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THE EFFECTS OF LYOTROPIC ANIONS ON CHARGE MOVEMENT, CALCIUM CURRENTS AND CALCIUM SIGNALS IN FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Intramembrane charge movement and Ca^{2+} currents were monitored in voltage-clamped segments of frog skeletal muscle fibres using the triple-Vaseline-gap technique. Calcium signals were measured in current-clamped fibres using either of the indicators Arsenazo III or Antipyrylazo III.

2. Non-linear capacitative currents (charge 1) were obtained using a subtraction procedure which employed either a −20 mV control pulse from a holding potential of −100 mV or alternatively a control pulse to +80 mV in depolarized fibres. The amount of charge mobilized depended on voltage according to a two-state Boltzmann function. The total charge (Q_{max}) was increased by *ca* 100% and the steepness parameter (k) by *ca* 70% when a +80 mV control pulse was used.

3. Thiocyanate (SCN^-) and other lyotropic anions reversibly shifted the voltage dependence of mobilized charge towards negative potentials. Q_{max} was not significantly affected. 'Off' charge tails were greatly prolonged by lyotropic anions.

4. Extracellularly applied lyotropic anions affected the dihydropyridine-sensitive Ca^{2+} current by shifting the I–V relation toward more negative voltages and delaying deactivation of the tail currents.

5. The effects of lyotropic anions did not depend on whether the anion was introduced intracellularly or extracellularly.

6. Extracellular SCN^- reversibly increased the peak amplitude and rate of rise of Ca signals, and decreased the latent period between stimulation and onset of the Ca signal.

7. It is concluded that lyotropic anions have similar effects on Ca^{2+} currents and on charge movement.

INTRODUCTION

Intramembrane charge movement has been related to the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in skeletal muscle (Schneider & Chandler, 1973; Almers,

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1978; Schneider, Ríos & Kovacs, 1981; Huang, 1988). Although the nature of the molecules that compose the 'charge' is unknown, dihydropyridine (DHP) receptors have been proposed to play a key role in the generation of charge movement (Ríos & Brum, 1987). If charge movement and DHP receptors are indeed so closely associated, it should be expected that factors such as extracellular ionic composition which affect DHP-sensitive Ca^{2+} currents would have similar effects on charge movement.

The mechanical activity of frog skeletal muscle fibres is greatly influenced by the presence of certain ions in the extracellular medium; for example, the replacement of chloride by thiocyanate (SCN^-) or other lyotropic anions brings about a marked potentiation of the amplitude of twitch tension and of the potassium contracture (Kahn & Sandow, 1955; Hodgkin & Horowicz, 1960). The present experiments examine the action of lyotropic anions on charge 1 (Chandler, Rakowski & Schneider, 1976) and on DHP-sensitive Ca^{2+} currents (I_{Ca}) (Sanchez & Stefani, 1978; Almers & Palade, 1981) in frog muscle. Consistent with previously reported effects on contraction, we found that SCN^- and other lyotropic anions facilitate the activation of charge movement. Similar effects were also observed on I_{Ca}, and intracellular calcium signals were substantially potentiated by SCN^-.

Preliminary results on the actions of lyotropic anions on charge movement, I_{Ca} and Ca signals have been published (Garcia & Sanchez, 1988; Garcia, Delay & Sanchez, 1989).

METHODS

The experiments were performed on short segments of twitch skeletal muscle fibres from the semitendinosus muscle of adult specimens of *Rana pipiens* or *Rana montezuma* for electrical measurements, and of *Rana catesbeiana* for optical experiments. Frogs were fed daily and kept at room temperature and were killed by decapitation. Charge movement experiments were conducted at 16–18 °C and experiments on I_{Ca} and Ca signals at 20–22 °C.

Voltage-clamp experiments

The triple-Vaseline-gap voltage clamp technique developed by Hille & Campbell (1976) was used with minor modifications as described elsewhere (Arreola, Calvo, Garcia & Sanchez, 1987). Fibres were dissected in a high K^+ depolarizing solution (solution A, Table 1) and laid in a chamber across three Vaseline seals, which divided the chamber into four pools. The cut ends of the fibre lay in the end pools, which contained an internal solution. The segment of the fibre lying in one particular inner pool could be voltage- or current-clamped; change of external solution for this segment was ensured by steadily flowing new solution into that pool while removing the excess, effectively exchanging the pool volume at least 50 times. Following changes to the internal solution in the end pools, a 60 min period was allowed for diffusion to the fibre segment which was to be clamped.

The generation of command signals to the clamp, data acquisition and data display were performed by a microcomputer with an interface for digitizing signals to 12-bit resolution at 250 kHz. Unless otherwise indicated, membrane current (I_m) was sampled at 4 kHz in charge movement experiments and at 600 Hz in I_{Ca} experiments. Each data record contained either 1000 or 5000 data points. Before the digitizing step, I_m was amplified and filtered with an active four-pole low-pass Bessel filter having a corner frequency of no more than half the sampling frequency.

Measurement of charge movement

Extracellular and intracellular solutions for charge movement experiments are shown in Table 1 (solutions C and E respectively). Movement of charge 1 was elicited by a 100 ms test pulse to a selected depolarizing potential. In order to measure non-linear charge movement, linear membrane currents must be subtracted from the unprocessed data record. In initial experiments we used a

P/4 procedure from a holding potential (E_h) of −100 mV; however, since Brum & Ríos (1987) have questioned the use of holding potentials in that range, and have suggested instead the use of E_h = 0 mV, we measured charge movement using one of two control pulses: either a −20 mV pulse from E_h = −100 mV, or a +80 mV pulse from E_h = 0 mV after clamping the fibre at this potential for 5 min. The fibre was allowed a 5 min period at E_h = −100 mV to recover from the depolarized

TABLE 1. Composition of solutions (mM)

External solutions, pH = 7·2 (MOPS)

	Na^+	K^+	TEA^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	$CH_3SO_3^-$	Cl^-
A	—	137·5	—	10·0	—	—	157·5	—
B*	—	—	108	10·0	—	—	128	—
C*	—	—	136	—	10	78	—	—
D	117	2·5	—	2·0	—	—	—	119·5

Internal solutions, pH = 7·1 (MOPS)

	Na^+	K^+	TEA^+	Cs^+	EGTA	Mg^{2+}	Cl^-	Glutamate	Aspartate	ATP
E	—	—	—	129	20	2	—	89	—	2
F	4	—	154	—	77	—	—	—	—	2
G	—	129·5	—	—	1	2	4	—	121·5	2

* Solutions B and C also contained tetrodotoxin (10^{-7} M)

state. The series of experimental test pulses was then begun. Averages of at least three consecutive records were taken at the same potential. Linear membrane charge components were subtracted off-line after appropriate scaling of control records. If necessary, a sloping base line substraction procedure was applied to experimental records as described by Brum & Ríos (1987). Linear capacitance was calculated by applying a +10 mV pulse from E_h = −100 mV. No attempts were made to separate charge 1 into components.

Measurement of Ca^{2+} currents

Calcium currents (I_{Ca}) were measured with a Ca^{2+}-containing solution (solution B) in the A pool and solution E in the end pools (Table 1). I_{Ca} was elicited following application of a 1 s depolarizing test pulse. Linear components were subtracted using a hyperpolarizing control pulse only. One minute was allowed between pulses in order to permit recovery of I_{Ca}.

Solutions used in electrical measurements

The solutions employed for measurements of charge movement and I_{Ca} were composed as shown (Hille & Campbell, 1976) in Table 1 (solutions A, B, C, E and F). They contained impermeant ions, except in I_{Ca} measurements, where Ca^{2+} ions were present (solution B). Tetrodotoxin (TTX) was used to block sodium channels. Movement artifacts were avoided in these measurements of electrical characteristics by use of a high concentration of intracellular EGTA. Extracellular and intracellular solutions were buffered with 3-*N*-morpholino propanesulphonic acid (MOPS, 4 mM) at pH 7·2 and 7·1 respectively. Lyotropic anions in test solutions were added by isosmotic replacement of the major anion in the extracellular or intracellular solutions. Unless otherwise stated, the concentration of SCN^- used was 12 mM. All chemicals were obtained from either Sigma Chemical Co. or Aldrich Chemical Co.

Measurement of Ca signals

The description of the general methods for to the optical measurement of Ca signals has been published (see Delay, Ribalet & Vergara, 1986). The fibre segments were current-clamped and stimulated to generate an action potential. The compositions of the solutions used are given in Table 1 (solutions D and G). Addition of SCN^- was made to the extracellular solution only, as described above. Two optical absorbance Ca^{2+} indicator dyes were used in these experiments: either 1 mM-Arsenazo III (ArIII) or 2 mM-Antipyrylazo III (ApIII) was added to the intracellular solution. Calcium signals were digitized at 16·6 kHz.

Averages of data values which appear in text or tables are expressed as means ± standard error of the mean. The fitting of numerical formulae to experimental data employed a non-linear least-squares algorithm.

RESULTS

Intramembrane charge movement

Non-linear membrane currents can be recorded in skeletal muscle in the absence of permeant ions after subtraction of linear current components with an appropriate control membrane current record. Figure 1*A* and *B* shows membrane current records at the potentials indicated. Two different control records were used for scaling and subtracting from the same test records. In panel *A*, membrane currents were subtracted using the current record obtained with a pulse of −20 mV from E_h = −100 mV; in panel *B*, the records were obtained by subtracting the control record obtained with a control pulse of +80 mV from E_h = 0 mV. The presence of non-linear currents is apparent with both types of subtraction procedures, though obvious differences are present. A fast initial component which has been related to gating of sodium channels (Vergara & Caputo, 1983) is present in the records corresponding to −40, −20 and 0 mV of *A* but is absent in the records of *B*. The time course of membrane current at the beginning and at the end of voltage clamp steps is much slower in *B* than in *A*. Finally, the amount of charge measured as the area under the current transients is clearly larger when the control pulse of +80 mV is used.

The total charge moved following the onset of the voltage-clamp steps ('on' charge) from the experiment in Fig. 1*A* and *B*, obtained by integration of the areas under the current transients, is shown in Fig. 1*C* as a function of the voltage step *V*. The points were fitted to a two-state Boltzmann distribution:

$$Q = Q_{max}/[1+\exp((-V+\bar{V})/k), \quad (1)$$

where Q_{max} is the maximum charge per unit linear capacitance, $\bar{V}$ is the mid-point and k a measure of the steepness. In agreement with Brum & Ríos (1987), the amount of charge mobilized at each potential was larger when the subtraction procedure employed a +80 mV pulse record (●); k was also larger though $\bar{V}$ was unchanged. This indicates that the control record for −20 mV contains more charge than the control record for +80 mV, as is clear from the inset to Fig. 1*C* where the former record has been scaled and subtracted from the latter.

Since the solutions were designed to record charge movement with minimum contamination of currents through ion channels, the remaining currents are expected to be essentially capacitive, ensuring the equality of 'on' and 'off' charges. Figure 2*A* is a plot of 'off' charge against 'on' charge from the experiment of Fig. 1*A* (○) and *B* (●). The straight line corresponds to equality of charges. As expected, the experimental points lie very close to the line when the subtraction procedure employed the −20 mV control pulse record. However, the 'on' charge was larger than the 'off' charge when the +80 mV control pulse record was used. In agreement

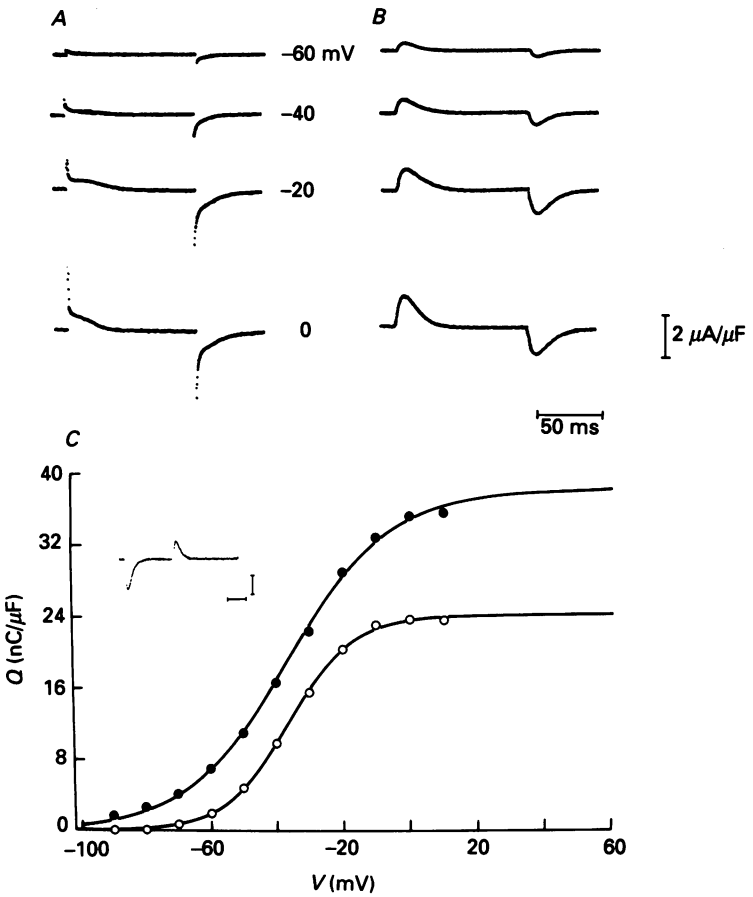

Fig. 1. The effects of holding potential on the voltage dependence of 'on' charge movement. *A* and *B*, intramembrane charge movement in reference saline (solution C) at the indicated potentials, each of which refer to the two adjacent traces. Records are shown from a single experiment, following subtraction of a scaled control in response to a -20 mV voltage step from $E_h = -100$ mV (*A*), and to 80 mV from $E_h = -0$ mV (*B*). *C*, ○ and ● represent the amounts of charge displaced by 'on' transients in *A* and *B* respectively as a function of the voltage step *V*. Smooth curves represent best fits to eqn (1) with $Q_{max} = 23{\cdot}83$ nC/μF, $\bar{V} = -36{\cdot}37$ mV and $k = 9{\cdot}70$ mV for ○, and $Q_{max} = 37{\cdot}52$ nC/μF, $\bar{V} = -36{\cdot}64$ mV and $k = 15{\cdot}51$ mV for ●. The inset shows the difference between scaled membrane currents produced by a -20 mV pulse from $E_h = -100$ mV and currents produced by a pulse to $+80$ mV from $E_h = 0$ mV. The calibration bars correspond to 40 ms for the abscissa and 0·3 μA/μF for the ordinate. Notice the inequality of 'on' and 'off' charges.

with this, the time integrals of the currents returned to zero only when the control pulse of -20 mV was used for subtraction (Fig. 2*B* and *C*). Since the test records are the same in both cases, the area under the 'off' transient during the *control* pulse to $+80$ mV is larger than the area under the corresponding 'on' transient. This was confirmed by integrating the transients; the ratio of 'off' to 'on' charge was $1{\cdot}00 \pm 0{\cdot}01$ for the -20 mV pulse record and $1{\cdot}15 \pm 0{\cdot}09$ for the $+80$ mV pulse record

in the same experiments ($n = 6$). This is also evident in Fig. 1*C* (inset). Larger 'off' areas can be explained if an ion channel remains open in depolarized fibres under these conditions, as Brum & Ríos (1987) have proposed. Evidence for the activation of this membrane conductance was also observed, following subtraction of

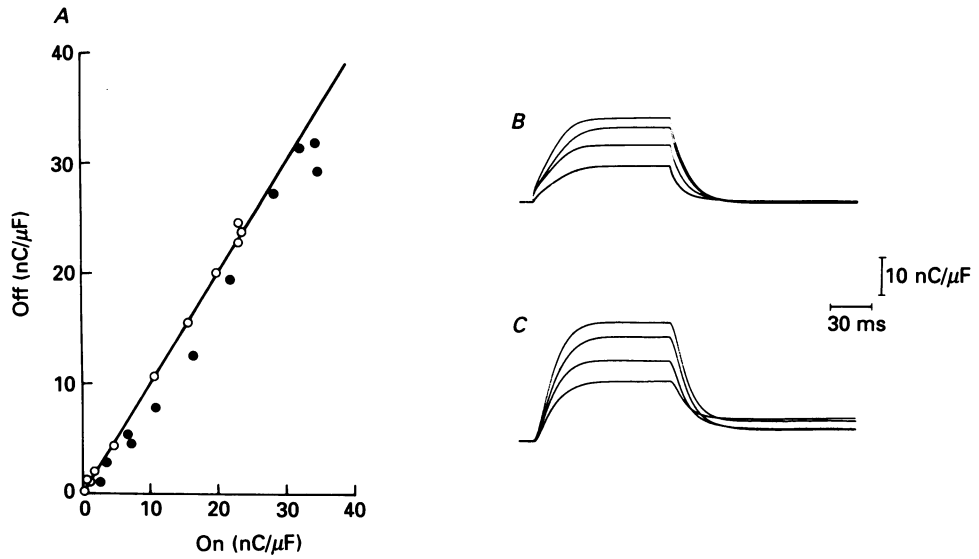

Fig. 2. The effects of holding potential on the relation between magnitudes of 'on' and 'off' charge. *A*, ○ and ● represent charges displaced after subtraction of a scaled control record to −120 mV from $E_h = -100$ mV, and of a scaled control record to +80 mV from $E_h = 0$ mV, respectively. The straight line corresponds to equality of charges; the filled symbols clearly deviate from this line. *B* and *C* show continuous integrals of the transients obtained using the two control records. Same experiment as in Fig. 1.

the control pulse of −20 mV, as an increase in 'off' areas after large depolarizations. The use of Co^{2+} or other divalent cation eliminates the inequality of charges, probably by blocking Ca^{2+} channels (Horowicz & Schneider, 1981). However, since Co^{2+} has strong surface charge effects on excitable membranes (Hille, Woodhull & Shapiro, 1975) and anions have been proposed to potentiate tension through changes in surface potential, we avoided the use of Co^{2+} and other similarly functioning blockers. Instead, we replaced Ca^{2+} by Mg^{2+} which has weak surface charge actions and is only very slightly permeant. We also restricted our measurements to membrane potentials below +20 mV where charge movement predominates.

Lyotropic anions and charge movement

The primary action of lyotropic anions on charge movement was to shift the voltage dependence of charge activation and slow the time course of the 'off' currents upon repolarization. Figure 3*A* and *B* shows the effects of 12 mM-SCN^- on charge movement. Panel *A* shows the movement of 'on' charge in the reference solution at the potentials indicated; the subtraction procedure employed a control record taken using a −20 mV pulse. The corresponding results using 12 mM-SCN^- are shown in panel *B*. Charge movement activates at more negative potentials, and 'off' tails

exhibited a more prolonged time course in the presence of SCN^- over the whole potential range.

Figure 3*C* shows the non-linear charge moved by step depolarizations to test potentials. Open symbols correspond to control values and filled symbols to values

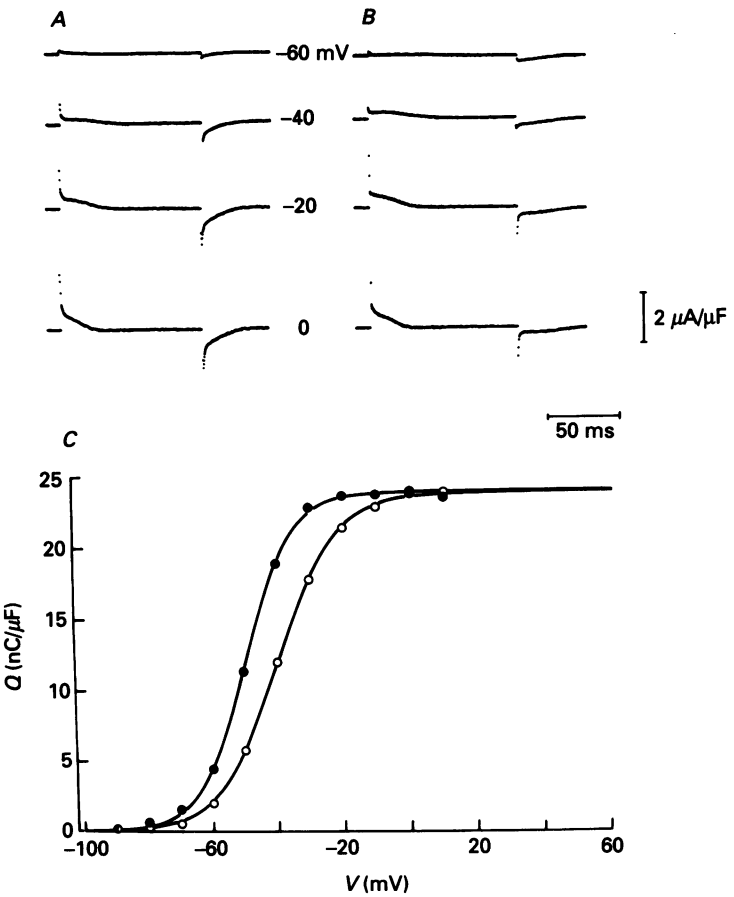

Fig. 3. The effect of extracellularly applied SCN^- upon the voltage dependence of charge movement. The records show membrane currents during voltage steps to the potentials indicated, under control conditions (*A*) and in the presence of extracellularly applied SCN^- (*B*), following subtraction of a control record obtained with a −20 mV pulse from $E_h = -100$ mV. *C*, ○ and ● represent the amounts of charge displaced by 'on' transients in *A* and *B* respectively. Smooth curves are best fits of eqn (1) with $Q_{max} = 23{\cdot}48$ nC/μF, $\bar{V} = -39{\cdot}87$ mV and $k = 8{\cdot}88$ mV for ○, and $Q_{max} = 23{\cdot}58$ nC/μF, $\bar{V} = -49{\cdot}60$ mV and $k = 7{\cdot}10$ mV for ●.

in the presence of SCN^-; continuous lines correspond to best fits of eqn (1) to the experimental values. The most significant effect of SCN^- was a shift in the voltage dependence of charge movement of *ca* −10 mV. A slight increase was found in the steepness, but the maximum charge remained essentially unchanged.

Table 2 shows averages of the best-fit parameters of eqn (1) for control experiments and experiments in the presence of SCN^- or other lyotropic anions in the extracellular

solutions. For results with the +80 mV subtractive pulse, the tabulated parameters were averaged over the corresponding best-fit values for the 'on' charge data only; for results with the −20 mV subtractive pulse, both 'on' and 'off' data were used. By ΔV is meant $\bar{V}_{anion} - \bar{V}_{control}$, i.e. the average shift in the mid-point due to the test

TABLE 2. Effects of lyotropic anions on charge movement

	Control −20 mV			Control +80 mV			
Anion	Q_{max} (nC/μF)	$\Delta\bar{V}$ (mV)	k (mV)	Q_{max} (nC/μF)	$\Delta\bar{V}$ (mV)	k (mV)	n
SO_4^{2-}	25·58±1·07	0	8·33±0·27	50·83±3·72	0	14·30±0·80	20
Br^-	22·69±0·51	−2·66±0·15	7·15±0·22	45·56±1·80	−3·10±0·18	13·38±0·67	4
NO_3^-	23·01±0·67	−5·21±0·10	7·38±0·32	57·69±9·10	−5·15±0·20	15·56±0·77	5
I^-	24·38±0·77	−7·49±0·26	6·23±0·28	65·71±8·90	−7·53±0·23	10·98±0·43	3
SCN^-*	27·84±2·63	−9·82±1·62	8·35±1·18	50·07±9·29	−10·54±2·02	11·58±1·09	4
SCN^-†	20·11±2·89	−9·61±2·51	10·08±0·72	40·41±4·29	−9·28±2·05	12·48±0·43	4

* SCN^- was applied extracellularly, † SCN^- was applied intracellularly.

anion relative to SO_4^{2-}; a systematic shift in the voltage dependence of the charge movement is evident in presence of lyotropic anions. Furthermore, the results were qualitatively similar whether the subtraction procedure used a control record for a +80 mV pulse from $E_h = 0$ mV or a −20 mV pulse from $E_h = -100$ mV. Consistent with previous results on the effects of these anions upon contraction, SCN^- most strongly altered the charge movement of all the anions tested here. The parameter n refers to the number of experiments performed.

In previous voltage-clamp experiments I_{Ca} has been recorded in isotonic internal EGTA (Almers & Palade, 1981; Arreola *et al.* 1987). For comparison, we performed several charge movement experiments with a similar solution (Table 1, solution F), using a P/4 procedure from $E_h = -100$ mV to subtract linear membrane current components. In six experiments the best-fit parameters for the reference solution were: $Q_{max} = 38{\cdot}79 \pm 4{\cdot}38$ nC/μF, $\bar{V} = -22{\cdot}99 \pm 3{\cdot}02$ mV and $k = 7{\cdot}63 \pm 1{\cdot}30$ mV. After SCN^-, the parameters were: $Q_{max} = 30{\cdot}42 \pm 4{\cdot}76$ nC/μF, $\bar{V} = -37{\cdot}00 \pm 4{\cdot}00$ mV and $k = 10{\cdot}65 \pm 1{\cdot}30$ mV. The relative displacement of the charge movement activation by SCN^- was similar to the value obtained in experiments with 20 mM-internal EGTA, although $\bar{V}$ was more positive in both control experiments and after SCN^-. This shift can probably be related to the surface charge effects caused by the absence of divalent cations in this internal solution.

'Off' tails in the presence of SCN^-

The appearance of prolonged decays in charge movement upon repolarization has been observed in the presence of perchlorate (Huang, 1987; Csernoch, Kovacs & Szücs, 1987) as well as SCN^- and other lyotropic anions (Fig. 3). Related results with SCN^- are shown in Fig. 4. Panel *A* shows superimposed membrane current records in control solution (solution B) following a step to −30 mV from $E_h = -100$ mV; subtraction of the linear component employed a −20 mV control pulse record. The duration of voltage-clamp pulses varied from 40 to 140 ms in 20 ms steps. The 'off' tails decayed monotonically for all pulse durations. In contrast, the time course of 'off' tails in presence of SCN^- decayed with a slower time course, but showed an

initial fast decay, followed by an increase in the 'off' tail amplitude, which then decayed with a very slow time course. The amount of the 'on' charge increased as expected from the shift in the activation curve (Fig. 3*C*). Similar results were obtained in two further experiments at the same potential (see also Fig. 3*A* and *B*). Figures 4*C* and *D* shows 'off' tails after a 100 ms depolarization from the same experiment.

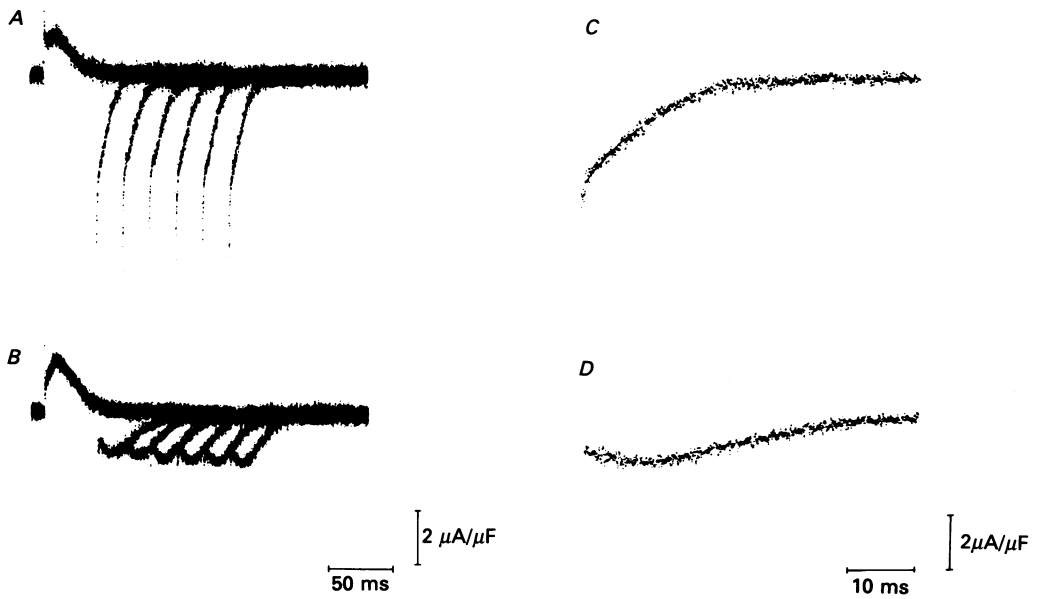

Fig. 4. The effects of extracellularly applied SCN^- on 'off' charge movement. Left, superimposed charge movement records obtained during voltage steps to −30 mV with different pulse durations. *A* shows the control record, and *B* the record in the presence of SCN^-. Right, *C* and *D* show records of 'off' charge after a 100 ms step. Sampling rate 5 kHz. Same experiment throughout.

Intracellular effect of SCN^- on charge movement

In order to determine whether the effects of SCN^- could be accounted for by changes in the membrane surface charge, we applied SCN^- intracellularly by diffusion through the cut ends of muscle fibres in the Vaseline-gap chamber. Figure 5*A* and *B* shows that the effects of intracellularly applied SCN^- are similar to those of extracellularly applied SCN^-: charge movement activates at more negative potentials and the time course of 'off' tails is greatly prolonged. The data are summarized in Fig. 5*C*; the average shift in $\bar{V}$ is not significantly different from that of extracellularly applied SCN^- (Table 2). It therefore seems unlikely that the effects of lyotropic anions depend upon which side of membrane the anion is applied.

Lyotropic anions and Ca^{2+} channels

SCN^- modifies the voltage dependence of I_{Ca} in skeletal muscle. Figure 6*A* and *B* shows control records of membrane currents during step depolarizations to the potentials indicated (solution B). In panel *A*, the depolarizations generate a large,

fast inward current that is probably carried through fast DHP-insensitive Ca^{2+} channels (Arreola *et al.* 1987). These currents are followed by a slow inward current (I_{Ca}) whose time to peak decreases with larger depolarizations (steps to 10 and 30 mV). I_{Ca} has been shown to be DHP sensitive (Almers & Palade, 1981). Panel *B*

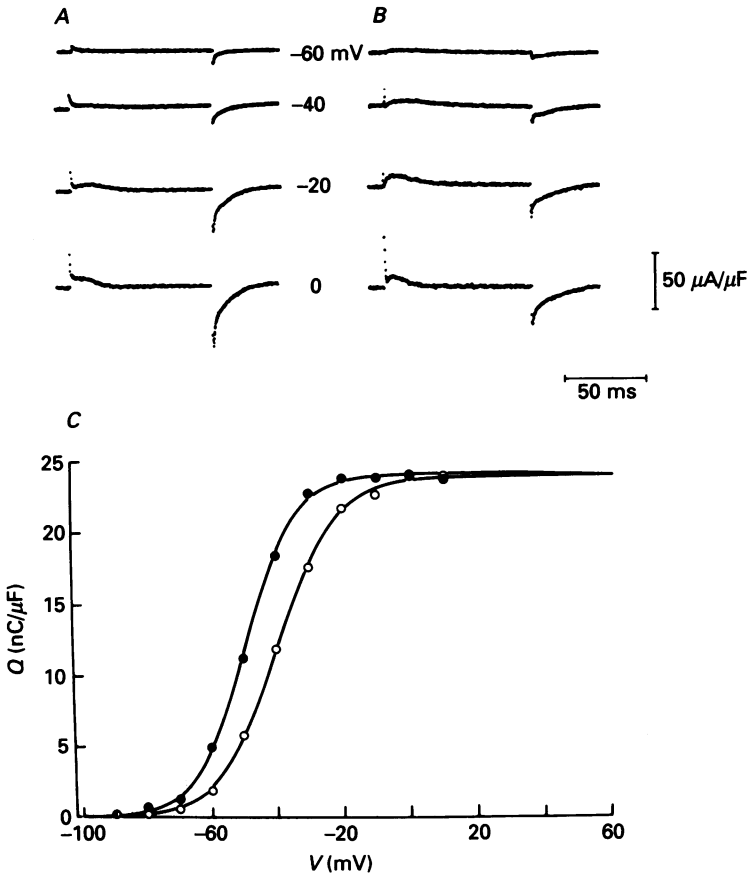

Fig. 5. The effect of intracellularly applied SCN^- on the voltage dependence of charge movement. Membrane currents are shown during voltage steps to the potentials indicated under control conditions (*A*) and in presence of intracellularly applied SCN^- (*B*), following subtraction of a scaled control record obtained with a −20 mV pulse from $E_h = -100$ mV. *C*, ○ and ● represent the amounts of charge displaced by 'on' transients in *A* and *B* respectively. Smooth curves are best fits of eqn (1) with $Q_{max} = 23{\cdot}49$ nC/μF, $\bar{V} = -39{\cdot}72$ mV and $k = 8{\cdot}92$ mV for ○, and $Q_{max} = 23{\cdot}68$ nC/μF, $\bar{V} = -49{\cdot}41$ mV and $k = 7{\cdot}58$ mV for ●.

shows records obtained with the same pulse protocols after the addition of 12 mM-SCN^- to the extracellular solution (solution B) in the same experiment. The time course of I_{Ca} becomes faster in SCN^- for a given voltage step, as is seen, for example, following the step to −10 mV. I_{Ca} is also accompanied by an outward current at large depolarizations (step to 30 mV) that is not present under control conditions. In addition to changes in the time course of I_{Ca}, SCN^- resulted in a shift towards more negative potentials of the peak *I–V* relation (Fig. 6*D*). The change in the extrapolated reversal potential in this graph is probably related to the appearance of an outward

current in the presence of SCN^-. Though the nature of this current was not resolved here, it is not likely to be carried by SCN^- through Cl^- channels, since tannic acid, which blocks Cl^- currents in frog skeletal muscle (Woll, Leibowitz, Neumcke & Hille, 1987), did not reduce the outward current (data not shown). Similar effects on I_{Ca} were observed with other lyotropic anions (data not shown).

TABLE 3. Effects of SCN^- on Ca^{2+} currents

	Solution	
	Control	SCN^-
Peak I_{Ca} ($\mu A/cm^2$)	$-53{\cdot}77 \pm 6{\cdot}90$	$-37{\cdot}27 \pm 4{\cdot}20$
Time to peak (ms)	512 ± 80	330 ± 36
ΔV_{peak}	0	$-21{\cdot}79 \pm 2{\cdot}65$
n	14	14

Figure 6*C* illustrates the action of SCN^- on the time to peak for the experiment of Fig. 6*A* and *B*. The smooth lines correspond to best fits to a single exponential plus a constant. The time to peak decreases in presence of SCN^- over the whole potential range and reaches a lower limiting value (see time to peak in Table 3).

Table 3 summarizes the effects of SCN^- on I_{Ca}. Peak I_{Ca} refers to the maximal I_{Ca} current observed over all voltage steps, ΔV_{peak} to the change in the voltage at which the maximal value of I_{Ca} was observed (Fig. 6*D*), and n to the number of experiments performed at each condition. In the presence of extracellular SCN^-, I_{Ca} decreased in magnitude, developed a faster time course and activated at more negative potentials.

The effects of extracellular SCN^- on I_{Ca} are reversible (see Table 4 below), eliminating the possibility that the shifts in activation may occur spontaneously. In three further experiments, we determined the membrane potential that elicited maximal I_{Ca} without SCN^- by obtaining current–voltage relations near 0 mV for a period of 40 min; no spontaneous shifts of this potential were observed.

Intracellular effects of SCN^- on I_{Ca}

Intracellular SCN^- produced effects on I_{Ca} similar to those following extracellular application. Figure 7 shows records of I_{Ca} for different voltage steps. In panel *A* (control), a small depolarization barely elicited an inward current; the same pulse in presence of SCN^- elicited significant I_{Ca} (panel *D*). As with extracellular SCN^-, I_{Ca} became faster and its time to peak decreased with intracellular SCN^-. For these data, peak I_{Ca} was $-77{\cdot}1\ \mu A/cm^2$ in control solution and $-60{\cdot}53\ \mu A/cm^2$ in SCN^-; the change in the voltage to peak I_{Ca} was -20 mV; and the time between onset of the voltage step and the peak I_{Ca} was 334 and 257 ms for panels *C* and *F*, respectively. These values are not greatly different from those shown in Table 3 for extracellular SCN^-.

Tails of 'off' current and I_{Ca}

The application of SCN^- to either side of the membrane has kinetic effects on charge movement and on Ca^{2+} currents. These are clearly observed as slowings in the time courses of the tail currents corresponding to both charge movement and I_{Ca}.

Figure 8*C* and *D* shows superimposed records of I_{Ca} tail currents before and after SCN^- (arrows) at two different membrane potentials in the experiment of Fig. 6. SCN^- prolongs these tail currents, which last over 100 ms in panel *D*. In comparison, 'off' current tails at the same membrane potential, taken from the experiment of

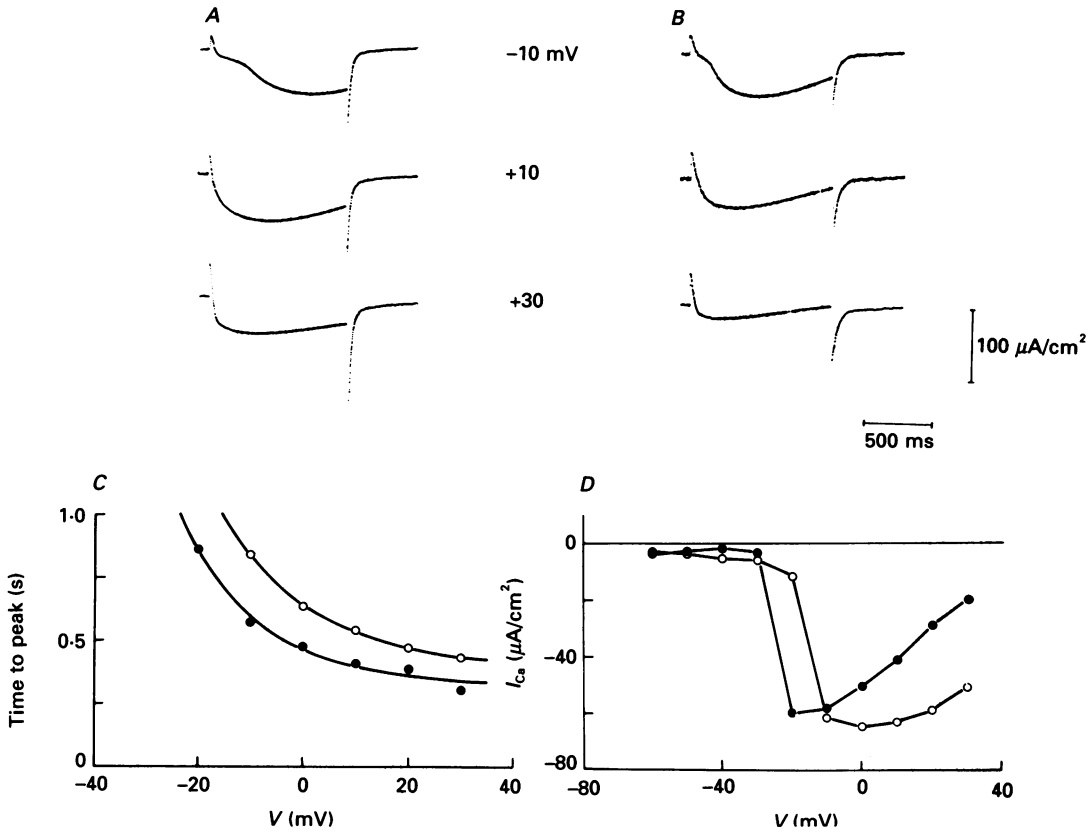

Fig. 6. The effects of extracellularly applied SCN^- upon membrane currents. The records show currents during voltage steps to the potentials indicated under control conditions (*A*) and in presence of extracellularly applied SCN^- (*B*), at a holding potential of −90 mV. *C*, time to peak of I_{Ca} as a function of membrane potential. *D*, peak inward currents plotted against membrane potential. For *C* and *D*, ○ correspond to control conditions, and ● to extracellularly applied SCN^-.

Fig. 3, are shown in Fig. 8*A* and *B*. Again the time course of the 'off' tails is prolonged by SCN^- (arrows), although the time course of Ca^{2+} tail currents is about one order of magnitude slower than the 'off' tails of charge movement. Also, in contrast to the action of SCN^- on 'off' tails shown in Fig. 8*A* (see also Figs 3 and 4), we did not observe changes in I_{Ca} tail currents other than the prolongation of the time course.

Table 4 summarizes the concentration dependence and reversibility of the effects of external SCN^-. Two experiments were performed using the indicated concentrations of external SCN^-; the time sequence is from left to right. The columns

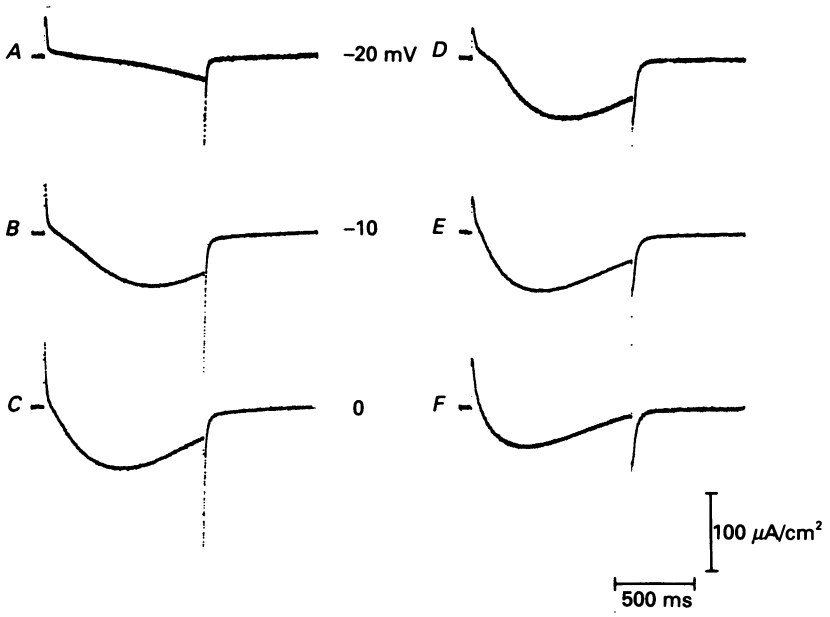

Fig. 7. The effect of intracellularly applied SCN^- upon membrane currents. The records show currents during voltage steps to the potentials indicated under control conditions (*A–C*) and in the presence of intracellularly applied SCN^- (*D–F*), at a holding potential of −90 mV.

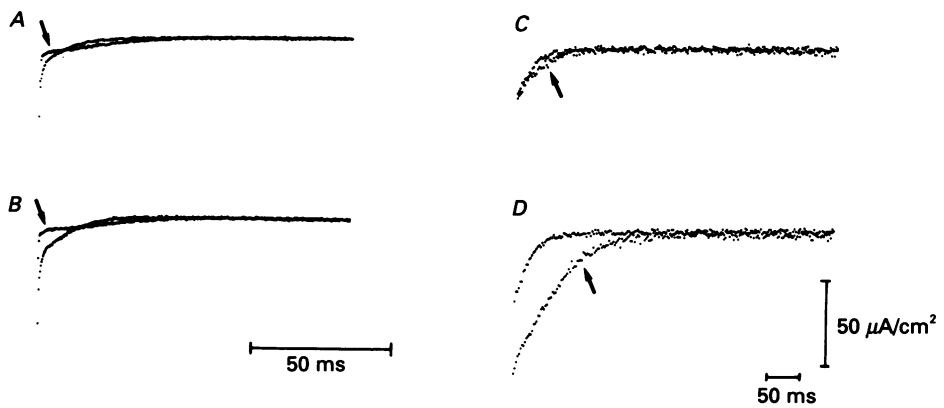

Fig. 8. The effects of extracellularly applied SCN^- upon time course of tail currents of 'off' charge movement and I_{Ca}. Left, superimposed 'off' tail currents produced by returning to the holding potential of −100 mV from a step to −30 mV (*A*) or to −20 mV (*B*). Same experiment as Fig. 3. Right, superimposed records of deactivation of I_{Ca} resulting from steps to the same potentials as in *A* and *B* (*C* and *D*, respectively). The holding potential was −90 mV. Same experiment as in Fig. 6. Arrows indicate records in presence of extracellularly applied SCN^-. Note that the time scales in *A* and *B* differ from those in *C* and *D*.

below give averaged parameter values for each concentration. Q_{max}, k and $\bar{V}$ refer to the best fits of charge movement data to eqn (1). $\Delta\bar{V}$ gives the shift in $\bar{V}$ from the initial control value. I_{Ca} refers to the maximal Ca^{2+} current observed over all voltage steps, and ΔV_{peak} has the same meaning as in Table 3. The parameters $\bar{V}$ and k for

TABLE 4. Effects of SCN^- on charge movement and Ca^{2+} currents

$[SCN]_o$ (mM)	0	6	12	18	0
Q_{max} (nC/μF)	27·3	24·2	23·1	20·6	17·0
k (mV)	11·07	4·78	6·74	5·69	13·58
$\bar{V}$ (mV)	−30·6	−41·7	−43·4	−42·8	−30·6
$\Delta\bar{V}$ (mV)	0	−11·1	−12·7	−12·1	0·04
I_{Ca} (μA/cm^2)	−50·2	−82·2	−61·7	−37·0	−35·9
ΔV_{peak} (mV)	0	−15	−15	−20	−10

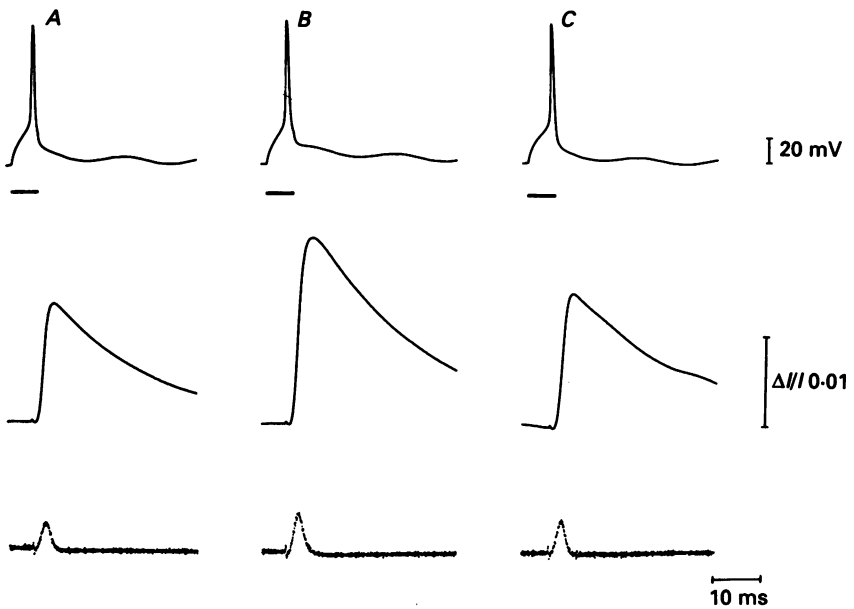

Fig. 9. The effects of SCN^- upon Ca signals recorded with ArIII in current-clamped fibre segments. The upper records show the action potential; the middle records, the Ca signal; and the lower records, the time derivatives of the Ca signal. *A*, recorded under control conditions; *B*, in presence of 12 mM extracellularly applied SCN^-; *C*, after return to control conditions. The Ca signal is calibrated as $\Delta I/I$ where I is the resting light level at 660 nm (Delay *et al.* 1986).

the charge movement data show little change beyond 6 mM-SCN^- and demonstrate good reversibility upon return to control conditions; Q_{max}, besides a tendency to decrease over the 2 h duration of the experiments, shows no strong dependence on SCN^- concentration. The main effect of SCN^- upon I_{Ca}, the shift in the membrane

potential at which maximal Ca^{2+} current was observed, likewise has no evident dependence upon SCN^- concentration above 6 mM, and shows substantial recovery following the wash-out step.

Effect of SCN^- on Ca signals

Figure 9 shows the effect of SCN^- upon Ca signals in a fibre loaded with the optical indicator ArIII and stimulated by action potential. Panel *A* shows the

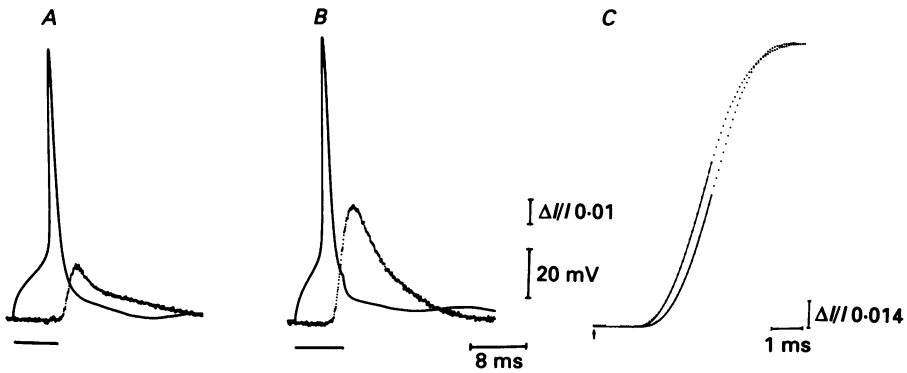

Fig. 10. *A* and *B*, the effect of SCN^- upon Ca signals recorded with ApIII. *A* shows the action potential and Ca signal recorded from a fibre segment using the Ca^{2+} indicator ApIII under control conditions. Panel *B*, same as in *A*, with 12 mM extracellularly applied SCN^-. The Ca signal calibration, which is approximate but consistent over the two panels, is given in terms of $\Delta I/I$ where I is the resting light level at 710 nm. *C*, the effect of SCN^- upon the rising phase of the ArIII Ca signal. The rising phases of the ArIII Ca signals in presence and absence of SCN^- are shown scaled to the same peak amplitude and superimposed with the respective best fits to eqn (2). Both traces were fitted using the parameter $A = 10{\cdot}6823$; the earlier trace, corresponding to 12 mM extracellularly applied SCN^-, has parameters $B = 2{\cdot}56756$ and $C = 2{\cdot}63619$; the later (control) trace has $B = 2{\cdot}27795$ and $C = 3{\cdot}40508$. The vertical scale is given in terms of $\Delta I/I$ where I is the resting light at 660 nm. The calibration bar applies to the later (control) trace; the earlier (SCN^-) trace was scaled to the same height as the later trace, by a factor of 1·54.

control record; the upper trace shows the action potential, the middle trace the Ca signal, and the lowest, the time derivative of the Ca signal. In panel *B* the fibre is exposed to extracellular SCN^-. The action potential is minimally affected, but the amplitude of the Ca^{2+} signal shows a substantial increase. The time derivative of the Ca^{2+} signal is also increased, consistent with an increase in the rate of Ca^{2+} release. In panel *C* (control solution) the potentiating effect is substantially reduced, demonstrating reversibility.

In order to rule out any sensitivity as a result of artifacts of ArIII to alterations in the intracellular environment, the experiments were repeated using the indicator ApIII. Figure 10 shows an example of these results. Panel *A* shows the action potential and the consequent ApIII signal in control conditions; panel *B*, in presence of SCN^-, again shows a dramatic increase in peak Ca^{2+} signal amplitude.

These results were consistent over several fibres; SCN^- increased the ArIII Ca^{2+} transient amplitude by a factor of $1{\cdot}86 \pm 0{\cdot}15$ and its time derivative by $1{\cdot}71 \pm 0{\cdot}17$

($n = 5$), and increased the Ap III counterparts by $1{\cdot}99 \pm 0{\cdot}10$ and $1{\cdot}75 \pm 0{\cdot}18$ ($n = 4$), respectively. The action potential amplitudes were not significantly altered by SCN^-.

Figure 10*C* shows control and SCN^--potentiated Ar III Ca signals scaled to the same peak amplitude. The leading edge of the Ca signal is evidently shifted to earlier times by SCN^-. Each trace was fitted to the function

$$f(t) = A(1-\exp(-t/B))^C, \quad (2)$$

over the rising edge of the Ca^{2+} signal (Vergara & Delay, 1986). The parameters A, B and C have no intrinsic significance; the functional form was chosen only to obtain an accurate parametrization of the signal to allow a good estimate of the shift in time. The best fits, shown superimposed on the points in Fig. 10*C*, have the parameters given in the legend. The average time shift, calculated from the functional form, is reduced by 0·24 ms in presence of SCN^-.

DISCUSSION

Measurement of charge movement

Measurement of non-linear charge movement in polarized fibres (charge 1) requires the subtractive correction of current records using a reference record taken over a voltage range where currents are essentially linear (Schneider & Chandler, 1973). Control records taken using hyperpolarizing pulses from a holding potential near -100 mV are likely to be contaminated by a non-linear charge (charge 2; Ríos & Brum, 1987). Consistent with this, we found that charge 1 was significantly greater if the alternative subtractive procedure was followed, in which a positive pulse was used in a depolarized fibre. However, our data records showed that 'off' charge was larger than 'on' charge, probably due to the opening of an ion channel during the control pulse. Brum, Fitts, Pizarro & Ríos (1988) reported that the contribution of this conductance to 'on' transients can be largely eliminated by the application of a negative pre-pulse, but apparently even with this precaution the channel can be activated during pulses to $+80$ mV, contaminating the 'off' transients and leading to inequality of moved charge (see Fig. 4*A* of Brum *et al.* 1988). To rule out sensitivity of our results to the specific subtractive procedure, we used both types of control records in the charge movement experiments.

Lyotropic anions, charge movement and I_{Ca}

Our experiments show that lyotropic anions have profound effects on charge movement in frog skeletal muscle. The voltage dependence of activation of charge 1 shift towards more negative potentials following the sequence: $SCN^- > I^- > NO_3^- > Br^- > SO_4^{2-}$. Furthermore, lyotropic anions markedly slow the 'off' charge movement upon repolarization, though no effect of such magnitude was seen in the 'on' transients. The experiments also show that SCN^- and other lyotropic anions shift the current–voltage relation of Ca^{2+} channels towards more negative potentials and slow the deactivation kinetics of tail currents upon repolarization.

A number of these effects are in common with those of the lyotropic or chaotropic anion perchlorate (ClO_4^-), which likewise shifts the voltage dependence of force activation and charge movement to more negative potentials, prolongs the time

course of the 'off' charge movement, and is effective both intracellularly and extracellularly (Gomolla, Gottschalk & Lüttgau, 1983; Huang, 1986, 1987; Csernoch *et al.* 1987). The similarity is only partial, however. For example, ClO_4^- appears to have more specific targets than does SCN^-, since the former alters only the tetracaine-sensitive component of charge movement, and SCN^- affects both the tetracaine-sensitive and insensitive components (Huang, 1986). Feldmeyer & Lüttgau (1988) have observed no shift in the voltage dependence of I_{Ca} activation in contracting fibres. Again, ClO_4^- does not affect the threshold for the delayed rectifier K^+ current (Gomolla *et al.* 1983), but SCN^- and other anions have almost identical effects upon these channels and upon the mechanical threshold (Kao & Stanfield, 1968) and similar though less pronounced effects on activation and inactivation of Na^+ currents (Dani, Sanchez & Hille, 1983).

The properties of ClO_4^- are believed to be incompatible with surface charge models that assume a uniform density of charge, and suggest that ClO_4^- specifically interferes with the gating mechanism that releases Ca^{2+} from the SR (Gomolla *et al.* 1983). The lyotropic series of anions, however, have been regarded as less specific in their actions, and Huang (1986) has proposed that SCN^- has a non-specific effect upon charge movement, analogous to effects upon Na^+ and K^+ currents. However, in analogy to the case of ClO_4^-, simple shifts along the voltage axis are unlikely to explain the alterations in kinetics of 'off' charge and I_{Ca} by SCN^-, and the equivalence of intracellular and extracellular actions of SCN^- argues against this possibility as well. It is of course expected that non-specific surface charge effects also contribute to the actions of the lyotropic anions.

In addition to the changes in Ca^{2+} currents by SCN^- mentioned above, we observed decreases in the times to peak of I_{Ca} at all membrane potentials, and decreases in the extrapolated reversal potentials of Ca^{2+} currents. These effects might result from alterations by SCN^- to the selectivity, activation and/or inactivation properties of Ca^{2+} currents by SCN^-. A decrease in selectivity of Ca^{2+} currents has been reported under certain conditions; for example, monovalent cations can readily permeate through Ca^{2+} channels in frog muscle when external Ca^{2+} is reduced below 1 μM (Almers, McCleskey & Palade, 1984).

The apparent SCN^--sensitive outward current observed here would tend to result in an earlier time to peak of I_{Ca}, and possibly also to a decrease in the extrapolated reversal potential as well. The shift in the peak of the I_{Ca}–V relation (Fig. 6*D*) may therefore be overestimated in these experiments.

Since anions have kinetic effects on Ca^{2+} currents and are equally effective when applied intracellularly, we suggest, in analogy with the effects on charge movement, that lyotropic anions interfere directly with the gating processes of Ca^{2+} currents.

Dihydropyridine receptors and charge movement

We have shown striking similarities between the effect of lyotropic anions on charge movement and on DHP-sensitive Ca^{2+} currents: SCN^- slows both sets of deactivation kinetics and shifts mobilized charge and the I_{Ca}–V relation to more negative voltages, whether SCN^- is applied intracellularly or extracellularly. However, deactivation of 'off' charge is still roughly one order of magnitude faster than deactivation of I_{Ca}. This has implications for the hypothesis that the charge movement is associated with

gating of Ca^{2+} currents. Suppose the gating current were to have one open and several closed states, a kinetic scheme consistent with but not limited to that of Sanchez & Stefani (1983). At the end of the voltage step, the Ca^{2+} current would be deactivated as soon as the gating charge were to move from the open state, though the gating charge would continue to move through the closed states. That is, even with conservative assumptions about the kinetic scheme of the gating charge, it is expected that closing of the channel would precede charge movement (Armstrong, 1981). Since this was not observed, most of the charge movement is probably not associated with the gating of Ca^{2+} currents.

The fact that lyotropic anions have very similar actions on charge movement and on Ca^{2+} currents can probably be explained following the proposal of Ríos & Brum (1987) that DHP receptors are the voltage sensors of charge movement. Ríos and Brum supported their view with the fact that a close correlation was found between the amount of charge blocked by nifedipine and the decrease in calculated peak release of Ca^{2+} by the SR in partially depolarized fibres (Brum & Ríos, 1987). The strong voltage dependence of both the blockade of DHP-sensitive Ca^{2+} currents (Sanguinetti & Kass, 1984) and of charge movement (Ríos & Brum, 1987) is also consistent with this view. A severe impairment in E–C coupling has been correlated with the absence of DHP-sensitive Ca^{2+} currents in dysgenic mouse skeletal muscle (Beam, Knudson & Powell, 1986), and contraction and Ca^{2+} currents both recover after co-culture with normal spinal cord neurons or by injection of complementary DNA encoding DHP receptors (Rieger, Bournaud, Shimahara, Garcia, Pinçon-Raymond, Romey & Lazdunski, 1987; Tanabe, Beam, Powell & Numa, 1988). In spite of these similarities, some inconsistencies are also present: high concentrations of nifedipine, capable of blocking Ca^{2+} currents entirely (Almers & Palade, 1981; Lamb & Walsh, 1987) not only fail to block contraction but actually decrease the mechanical threshold (McCleskey, 1985) and potentiate twitch tension (Arreola *et al.* 1987; Dulhunty & Gage, 1988). Besides, in fully polarized fibres, low concentrations of nifedipine that block I_{Ca} by *ca* 50% (Lamb & Walsh, 1987) do not decrease the estimated Ca^{2+} release (Ríos & Brum, 1987). These differences are not fundamental and, as pointed out by Lamb & Walsh (1987), it is possible that two very similar, though not identical, populations of DHP-sensitive molecules co-exist in transverse tubule system membranes, one associated with charge movement and the other with Ca^{2+} currents.

Lyotropic anions and Ca signals

Extracellularly applied SCN^- increases the amplitude and rate of rise of the Ca signal, which can be interpreted as increases in the amount and rate of release of Ca^{2+} respectively. These actions are consistent with previous observations on the mechanical effects of lyotropic anions on twitch tension (Kahn & Sandow, 1955; Hodgkin & Horowicz, 1960; reviewed in Horowicz, 1964). Since it is unlikely that I_{Ca} contributes significant intracellular free Ca^{2+} following an action potential, it is more reasonable to conclude that the observed increase in intracellular free Ca^{2+} is due to increased liberation from the SR. This is consistent with models in which charge movement couples to Ca^{2+} release, especially given the observed effects of SCN^- on the voltage dependence of charge 1. SCN^- may therefore share with ClO_4^- the

property of modifying the calcium release process by acting on the charge movement step of the release process (Gomolla *et al.* 1983; Csernoch *et al.* 1987).

Since SCN^- shifts the voltage dependence of the charge to more negative potentials, the voltage threshold for calcium release is expected to decrease; for 2 mM-ClO_4^- the observed decrease is about 20 mV (Fig. 7 of Csernoch *et al.* 1987). It is expected therefore that the threshold voltage for Ca^{2+} release will be reached earlier in the action potential in presence of SCN^-, and that the Ca signal will depart from baseline at an earlier time relative to the onset of the action potential. To estimate the decrease in the latency of the Ca signal, we may assume that the action potential rises at a rate of about 200 mV/ms (Fig. 9). If the threshold were decreased by 20 mV by SCN^-, the action potential would arrive at this threshold $20/200 = 0{\cdot}1$ ms earlier. Since reduction of the Ca signal latency by 0·1 ms cannot explain the observed 0·24 ms (Fig. 10*C*), it is possible that SCN^- facilitates the coupling between charge movement and Ca^{2+} release in a manner different to ClO_4^-.

The amount of Ca^{2+} release in an action potential is clearly increased by SCN^-. Two mechanisms may contribute to this increase. First, since the threshold voltage for Ca^{2+} release is lowered, the Ca^{2+} release may begin earlier and continue for longer, increasing the total amount released. Second, since SCN^- prolongs the time course of the relaxation of the 'off' charge movement, the remaining charge might be expected to prolong the Ca^{2+} release. Since our results show no striking effect of SCN^- upon time course of the falling phase of the Ca signal, the second possibility appears less likely.

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