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A CALCIUM- AND VOLTAGE-DEPENDENT CHLORIDE CURRENT IN DEVELOPING CHICK SKELETAL MUSCLE

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SUMMARY

1. Depolarization of embryonic chick myotubes from negative potentials elicits a rapid spike followed by a long-duration after-potential. The ionic basis of the long-duration after-potential was examined by making intracellular recordings from cultured myotubes, and by making whole-cell patch-clamp recordings from myoblasts and myoballs.

2. The peak potential of the long-duration after-potential varied with the chloride gradient, suggesting that a conductance increase to chloride is involved in generating the after-potential. However, a calcium current was also implicated, since lowering the extracellular calcium or replacing extracellular calcium with cobalt abolished the after-potential.

3. When extracellular calcium was replaced with strontium or barium, short-duration spikes similar to calcium spikes were observed, but only strontium was able to support activation of long-duration after-potentials. Intracellular injection of calcium or strontium into myotubes bathed in calcium-free extracellular solutions restored the ability of depolarization to evoke an after-potential. Intracellular injection of magnesium, barium, nickel or cobalt did not restore this ability. These experiments strongly suggested that the long-duration after-potential was due to a calcium- and voltage-activated chloride current.

4. Whole-cell voltage-clamp recordings from myoballs and myoblasts showed that a large chloride conductance could be activated by depolarization when the internal free calcium concentration was buffered at levels greater than 10^{-7} M. At $2{\cdot}5 \times 10^{-7}$ M-calcium, the voltage dependence of activation was steepest in the range of -30 to -20 mV and the activation kinetics varied with the membrane potential. The time to half-maximal activation ranged from 0·1 s at positive potentials to greater than 1 s at more negative potentials. The time constant for deactivation was approximately 1 s at -50 mV. No inactivation was observed.

5. The selectivity of the chloride current was measured by substituting other anions for chloride. The following permeability series was found: $I^- > NO_3^- > Br^- > Cl^-$ > acetate > $F^- > SO_4^-$ = glucuronate. Thus anion permeability decreased as the hydration radius increased.

6. Measurements of the resting potential of developing myoblasts and myotubes under 'physiological' conditions (37 °C, bicarbonate buffer) suggest that the after-potential acts to depolarize these cells 10–20 mV above their resting potential (approximately -60 mV) for several seconds.

7. We discuss the possibility that the long-duration after-potential may be involved in triggering myoblast fusion and in the generation of bursts of spontaneous contractions in developing myotubes.

INTRODUCTION

Depolarization of developing chick myotubes gives rise to a spike followed by an after-potential that can last for many seconds. When intracellular recordings are made with microelectrodes containing 3 M-KCl, the after-potential manifests itself as a large depolarization (a long-duration spike, LDS). The ionic basis of the LDS, which is observed both *in vivo* (Kano, 1975) and *in vitro* (Li, Engel & Klatzo, 1959; Kano, Shimada & Ishikawa, 1972; Kano & Shimada, 1973), is not completely understood. Fukuda (1974) presented evidence that the LDS is due to an increase in chloride conductance. In support of this idea, voltage-clamp studies of colchicine-treated myotubes (myosacs) revealed a slowly activating, long-lasting current whose magnitude varied with the external chloride concentration (Fukuda, Fischbach & Smith, 1976). The potential at which this current could first be activated was very similar to the threshold for activation of the LDS. Evidence gathered by others suggests that the situation is more complex. The LDS can be blocked with manganese or cobalt (Kano & Shimada, 1973; Fukuda, 1974; Kano, 1975), and repeated suggestions have been made that the current underlying the LDS might be carried mainly by calcium ions (Kano & Shimada, 1973; Kano, 1975; Saito & Ozawa, 1986*a*).

The two sets of observations do not necessarily conflict. Chloride currents activated by a rise in intracellular free calcium have been recently reported in a variety of tissues including frog oocytes (Barish, 1983; Miledi & Parker, 1984), spinal and sensory neurones (Owen, Segal & Barker, 1984, 1986; Mayer, 1985), lacrimal gland cells (Evans & Marty, 1986), and a pituitary cell line (Korn & Weight, 1987). The hypothesis that the LDS is due to a calcium-activated chloride current leads to several predictions: (1) the peak amplitude of the LDS should vary with the chloride equilibrium potential; (2) the LDS should be abolished when calcium is not present in the extracellular solution; (3) intracellular calcium injection should be able to activate an LDS even in the absence of extracellular calcium; and (4) buffering the intracellular free calcium to a low level should prevent activation of a calcium-dependent chloride current. The experiments reported here were designed to test these predictions. We show that all the predictions are met, and go on to characterize the calcium-activated chloride current under voltage clamp with defined intracellular calcium levels. Some of these results have been presented in preliminary form (Hume & Thomas, 1988).

METHODS

Cell culture

Standard chick embryo muscle cell cultures were prepared as described previously (Hume & Honig, 1986). Briefly, pectoral muscle was dissected from 11-day-old chick embryos, minced, and incubated in a calcium- and magnesium-free saline (Puck's saline) for 20 min at room temperature. The tissue was then spun down for 5 min, resuspended in culture medium, and triturated until the solution was cloudy. Cell density was determined with a haemocytometer after the suspension was

filtered through lens paper to remove debris. Cells were plated onto gelatin-coated tissue culture dishes (Corning) at 150000 per 35 mm dish. The culture medium, Eagle's MEM with Earle's salts (Gibco), was supplemented with 10% heat-inactivated horse serum (Gibco), penicillin–streptomycin (50 units/ml, 50 μg/ml), and conalbumin (40 μg/ml; Sigma). Cultures were maintained in a humidified incubator at 37 °C with an atmosphere of 95% air, 5% CO_2. At 4 days the cultures were treated with medium containing 10^{-5} M-cytosine arabinoside (Sigma) for 4 days to kill dividing fibroblasts. Cultures were fed with normal medium at 8 days. We made recordings from mononucleate myoblasts at 1–2 days and from multinucleate myotubes at 6–10 days.

Myoballs (spherical, multinucleate muscle cells) were made in an identical manner up to the cell plating stage. Cells were preplated at 750000 per 35 mm dish onto uncoated tissue culture dishes and incubated at 37 °C for 2–3 h. The culture dishes were then swirled several times and the medium of each dish was transferred to a fresh, uncoated tissue culture dish. This procedure greatly reduced the number of fibroblasts, which adhere more rapidly to the dishes than the muscle precursor cells. The high cell density was used to promote the formation of clusters of muscle precursor cells. The reduction in fibroblasts and the use of uncoated dishes made the culture dish surface poorly adhesive, which inhibited muscle cell elongation. Three- to six-day-old myoballs were used for recording.

Intracellular recording

Intracellular recordings from elongate, multinucleate myotubes were made using conventional glass microelectrodes filled with 3 M-KCl or 3 M-potassium acetate as described previously (Hume & Honig, 1986). The high input resistance of muscle cells bathed in the blocking solutions allowed the membrane potential to be varied between +20 and −100 mV by passing very small currents (< 1 nA). The resistance of electrodes was nearly constant when such small currents were passed; thus in our experiments we used a single microelectrode with a balanced bridge circuit both to record voltage and to pass current (unless noted otherwise). The bridge circuit was balanced just prior to penetration of each cell. Experiments were performed at room temperature (20–22 °C) unless otherwise noted. To make recordings at 37 °C a thermoelectric temperature controller was used. In these recordings fresh external solution was continuously passed through the dish to minimize evaporation.

Whole-cell patch-clamp recording

Standard techniques were employed in order to form high-resistance seals with pipettes onto the membrane of myoblasts or myoballs and to gain access to the cell interior (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Because myoballs are quite large, we allowed at least 5 min to pass between gaining access to the cell interior and the beginning of recording, in order to fully dialyse the cell. Polished pipettes had resistances of 2–4 MΩ. We recorded from myoballs whose diameters ranged from 15 to 30 μm.

Solutions

Just prior to recording, each culture dish was washed at least three times over a period of 5 min with the appropriate external solution in order to replace the incubating media. Each wash exchanged about 3 ml. Recording was then performed over a period of up to several hours. Unless otherwise noted, solutions contained HEPES (as buffer) and 30 μM Phenol Red (as indicator) in order to maintain the pH between 7·2 and 7·4. The pH was adjusted with the appropriate hydroxide as indicated in Tables 1 and 2. Our standard physiological solution contained (in mM): 132 NaCl, 5 KCl, 5·4 $CaCl_2$, 1·6 $MgSO_4$, 1·3 $NaHPO_4$, 6·3 glucose, 12·5 HEPES, and 4 NaOH. In a few experiments we recorded from cells bathed in the culture medium (MEM) in order to more closely approximate the normal conditions under which muscle cells develop. In these experiments the pH was maintained by bubbling the solution with 5% CO_2. The composition of the blocking solutions is shown in Table 1, and the patch-clamp solutions in Table 2.

RESULTS

In our initial experiments, intracellular recordings were made from chick myotubes bathed in an extracellular solution with ion concentrations similar to that of plasma

(standard external solution). For these experiments we used microelectrodes filled with 3 M-KCl. Under these conditions, brief depolarizing current pulses from the resting potential usually elicited a rapid spike followed by a long-duration spike (LDS) that lasted 10–60 s. If the initial membrane potential was adjusted to −70 mV by passing a constant current through the recording electrode, all of the myotubes tested (20/20) exhibited an LDS in response to depolarization. There was a dramatic increase in the input conductance during the LDS, and the return to baseline was accompanied by the gradual decline of this conductance (Fig. 1).

TABLE 1. Composition of blocking solutions (in mM)

	Blocking solutions								
Solution	1	2	3	4	5	6	7	8	9
NaCl	110	—	—	—	110	110	110	110	110
Sodium acetate	—	110	—	—	—	—	—	—	—
Sodium glucuronate	—	—	110	110	—	—	—	—	—
NaOH	5	5	1	1	5	5	5	5	5
KCl	4	—	—	—	4	4	4	4	4
Potassium acetate	—	4	—	—	—	—	—	—	—
KOH	—	—	4	4	—	—	—	—	—
Glucuronic acid	—	—	4	26	—	—	—	—	—
TEA-Cl	20	20	20	—	20	20	20	20	20
TEA-OH	—	—	—	20	—	—	—	—	—
$MgCl_2$	1	1	1	—	4	4	1	1	1
$MgOH_2$	—	—	—	1	—	—	—	—	—
$CaCl_2$	4	4	4	4	1	0·1	—	—	—
$SrCl_2$	—	—	—	—	—	—	4	—	—
$BaCl_2$	—	—	—	—	—	—	—	4	—
$CoCl_2$	—	—	—	—	—	—	—	—	4
Tetrodotoxin	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
HEPES	12·5	12·5	12·5	12·5	12·5	12·5	12·5	12·5	12·5
Phenol Red	0·03	0·03	0·03	0·03	0·03	0·03	0·03	0·03	0·03
Glucose	10	10	10	10	10	10	10	10	10

Names of blocking solutions: 1, standard; 2, 79% acetate; 3, 79% glucuronate; 4, 94% glucuronate; 5, 1 mM-calcium; 6, 0·1 mM-calcium; 7, strontium; 8, barium; 9, cobalt.

Chloride dependence of the long-duration spike (LDS)

In order to better isolate the LDS from other concurrent responses, we used a solution containing the voltage-dependent channel blockers tetrodotoxin (TTX) and tetraethylammonium (TEA) to suppress sodium and potassium currents respectively (standard blocking solution). In this solution, brief depolarizing pulses still elicited a rapid spike followed by an LDS in almost all myotubes stimulated from −70 mV. Thus, prior activation of the sodium spike was not necessary for the LDS.

To test whether the LDS was sensitive to external chloride, the peak potential of the LDS was measured in several chloride-substituted blocking solutions. In most cells we found that the peak amplitude of the LDS gradually became more positive with each of the first few stimuli (see below). However, after 5–10 stimuli the peak of the LDS stabilized at a steady level, which was taken to be the 'actual' peak potential. The mean peak potential of the LDS of cells in the blocking solution was

−20·4 mV (S.E.M. = 2·0, n = 25). The peak potential shifted to −4·2 mV (S.E.M. = 3·0, n = 25) when 79% of the chloride was replaced with the larger anion acetate, and to +12·3 mV (S.E.M. = 2·0, n = 15) when the even larger anion glucuronate was used (Fig. 2). The average peak potential was still more positive, +32·3 mV (S.E.M. = 2·3,

TABLE 2. Composition of patch-clamp solutions (in mM)

	Internal solutions								
Solution	1	2	3	4	5	6	7	8	9
KCl	—	—	—	—	—	—	—	155	5
Potassium glucuronate	—	—	—	—	—	—	—	—	140
NaCl	—	—	—	—	—	—	—	5	5
TEA-Cl	110	108	106	104	102	70	—	—	—
TEA-F	—	—	—	—	—	—	70	—	—
KOH	—	—	—	—	—	—	—	—	10
TEA-OH	83	83	83	83	83	43	43	—	—
H_4BAPTA	20	20	20	20	20	10	10	—	—
K_2EGTA	—	—	—	—	—	—	—	10	10
$CaCl_2$	8	10	12	14	16	3	4	—	—
HEPES	10	10	10	10	10	10	10	10	10
Glucose	—	—	—	—	—	140	110	10	10
Free Ca^{2+} (10^{-7} M)	0·70	1·07	1·61	2·50	4·29	?	?	< 0·1	< 0·1

	External solutions						
Solution	Cl^-	Br^-	I^-	Acetate	SO_4^{2-}	NO_3^-	Glucuronate
TEA-Cl	160	—	—	—	30	—	30
TEA-Br	—	150	—	—	—	—	—
TEA-I	—	—	160	—	—	—	—
TEA acetate	—	—	—	140	—	—	—
TEA-OH	4	4	3·5	3	143	143	118
Glucuronic acid	—	—	—	—	—	—	115
HNO_3	—	—	—	—	—	140	—
H_2SO_4	—	—	—	—	70	—	—
$CoCl_2$	5	5	5	5	5	5	5
HEPES	10	10	10	10	10	10	10
Glucose	25	5	20	5	20	10	5
Junction potential (mV)	−8·7	−9·0	−9·5	+1·6	+5·0	−6·7	+2·0

All solutions were adjusted to a pH of 7·2 using TEA-OH or KOH and an osmolarity of 300 mosM using glucose. Liquid junction potentials were measured by filling a patch pipette with internal solution 6, adjusting the potential to 0 mV in internal solution, then measuring the potential in external solution. A 3 M-KCl broken-tip electrode was used for reference. The free calcium levels for internal solutions 6 and 7 are not given because 10 mM-BAPTA does not accurately control the internal calcium in myoballs.

n = 15), when 94% of the external chloride was replaced with glucuronate. Since the shift in peak potential was less for acetate than for glucuronate, it is likely that acetate was significantly more permeable than the larger anion glucuronate during the LDS. A possible complication of these experiments is that these substitute anions may have entered the cells and altered the internal pH. This is of concern, since pH is known to have a dramatic effect on some chloride currents (Hutter & Warner, 1967*a*). Voltage-clamp experiments in which we directly controlled internal ion concentration and pH clearly demonstrate the lesser permeability of these larger anions relative to chloride (see selectivity section below).

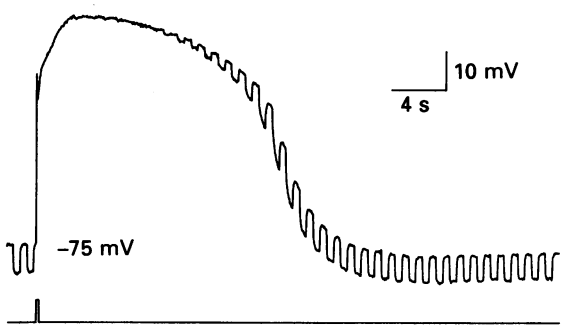

Fig. 1. Activation of the long-duration spike resulted in a dramatic increase in the input conductance of myotubes. The membrane potential of this myotube was initially adjusted to −75 mV by passing a steady hyperpolarizing current through the intracellular microelectrode. Hyperpolarizing current pulses (500 ms, −0·4 nA) were given every second in order to monitor the input conductance of the cell. A 200 ms, 2 nA depolarizing current pulse was given in order to activate the long-duration spike (LDS). The cell was bathed in the normal solution.

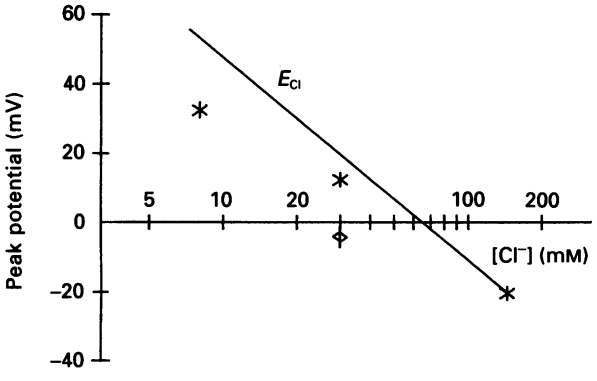

Fig. 2. The peak potential of the LDS shifted to more positive values as the chloride concentration in the external solution was reduced. Each point represents the average of the peak potential of the LDS from ten to twenty-five cells. The normal-, intermediate- and low-chloride blocking solutions contained 144, 30 and 8 mM-chloride respectively. Chloride was replaced with the larger anions acetate (◇) or glucuronate (×). The line indicates the shift predicted from the assumption that the peak of the LDS depends only on the chloride equilibrium potential (E_{Cl}). Error bars represent the S.E.M.

The initial increase in peak potential over the first few stimuli was probably the result of a gradual rise in the internal chloride concentration due to leakage from the 3 M-KCl electrode. Consistent with this idea was the observation that when myotubes were impaled with microelectrodes filled with 3 M-potassium acetate, the peak potential of the LDS generally became more negative over time, suggesting that the less permeant anion acetate may have been displacing internal chloride. The average peak potential for cells bathed in the standard blocking solution and impaled

with potassium acetate electrodes was −46·5 mV (S.E.M. = 2·6, n = 10). The shifts in peak potential with glucuronate substitution were in the expected direction, but not quite as large as the calculated changes in E_{Cl} from the Nernst equation. This most probably was due to additional conductances that were activated by the depolarization. For instance, the concentration of TEA we used blocks most, but not all, of the voltage-dependent potassium conductance.

Extracellular calcium dependence of the LDS

It had been reported that either manganese or cobalt could eliminate the LDS when added to the extracellular solution (Kano & Shimada, 1973; Fukuda, 1974; Kano, 1975). This result could be interpreted in two ways. Either the transition metals were blocking voltage-dependent calcium channels, or they were directly affecting the chloride conductance. We first verified that replacing calcium with cobalt eliminated the LDS and then tested the effects of simply lowering the external calcium on the LDS. In a blocking solution that contained 1 mM-calcium and 4 mM-magnesium, twenty out of twenty myotubes produced an LDS upon depolarization from −70 mV. When the calcium was lowered to 0·1 mM, none of twenty myotubes tested produced an LDS (Fig. 3). Since the LDS depended on the extracellular calcium concentration being above a minimal level, the disappearance of the LDS with manganese or cobalt is most probably explained by their ability to block voltage-dependent calcium channels.

We next asked whether the LDS could be activated subsequent to strontium or barium spikes, since these ions permeate many calcium channels. When the normal concentration of calcium (4 mM) in the blocking solution was replaced with strontium, depolarization from −70 mV activated a rapid spike and an LDS very similar to those seen with external calcium. However, when the same experiment was repeated with external barium, short barium spikes (100–500 ms duration) were observed in many of the cells, but an LDS was never seen (Fig. 3). This result indicated that it was most probably calcium entry into cells and not current through the calcium channels (at least when carried by barium) that is necessary for activation of the LDS. Such a result is consistent with a number of other calcium-dependent processes which are less sensitive to barium, e.g. calcium-dependent potassium channel activation and calcium-dependent calcium channel inactivation (Meech, 1974; Eckert & Tillotson, 1981).

Intracellular calcium dependence of the LDS

To test the role of intracellular calcium in activating the LDS, we eliminated calcium influx by bathing myotubes in the cobalt blocking solution. Intracellular calcium was manipulated in these experiments by including calcium in a second intracellular microelectrode. In the first set of experiments, myotubes were impaled with two microelectrodes, one containing 3 M-KCl, and the other containing 20 mM-$CaCl_2$. Passing depolarizing current through the KCl electrode gave only a passive response, while passing depolarizing current through the calcium electrode (which would be expected to inject calcium into the cell) elicited an LDS in all thirteen cells tested (Fig. 4). This protocol did not allow us to separate the calcium dependence from any

voltage dependence of the response, since it was necessary to depolarize cells in order to inject calcium. However, a variation on this basic experiment allowed us to show that the LDS depended on voltage as well as on the internal calcium concentration.

Myotubes were again impaled with two microelectrodes, but in this case one was

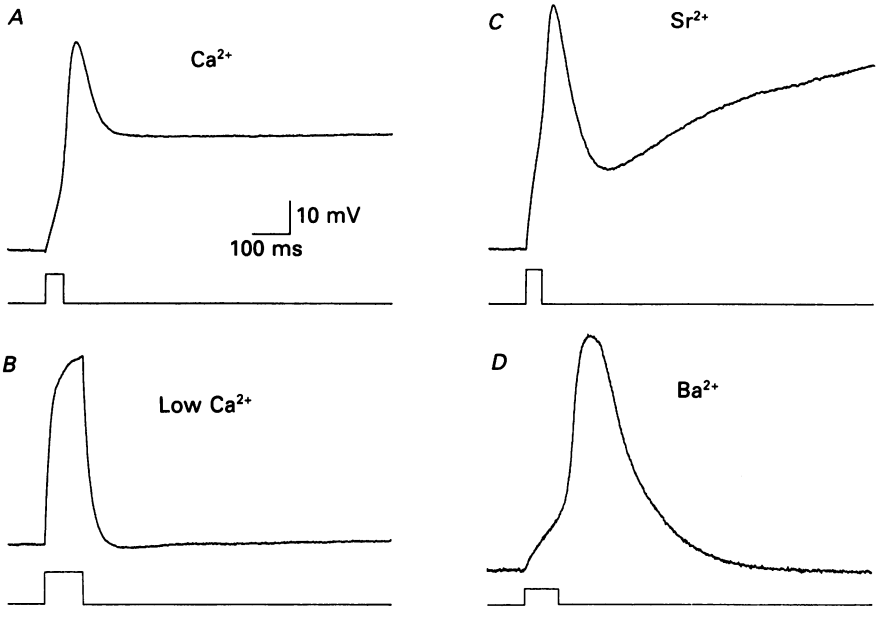

Fig. 3. Millimolar concentrations of calcium and strontium support activation of the LDS, while barium does not. Each cell was initially adjusted to −70 mV, then stimulated with a depolarizing current pulse (duration indicated by lower trace). *A*, a 50 ms, 0·5 nA pulse activated a calcium spike and an LDS from this cell bathed in 4 mM-calcium (standard) blocking solution. *B*, a 100 ms, 1 nA pulse failed to activate an LDS from this cell bathed in 0·1 mM-calcium blocking solution. *C*, a 50 ms, 1 nA pulse activated a strontium spike and an LDS from this cell bathed in 4 mM-strontium blocking solution. *D*, a 100 ms, 0·2 nA pulse activated a barium spike but not an LDS from this cell bathed in 4 mM-barium blocking solution.

filled with 3 M-KCl plus 20 mM-$MgCl_2$ (in order to test the specificity of the divalent cation), and the other was filled with 3 M-KCl plus 20 mM-$CaCl_2$. Fibres were first penetrated with the Mg–KCl electrode. Depolarization with this electrode from −70 mV did not elicit an LDS in any of the five cells tested. However, we found that once the Ca–KCl electrode had been in a cell for a minute or more, passing depolarizing current through either the Ca–KCl or the Mg–KCl electrode elicited an LDS in all five myotubes (Fig. 5). Thus under these conditions an LDS could be elicited by depolarization alone, i.e. without a simultaneous injection of calcium. We interpret this experiment as follows: the high osmotic strength of the 3 M-KCl led to a flow of solution from the pipette into the myotube. When the electrode contained calcium as well as KCl, the calcium was swept along by bulk flow, leading to a persistent rise in intracellular free calcium. At this elevated calcium level, depolarization alone was sufficient to activate the LDS. In support of this idea, within a minute or two after

the Ca–KCl electrode was removed, an LDS could no longer be elicited by depolarization through the Mg–KCl electrode. These results also demonstrate that magnesium was unable to activate the LDS. In additional experiments using this same protocol we found that intracellular loading with barium, cobalt, or nickel could not restore the ability of cells to display a voltage-dependent LDS, but that as expected from the earlier results, strontium loading was able to restore this ability.

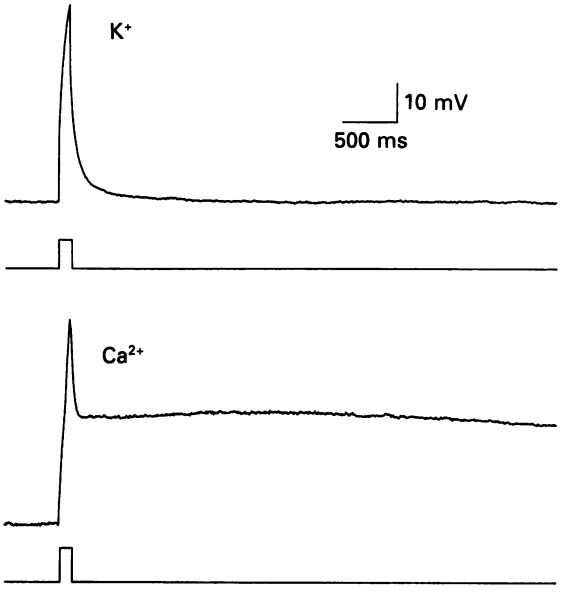

Fig. 4. Intracellular injection of calcium activated the LDS. This cell was bathed in a blocking solution containing 4 mM-cobalt (no calcium) in order to study the effect of intracellular calcium on the LDS. The cell was then impaled with two microelectrodes, one containing 3 M-KCl and the other containing 20 mM-$CaCl_2$. A 100 ms, 1 nA depolarizing pulse through the KCl electrode did not elicit an LDS. However, when the cell was depolarized to the same potential with the $CaCl_2$ electrode, an LDS was activated. In each case, the membrane potential was initially adjusted to −70 mV.

A calcium-dependent chloride current

Although ejection from calcium-containing pipettes allowed us to show a calcium dependence of the chloride current, the intracellular calcium concentration was unknown. To study the properties of the chloride current at known calcium concentrations, we turned to recording from 3- to 6-day-old myoballs (fused muscle cells which have not elongated) using the whole-cell patch-clamp configurations. This technique allows for rapid exchange of the cytoplasm with the pipette solution (Fenwick, Marty & Neher, 1982). In order to isolate chloride currents, TEA was used exclusively as the intra- and extracellular monovalent cation, and cobalt was used as the extracellular divalent cation. We buffered the calcium concentration of the pipette solution with 20 mM-BAPTA (bis(*o*-aminophenoxy)ethane-N,N,N′,N′,-tetra-acetic acid; see below). When no calcium was added to the pipette solution (free

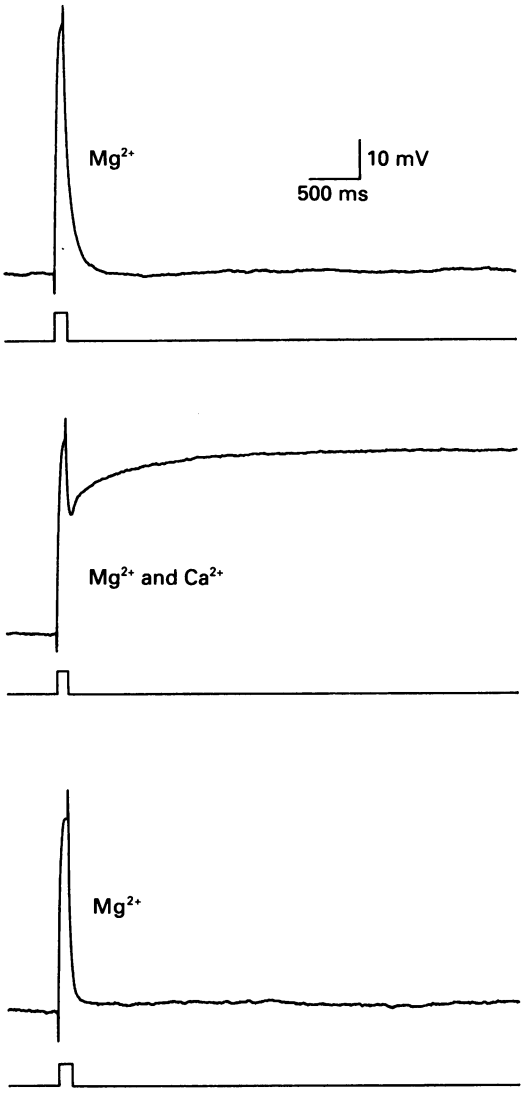

Fig. 5. The LDS is voltage dependent as well as calcium dependent. This cell was bathed in a blocking solution containing 4 mM-cobalt (no calcium) in order to examine the effect of intracellular calcium on the LDS. The cell was first impaled with a microelectrode containing 20 mM-$MgCl_2$ plus 3 M-KCl. Depolarization did not elicit an LDS (top trace). The cell was then impaled with a second microelectrode containing 20 mM-$CaCl_2$ plus 3 M-KCl. Depolarization through the Mg–KCl electrode now elicited an LDS (middle trace). Finally, the Ca–KCl electrode was removed from the cell, and depolarization no longer elicited an LDS (bottom trace). In each case, depolarization was achieved with a 100 ms, 1 nA pulse through the Mg–KCl electrode, with the membrane potential initially adjusted to −70 mV.

calcium $< 10^{-8}$ M), depolarizing steps up to +40 mV from a holding potential of −100 mV elicited little or no voltage-dependent current. However, when cells were dialysed with a solution that buffered the internal free calcium concentration at $2{\cdot}5 \times 10^{-7}$ M, depolarizing steps from −100 to −20 mV elicited a slowly increasing

inward current, and repolarization was accompanied by a much larger inward tail current (Fig. 6). With steps to more positive potentials, a clear reversal of the voltage-dependent current (near −10 mV) could be demonstrated. We also examined whether a calcium-dependent chloride current was present in mononucleate

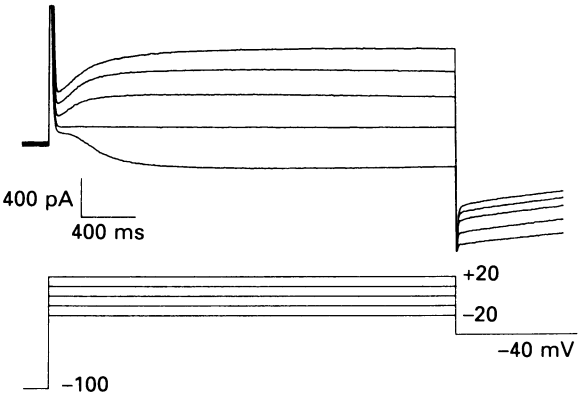

Fig. 6. Voltage-dependent currents from a myoball internally dialysed with $2{\cdot}5 \times 10^{-7}$ M-free calcium. Whole-cell voltage clamp of myoballs was used to study the calcium and voltage dependence of the chloride current. This cell was held at −100 mV and received 3 s steps to the potentials indicated, followed by a repolarization to −40 mV. This myoball was bathed in the TEA-Cl external solution, and dialysed with the TEA-Cl internal solution that buffered the free calcium concentration at $2{\cdot}5 \times 10^{-7}$ M. Leakage currents have not been subtracted.

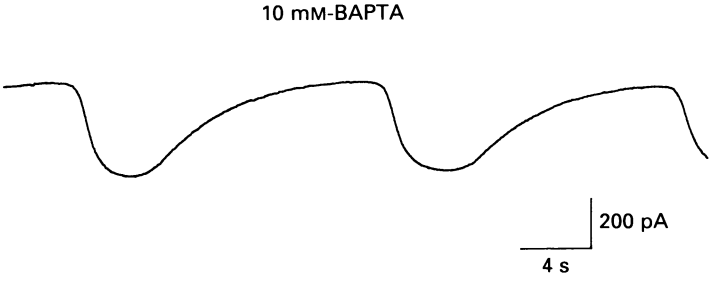

Fig. 7. Cyclic activation of the chloride current when internal calcium was buffered with 10 mM-BAPTA (internal solution 6). The potential of this myoball was clamped to −100 mV. After being dialysed for approximately 1 min with internal solution (Table 2), the holding current of this cell began to oscillate between −170 and −535 pA with a period of about 18 s. The oscillations lasted for several minutes. Given the ionic conditions, these oscillations are likely to represent cyclic activation of the calcium-dependent chloride current. Since the potential was held constant it appears that 10 mM-BAPTA is insufficient to clamp the intracellular calcium concentration. This cell was bathed in the TEA-I external solution but similar oscillations were seen in cells bathed in different external solutions.

myoblasts. We found that myoblasts possessed very little or no voltage-dependent chloride current when the internal free calcium concentration was buffered at 10^{-8} M, while over half of these cells had such a current when the internal free calcium concentration was buffered at $3{\cdot}2 \times 10^{-7}$ M.

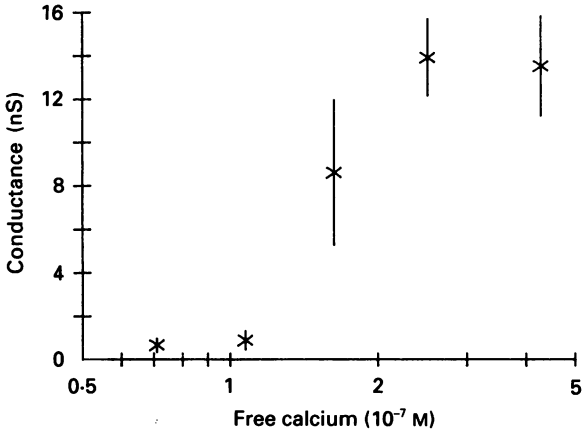

Fig. 8. Calcium dependence of the voltage-activated chloride current. The chloride conductance increased dramatically in the range of 1 to 3×10^{-7} M-internal free calcium. Currents were activated by 3 s step depolarizations to 0 mV from a holding potential of −100 mV. Conductance was calculated using Ohm's law, based on the reversal potential and the tail current present when the potential was repolarized to −40 mV. The conductance was normalized to 100 pF of cell capacitance in order to correct for variations in surface area. Each point represents data from four cells. Myoballs were dialysed with internal solutions 1–5 in Table 2, and bathed in the TEA-Cl external solution. Error bars represent the S.E.M.

TABLE 3. Anion selectivity of the calcium-dependent chloride current

Anion	[Anion](mM)	E_{rev}, mean ± S.E.M. (mV)	N	pAnion/pCl
Iodide	160	−58·0 ± 0·7	4	3·89
Nitrate	140	−42·3 ± 1·7	4	3·23
Bromide	150	−34·2 ± 1·4	5	1·56
Chloride	160	−24·6 ± 1·6	7	1
Acetate	140	+15·1 ± 4·2	7	0·18
Fluoride	Internal	−64·8 ± 1·7	5	0·11
Sulphate	70	+9·4 ± 1·8	9	0·03
Glucuronate	115	+10·3 ± 0·2	3	0·03

Anion permeability decreased as the hydration radius increased. Reversal potentials were estimated during activation of the current by varying the potential of step-depolarizations. If the reversal potential was negative to the activation range, then reversal was estimated during deactivation by varying the potential of step-repolarizations. Except when fluoride was being tested, myoballs were dialysed with internal solution 6 and bathed in the appropriate external solution in Table 2. Reversal potentials were corrected for liquid junction potentials between the internal and external solutions (Table 2). Because fluoride caused the cobalt to precipitate out of the external solution, we estimated fluoride permeability by bathing the cells in the chloride-containing external solution, and dialysing them with a 70 mM-fluoride internal solution (internal solution 7) that had a junction potential of −12·0 mV. Permeability ratios were calculated with the Goldman equation using the shift in reversal potential between the test anion and chloride.

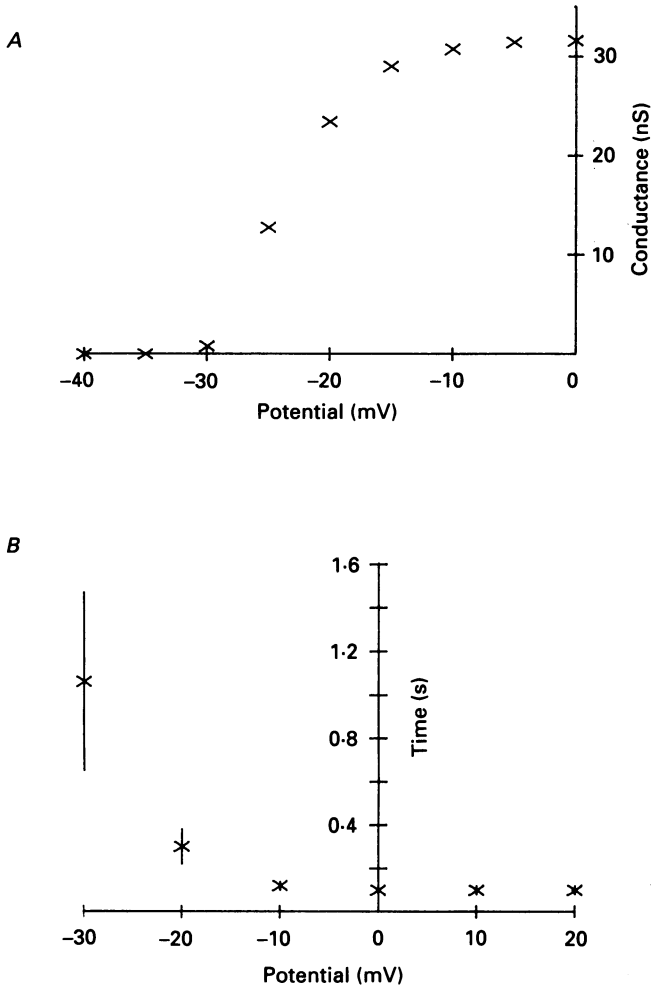

Fig. 9. Voltage and time dependence of the chloride conductance for $2{\cdot}5 \times 10^{-7}$ M-internal free calcium. *A*, conductance increased steeply between −30 and −20 mV for this cell. In some other cells the activation curve was shifted to the left by up to 5 mV. Currents were activated by 3 s step-depolarizations from −100 mV to the potentials indicated. The cell was then repolarized to −40 and the conductance was calculated from the amplitude of the tail currents. Thus there was an identical driving force for each measurement. *B*, the rate of activation was slowest at the foot of the activation curve, and reached a plateau around 0 mV. Cells were stimulated as in part *A*. The rate of activation was estimated by measuring the time to half-maximal activation from the beginning of the step. Error bars represent the S.E.M. For both parts *A* and *B*, cells were dialysed with internal solution 4 ($2{\cdot}5 \times 10^{-7}$ M-free calcium), and bathed with the TEA-Cl external solution.

In many cells depolarization beyond +40 mV activated a large increase in chloride current independent of the internal free calcium concentration. This current was clearly different from the calcium-dependent chloride current since single channels of very large conductance were readily observed above +40 mV. These channels are

probably the same as those described by Schwarze & Kolb (1984). Thus we decided to study the calcium-dependent chloride current at potentials up to +20 mV to avoid contamination with the large-conductance chloride channels.

In our initial experiments we used 10 mM-BAPTA, but we found that this was insufficient for controlling the intracellular calcium concentration. In some cells the lack of adequate calcium buffering was obvious. Approximately 1 min after rupturing the cell membrane, these cells (held at −100 mV) began to show cyclic activation and deactivation of a large inward current (Fig. 7). We suspect that this current reflects oscillations in intracellular calcium concentration leading to cyclic activation of the calcium-dependent chloride current, since it was not observed when cells were dialyzed with the same nominal intracellular free calcium level, but with 20 mM-BAPTA. In other cells buffered with 10 mM-BAPTA, the lack of control of intracellular calcium concentration had more subtle effects. The kinetics of the currents studied at the same nominal calcium level varied greatly from cell to cell, as did the voltage range of activation. When BAPTA was increased to a concentration of 20 mM, the kinetics and activation range of the chloride current were much more reproducible. Evans & Marty (1986) also noted that high buffer concentration was necessary to study a calcium-dependent chloride channel.

In order to examine the calcium dependence of the voltage-dependent chloride current, five calcium concentrations in the range of $0{\cdot}71$–$4{\cdot}29 \times 10^{-7}$ M were tested. We measured the conductance activated by a 3 s depolarizing step from −100 to 0 mV, and found that the chloride conductance was very steeply dependent on calcium concentration. Although there was little activation of the current at less than 10^{-7} M-calcium, it was maximally activated by 3×10^{-7}M. Such a steep dependence of activation on the calcium concentration implies a highly co-operative process, with the binding of several calcium ions required to open the channel. At internal free calcium concentrations above 5×10^{-7} M, voltage-dependent chloride currents activated by depolarization from −100 to 0 mV could still be observed, but often large 'leakage' currents, which reversed near the chloride equilibrium potential, developed at the holding potential. This result suggests that as the internal calcium concentration increased, the voltage dependence of activation (see below) shifted to more negative values.

Anion selectivity of the calcium-activated chloride current

We tested the selectivity of the chloride channels by measuring the reversal potential of voltage-activated currents. We made whole-cell recordings from myoballs bathed by solutions in which most of the chloride on one side of the membrane was replaced by another anion (Table 2). In most solutions a clear reversal of the voltage-dependent current from inward to outward occurred within the range of activation, but for some solutions the current was outward at all activating potentials (> −40 mV). In these cases we gave a step to 0 mV to activate the current, and then varied the magnitude of the repolarizing step so that we could estimate the reversal potential from the tail currents. The reversal potential measurements indicated that permeability was inversely proportional to the hydrated radius of the anion, with a selectivity series of $I^- > NO_3^- > Br^- > Cl^-$ > acetate > F^- > sulphate = glucuronate (Table 3). The calculated permeability for

the large anions sulphate and glucuronate was 0·03 times that for chloride, indicating that these anions are nearly or completely impermeant.

Voltage dependence of the chloride current

In order to study the voltage dependence of activation and the kinetics, we chose an internal free calcium concentration of $2{\cdot}5 \times 10^{-7}$ M. Cells were held at −100 mV and depolarized to various potentials for 3 s and then repolarized to a fixed potential. To eliminate any possible complications of a non-linear current–voltage relationship we estimated activation by measuring the initial amplitude of the tail currents. The foot of the activation curve varied between −35 and −30 mV depending on the cell, but in all cells the voltage dependence was very steep, with approximately 80% of the maximum conductance being activated within a 10 mV range (Fig. 9*A*).

We also measured the kinetics of the currents evoked at this calcium level. Following a step depolarization activation of the current was sigmoidal, so we chose to estimate the rate of activation by the time it took for the current to reach half of its maximum. This time was slowest for the most negative activating potentials, sometimes taking greater than 1 s. At more positive potentials the time to reach half-maximal current became shorter, eventually reaching a minimum value of approximately 100 ms (Fig. 9*B*). Once activated by depolarization, the chloride current could be maintained for more than 30 s without showing signs of inactivation. Upon repolarization to negative potentials the inward tail currents declined with a slow exponential time course (time constant = 1·5 s at −50 mV).

In summary, the calcium-dependent chloride current exhibits voltage-dependent activation at a fixed internal calcium concentration. The time course of activation ranges from approximately 100 ms to greater than 1 s. The kinetics and reversal potential of this current correspond well within the calcium-dependent, long-duration spike observed in unclamped myotubes.

Effect of the chloride current under physiological conditions

The physiological role of a depolarization-activated increase in chloride conductance in developing chick muscle is unknown. In order to assess its role, it is necessary to know the normal resting potential and chloride equilibrium potential of myoblasts and young myotubes. The normal value of the resting potential in developing chick muscle is controversial. Fischbach, Nameroff & Nelson (1971) presented data from intracellular recordings suggesting that the resting potential of myoblasts and small myotubes might be as little as −20 mV. However, Entwistle, Zalin, Bevan & Warner (1988) presented two independent lines of indirect evidence suggesting that the resting potential of myoblasts is much more negative (near −70 mV). To resolve this issue we made whole-cell current-clamp recordings from mononucleate myoblasts. For each cell we determined the zero-current potential, which should be equal to the resting potential. In these experiments there was no calcium added to the internal solution, so the free calcium concentration was less than 10^{-8} M. When the zero-current potential was measured immediately upon rupturing the cell membrane, the potential differed widely between cells and was often quite small. In mononucleate myoblasts the zero-current potential was as low as −6 mV, and averaged only −39·0 mV (S.E.M. = 5·6, $n = 17$). However, after

allowing 30 s for the internal solution to fully dialyse the cell, the zero-current potential reached a much more negative value of −68·8 mV (S.E.M. = 2·7, $n = 16$). The results were similar whether the internal solution was high (internal solution 8, $n = 10$) or low (internal solution 9, $n = 6$) in chloride, indicating that the membrane was permeable mainly to potassium. A corollary of these results is that the internal potassium in some myoblasts was unusually low. It seemed possible that the low resting potentials found by Fischbach *et al.* (1971) and by us are an artifact of the recording methods. The sodium–potassium pump is known to be highly temperature dependent, and all the recordings described so far were made at room temperature. Thus the normal mechanism that regulates intracellular potassium is inhibited under these experimental conditions. Myoblasts are quite elongate cells, and have a high surface area to volume ratio, so their internal potassium store should be more rapidly depleted at room temperature than is the case for larger myotubes. To test this idea, we made whole-cell current-clamp recordings from myoblasts at 37 °C. To approximate normal conditions, the cells were bathed in bicarbonate-buffered solution (MEM bubbled with 5% CO_2). We found that the resting potential of myoblasts was quite negative from the moment that whole-cell recordings were established (mean = −54·4 mV, S.E.M. = 4·3, $n = 7$), and did not differ greatly from the resting potential of multinucleate myotubes measured under the same conditions with intracellular recordings (mean = −63·5 mV, S.E.M. = 3·9, $n = 16$). It thus seems likely that under normal physiological conditions myoblasts, like myotubes, have a resting potential more negative than −50 mV.

A rough estimate of the chloride equilibrium potential in myotubes can be made by comparing the peak potentials of the LDS immediately following impalement with KCl and potassium electrodes. As noted earlier, KCl electrodes tend to increase internal chloride over time, while potassium acetate electrodes tend to decrease internal chloride over time. Because the increase in chloride conductance observed in blocking solution is very large compared to the other conductances, the peak of the LDS is very close to the chloride equilibrium potential. Thus measurements made with KCl and potassium acetate electrodes soon after penetration should place upper and lower limits on the actual chloride equilibrium potential. In cells initially adjusted to −70 mV, the peak potential of the first LDS elicited following impalement with a KCl electrode was on average −29·8 mV (S.E.M. = 1·2, $n = 34$), while the initial value soon after penetration with potassium acetate electrodes was −42·7 mV (S.E.M. = 2·9, $n = 10$). The chloride reversal potential under our standard recording conditions is therefore probably somewhere within this range. We were concerned that our standard recording conditions (room temperature, no bicarbonate in the blocking solution) might have caused internal chloride to take on an abnormal value. We therefore made a series of recordings from myotubes maintained at 37 °C in bicarbonate-buffered MEM. Under these conditions most myotubes initially adjusted to near −70 mV still displayed long-duration spikes upon depolarization. However, the amplitude and duration of the LDS was not as large as at room temperature. When recordings were made with potassium acetate electrodes, the mean duration of the LDS was 1·04 s (S.E.M. = 0·13, $n = 12$) and the LDS reached a mean peak potential of −55·9 mV (S.E.M. = 2·5, $n = 16$). It should be noted that under normal physiological conditions, the peak of the LDS is not as good an

indicator of the chloride equilibrium potential as it is in the blocking solution. This is because without TEA in the medium, depolarization activates a substantial potassium conductance in addition to the chloride conductance. For example, when cells were stimulated from an initial potential of −50 mV, the peak of the LDS was still 5–10 mV positive to −50 mV. This result indicates that the normal chloride reversal potential of myotubes was positive to −40 mV, as was suggested by the initial impalements of myotubes in the blocking solution. Because internal dialysis is rapid with whole-cell recording, we were not able to determine the chloride equilibrium potential of myoblasts under normal conditions. However, since myoblasts and myotubes have similar resting potentials, most probably their chloride equilibrium potentials are also similar. If so, activation of the chloride current will depolarize myoblasts as well as myotubes (see also Entwistle *et al.* 1988).

DISCUSSION

Previous investigations strongly suggested that an increase in chloride conductance is responsible for the long-duration spike (LDS) of developing chick muscle. However, a role for calcium ions was also implicated. The results reported here confirm that the chloride gradient drives the LDS, and demonstrate that calcium is necessary for activation of the response. Changing the external chloride concentration, or preventing a rise in intracellular chloride by using potassium acetate in the intracellular microelectrode both affected the peak potential of the response. On the other hand, lowering the external calcium concentration eliminated the response. In the absence of extracellular calcium, intracellular injection of calcium or strontium, but not magnesium, barium, nickel or cobalt restored the ability of depolarization to activate the response.

Whole-cell voltage-clamp studies of myoballs revealed the presence of a voltage-dependent current whose reversal potential depended on the chloride gradient, and whose magnitude was steeply dependent on the intracellular free calcium concentration. When the internal calcium was set at 10^{-7} M or lower, little chloride current could be evoked at any potential we tested. Between 1 and 5×10^{-7} M-internal free calcium it was necessary to depolarize cells in order to elicit chloride currents. In contrast, when internal calcium was set above 5×10^{-7} M, we saw not only voltage-dependent chloride currents, but in addition a large chloride 'leakage' conductance at our holding potential of −100 mV. A possible molecular explanation of these observations is suggested by studies of the large-conductance calcium-activated potassium channels. Barrett, Magleby & Pallotta (1982) showed that for a fixed potential, the fraction of the time that these channels spend in the open state increases with the internal free calcium concentration, and that for a fixed calcium concentration the probability of being open increases with depolarization. At sufficiently high calcium levels, these channels spend a substantial portion of the time in the open state, even at quite negative potentials. The calcium-dependent chloride channels of chick muscle may behave in a similar function over the sub-micromolar range of internal free calcium.

Similar calcium-dependent chloride conductances have been reported in other cell

types from a variety of organisms. For example, calcium-dependent chloride currents have been demonstrated in mouse spinal neurones (Owen *et al.* 1984, 1986), rat sensory neurones (Mayer 1985), frog oocytes (Miledi & Parker, 1984), and plant cells (Lunevsky, Zherelova, Vostrikow & Berestovsky, 1983). In some systems, the dependence on calcium has been tested directly by manipulating the internal free calcium concentrations. This technique has permitted investigation of the chloride current independent of the properties of the voltage-dependent calcium currents. Evans & Marty (1986) found that the calcium-dependent chloride current of rat lacrimal gland cells is almost fully activated at all potentials positive to -100 mV for 10^{-6} M internal free calcium. In contrast, they observed a significant voltage dependence of activation over an 80 mV range for 5×10^{-7} M-calcium. Evans & Marty (1986) and Korn & Weight (1987) both found that large inward leak currents often developed for internal calcium buffered above 5×10^{-7} M with EGTA. Our results were quite similar in that calcium levels greater than 3×10^{-7} M often gave large leakage currents, although we still saw some voltage-dependent current when calcium was set as high at 10^{-6} M. In all of the cases that have been examined so far, the kinetics of calcium-activated chloride currents are relatively slow, their time constants of activation being around several hundred milliseconds.

What physiological role might a calcium-activated chloride current play in myoblasts? Entwistle *et al.* (1988) have shown that three different processes which depolarize myoblasts can trigger their fusion, forming myotubes. The common feature appears to be that depolarization activates voltage-dependent calcium channels, and that a rise in internal free calcium is the actual trigger for fusion. Of relevance is their observation that one particular agent that promotes fusion, PGE_1 (prostaglandin E_1), seems to depolarize myoblasts by activating a choride-dependent process. Might the calcium-dependent chloride current we have studied be the current activated by PGE_1? Since activation of this chloride current requires a rise in internal calcium, it might seem that the myoblasts would be fusion competent even before the current was activated. However, the dose–response relation between intracellular free calcium and fusion competence has not yet been determined. The chloride current we have studied is activated at relatively low calcium concentrations, and has a steep calcium dependence. It could potentially be part of a regenerative cascade in which either a small initial increase in intracellular calcium (perhaps released from an internal store) or a transient depolarization is sufficient to trigger activation of the chloride current. However, a larger influx of calcium, triggered by the opening of calcium channels during the long-duration spike, may be necessary to activate the fusion program.

There are potential roles for the calcium-dependent chloride current in multinucleate myotubes as well. Developing myotubes undergo bursts of repetitive contractions *in vitro* (Fischbach *et al.* 1971) and *in vivo* (Hamburger & Balaban, 1963; Hamburger, Balaban, Oppenheim & Wenger, 1965). It is thought that these bursts are caused by cyclic waves of depolarization which induce calcium influx through voltage-dependent calcium channels, and possibly calcium release from the sarcoplasmic reticulum as well (Saito & Ozawa, 1986*b*). The processes that trigger the bursts are not known, but our work suggests a hypothesis. In addition to the

calcium-dependent chloride current, skeletal muscle is known to have a calcium-dependent potassium current. The calcium dependence of the potassium current has been studied in detail, and it is clear that substantially higher calcium levels are necessary to activate the potassium current than to activate the chloride current (Barrett *et al.* 1981). Thus an oscillatory cycle could potentially occur. A cycle could be triggered by a rise in intracellular calcium sufficient to activate the chloride current at the resting potential, or by a transient synaptic depolarization. The initial depolarization would be regenerative, further activating the chloride current and also opening voltage-dependent calcium channels. The chloride current would soon be maximally activated. As the internal calcium rose it would evoke contraction, but eventually the calcium level would rise enough to activate the calcium-dependent potassium conductance, which would repolarize the cell and terminate the burst. As intracellular calcium fell, the fibre would relax and when intracellular calcium fell sufficiently a new cycle could be initiated.

There are also interesting developmental changes in the chloride conductance of chick muscle. Poznansky & Steele (1984) have demonstrated that at 1 week prior to hatching, there is little detectable resting chloride conductance in chick fast-twitch muscle. However, by the time of hatching, 70% of the resting membrane conductance is due to chloride, as is also found in the adult (Lebeda & Albuquerque, 1975). This large chloride conductance is thought to stabilize the resting potential. Kano (1975) showed that, over the last week of embryonic development, the percentage of cells exhibiting an LDS first waxes, then wanes. By a few days after hatching, no cells show an LDS. Since we show here that a calcium-activated chloride current underlies the LDS, these two simultaneously occurring developmental changes could be related. For instance, might the resting internal free calcium concentration rise enough so that the chloride channels are fully activated at the resting potential? Our results indicate that an internal calcium level of above 5×10^{-7} M would be required to activate most chloride channels at -100 mV. Measurements made on skinned vertebrate muscle fibres indicate that this level of calcium is sufficient to elicit a significant fraction of the maximal tension in some preparations (Hellam & Podolsky, 1967; Stephenson & Williams, 1981), and measurements made from intact amphibian muscle fibres with calcium-sensitive microelectrodes suggest that the resting free calcium of skeletal muscle is only about 1×10^{-7} M (Tsien & Rink, 1980). It is therefore unlikely that the average free calcium level in resting chick muscle is as high as 5×10^{-7} M. An alternative explanation of the developmental changes is that the calcium-dependent chloride channels stop being expressed, and a new type of chloride channel replaces them. The idea that there are two separate chloride channels is supported by our data on anion selectivity. The selectivity series for the amphibian resting chloride conductance (Hutter & Warner, 1967*b*); Woodbury & Miles, 1973) and probably for the adult chicken chloride conductance as well (Morgan, Entrikin & Bryant, 1975), is $Cl^- > Br^- > NO_3^- > I^-$, which is opposite to the sequence that we determined for the calcium-activated chloride current.

A common feature of all of the proposed roles of the calcium-dependent chloride channel during development is that they make predictions about the way in which

intracellular calcium changes during physiological processes. Fluorescent indicators of intracellular free calcium concentration should provide a straightforward means of testing these predictions.

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