted (1975) should allow others to make approximate corrections, but because the chemicals used are not manufactured to established standards of purity it would be preferable to test individual chemical lots.

We are indebted to Mr. Hugh Larsen for expert technical assistance, to Mr. Thomas Witt of the Given Analytical Facility, University of Vermont, for measuring the calcium contamination of methylsulfate samples, and to Dr. Robert Woodworth of the Biochemistry Department for lending the 92-20 electrode and for several helpful discussions in the course of the work. We would also like to thank Dr. James W. Ross and the staff of Orion Research Inc., Cambridge, Mass., for their technical advice and their unfailing practice.

This work was supported by United States Public Health Service grant HL 14614. James L. Kenyon was supported by United States Public Health Service Training Grant NIH TO 1 GM 00439.

Received for publication 8 February 1977.

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