TWO VOLTAGE GATED POTASSIUM CURRENTS ON EMBRYONIC XENOPUS SKELETAL MUSCLE CELLS GROWING IN CULTURE

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BY

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ABSTRACT

At least 4 classes of voltage activated potassium currents have been identified on adult frog muscle using macroscopic voltage clamp techniques. This thesis deals with the identification and characterization of two potassium conductances present in the plasma membrane of embryonic Xenopus muscle cells growing in 24 hour old cultures. The tight seal whole cell recording technique was used in this study. One of the currents identified was a hyperpolarization gated inward potassium current thought to be mediated through anomalous rectifying potassium channels. This current activated when the membrane potential was hyperpolarized to values negative to the equilibrium potential for potassium and could be blocked by the external application of 1mM cesium chloride. A second depolarization activated outward current possesses characteristics similar to the delayed rectifier potassium current described on adult frog skeletal muscle preparations. This conductance activated upon membrane depolarizations positive to membrane potentials of -30mV and could be reduced by the external application of 50mM Tetraethylammonium. Kinetic and pharmacological properties of these currents have been examined.

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I INTRODUCTION

I.1 General Introduction

Ionic channels are macromolecular pores in the cell membranes of all excitable and most non-excitable cell types. Physiologically these channels respond to stimuli by permitting trans membrane passive permeations to selective ions. Mechanisms regulating the opening and closing (gating) of ion channels have been categorized into three groups. (1) Ion channels which gate in response to changes in membrane potential are referred to as voltage gated or voltage sensitive ion channels. On electrically excitable cells these voltage gated channels mediate action potentials and modulate the resting membrane potentials. In non-excitable cells voltage gated channels play important roles in signal transduction (Catterall,1988 ; Hille 1984). (2) Ligand gated ion channels respond to extracelluar neurotransmitter molecules or hormones. Some examples of these ionic channel types include the acetylcholine receptors, amino acid receptors and neuropeptide receptors (Catterall,1988). These channel types mediate local changes in ionic conductance when the receptor is bound by the appropriate transmitter and this in turn depolarizes or hyperpolarizes the cell

membrane (Catterall,1988). (3) In addition to regulation by voltage and ligands many ion channels are also regulated by intracellular second messenger systems. For example, single channel recordings on rat skeletal muscle cells have revealed two classes of calcium dependent potassium channels whose conductances appear sensitive to the internal free concentration of second messenger Ca^{+2} ions (Pallotta, Magleby and Barrett,1981 : Blatz and Magleby,1986).

For over one hundred and fifty years scientists have spoken of pores in cell membranes primarily to explain apparent changes in permeabilities. However, quantitative details of these permeability changes remained unsubstantiated until the early 1950's and the sodium theory of Hodgkin and Huxley. The initial breakthrough leading to this theory came almost 20 years prior with the discovery of the giant axon of the squid Loglio. By inserting a conducting wire down the length of the axon it was possible to control the membrane potential (Marmont, 1949). Under these conditions the axon could be said to be voltage clamped and it was possible to directly measure transmembrane flow of ions as well as the electromotive forces and permeabilities underlying them (Hille,1967, 1984).

The analysis of the ionic basis of the action potential by Hodgkin, Huxley and Katz in 1952 (Hodgkin and Huxley,1952(a)(b)(c), Hodgkin, Huxley and Katz, 1952), first revealed the kinetic complexity and ionic selectivity of permeability changes in nerve cells. Voltage clamp recording of membrane currents in the axon of the squid Loglio revealed that the propagated action potential could be accounted for in terms of two ionic channels, a voltage gated sodium channel and a voltage gated potassium channel. The action potential begins when a small depolarization opens voltage gated sodium channels permitting a passive movement of sodium ions into the cell. This passive influx of sodium ions reflects the electrochemical gradient of that ion. The result of this influx of positively charged sodium ions is an amplified depolarization of the neuronal cell, detected by sensors of the voltage gated potassium channels. Since the concentration of potassium ions inside the cell is higher than that of the extracelluar environment voltage gating of potassium channels permits the passive outward flow of potassium ions. The result of this outward movement of potassium ions is a repolarization of cell membrane potentials to original levels.

For a period it appeared that the

electrophysiological response of many other membranes might be explained in similar ways. However it soon became apparent that complex functions of many excitable membranes could not be explained in such simple terms. As voltage clamping techniques evolved it became apparent that no other action potential is as simple as that of the axon. We now realize that there are many distinct conductances in excitable membranes with differing gating, kinetics, and pharmacological properties. Furthermore, for each ionic species it is also known that a diversity of channel types exist and that such diversities appear both across species and within the same organism (for review see Hille,1984 and Rudy,1988). The electrical excitability of skeletal muscle, for example involves voltage and time dependent changes in the membrane permeability of numerous ionic species. The electrical excitation of frog skeletal muscle fibres involves changes in membrane permeability to sodium, potassium, chloride and calcium ions. The fundamental excitable elements underlying these permeability changes are the ionic channels.

While variability has been shown to exist among all types of ionic channels, by far the most impressive diversification exists within that class of channels where potassium is the major current carrier (Hagiwara

1983, Latorre,1989; Rudy,1988). Voltage dependent potassium channels have been studied in skeletal muscle preparations using macroscopic voltage clamp techniques (for review see Stefani and Chiarandini,1982). In the past adult frog skeletal muscle fibres have provided a good model system for the study of potassium channel types. These studies provided numerous kinetic and pharmacological diagnostic properties which today facilitate the separation and identification of potassium channels. To date a number of potassium channel types have been described on adult frog skeletal muscle fibres including a delayed rectifier, two calcium dependent slow potassium currents, and an inward anomalous rectifying potassium current. Each of these current types will be described separately below.

I.2 Outward Potassium Currents

I.2a Delayed Rectifier Potassium Currents

In their original description of the squid giant axon Hodgkin and Huxley (1952) described a depolarization dependent outward potassium conductance which activated with a delay, when compared with the activation of sodium channels at the same membrane potential. Since this original description on the squid

giant axon, delayed rectifier channels have been identified in the membranes of most excitable and many non-excitable cell types. Today the term delayed rectifier refers to a class of functionally similar channels rather than to a unique channel type (Hille, 1984). The delayed potassium conductances are responsible for the repolarization of action potentials in excitable membranes. Adrian, Chandler and Hodgkin (1970) were the first to describe a delayed rectifier current on adult frog skeletal muscle fibres. This current which activated when the membrane potential was more positive than -40mV rises rapidly to a maximum outward conductance then shows a slow time dependent inactivation. While the activation of this current appeared similar to the delayed rectification described in squid giant axon, on muscle cells these currents had a more rapid and complete inactivation under maintained depolarization (Constantin,1968; Stanfield,1970a; Argibay and Hunter,1973).

The reversal potential of the delayed rectifier current was shown to be mainly dependent on potassium ions but did not follow potassium completely. The channels appeared to be at least partially permeable to sodium, resulting in reversal potentials less negative than E_K (Adrian, Chandler and Hodgkin, 1970).

Hodgkin and Huxley (1952c) first demonstrated that the level of the membrane potential (holding potential) can influence membrane conductance. The holding potential at which 50% of the maximum possible outward conductance could be elicited (steady state inactivation) was reported to be -40mV for the delayed rectification on skeletal muscle. This value was approximately 20mV positive to the half activation for sodium currents (Adrian, Chandler and Hodgkin, 1970).

The pharmacology of delayed rectifier potassium conductances have been investigated. Delayed rectification in adult frog skeletal muscle can be blocked by the external application of tetraethylammonium (TEA) (Kao and Stanfield,1970; Stanfield,1970a for review see Stanfield,1983). An external TEA concentration of 50mM reduced the delayed rectification in frog skeletal muscle fibres by approximately 80%. External application of low concentrations of 4-aminopyridine (4AP) suppressed delayed potassium current in adult frog skeletal muscle fibres. The 4AP produced rapid and reversible reductions in delayed outward conductance (Molgo,1978; Fink and Luttgau ,1978; Castle and Haylett,1987; Gillespie, 1977). 3,4-Diaminopyridine (3,4DAP) has

also been shown to be a potent blocker of delayed rectification on squid giant axons, in the micromolar concentration range (Kirsch and Narahashi,1978). Subsequent studies revealed 3,4DAP to be a potent inhibitor of delayed rectification of adult frog skeletal muscle fibres (Cognard, Traore, Potreau and Raymond,1984; Sanchez and Stefani, 1983). Quinine, an alkaloid known to block sodium channels has also been shown to block delayed rectification on neuroblastoma cells (Fishman and Spector,1981). However to date no report of the effect of quinine on delayed rectification in skeletal muscle fibres has been presented.

Delayed rectifier potassium currents described in mammalian skeletal muscle fibres showed similar characteristics to those described above for frog. In preparations of rat skeletal muscle fibres a delayed rectifying current has been described with time constants of activation and inactivation comparable to those observed in frog (Adrian, Chandler and Hodgkin ,1970; Duval and Leoty,1978; Pappone,1980). Furthermore these studies have shown that time to reach peak delayed current decreased as the degree of depolarization increased. Similar to the delayed rectifier on frog muscle, rat fibres showed reversal

potentials close but positive to the calculated equilibrium potential for potassium.

I.2b Slow Outward Potassium Currents

In frog skeletal muscle fibres analysis of potassium tail currents provided the first evidence of a depolarization produced conductance which developed with a time course much slower than the delayed rectifier (Adrian, Chandler and Hodgkin,1970b; Stanfield,1970a). These early investigations revealed a conductance which reached maximum amplitude within 2 seconds (membrane potential of -30mV at 20°C). Maximum conductances observed were approximately one sixth the maximum value of delayed rectification measured at the same membrane potential. Following the maximum amplitude the slow conductance declined to steady state levels within 10 seconds and these were approximately one third the maximum value. The existence of the slow outward conductance has since been confirmed by others on frog skeletal muscle fibres (Almers and Palade,1981; Cognard, Potreau, Traore and Raymond,1984; Traore, Cognard, Potreau and Raymond,1986) and on preparations of rat skeletal muscle fibres (Barrett, Barrett and Dribin,1981; Hughes Schmid, Romey, Duval, Frelin and Lazdunski,1982). Aside from being kinetically distinct

from the delayed rectifier the slow potassium conductance showed equilibrium potentials which were approximately 10mV more negative than the delayed current (Adrian, Chandler and Hodgkin,1970b). This finding suggested that the slow potassium conductance was more selective towards potassium ions than was the delayed rectifier.

In addition to the differences in kinetic and reversal potential properties, pharmacological evidence supports the hypothesis that the slow potassium conductance is mediated by a class of potassium channels which are distinct from the delayed current. For example, on frog muscle, TEA an effective blocker of delayed rectification was found to be only slightly effective at reducing the slow potassium conductance (Stanfield,1970,1975; Almers and Palade,1981). Similar results have been observed on rat skeletal muscle fibres (Romey and Lazdonski,1984). 3,4 Diaminopyridine, a potent inhibitor of delayed rectification had shown little effect on the slow potassium conductance at similar external concentrations (Kirsch and Narahashi,1978; Sanchez and Stefani,1978). Furthermore 4AP which has been shown to be an inhibitor of the delayed rectifier (Gillispie,1977) had no effect on the slow potassium conductance present in frog skeletal

muscle fibres (Fink and Luttgau,1978; Castle and Haylett,1987).

It has been proposed that the slow outward potassium conductance may be composed of two distinct ionic components both of which are controlled by the intracellular free calcium concentration (Stanfield, 1983). Calcium activated potassium currents were first identified on molluscan neurons (Meech,1974) and since described on a number of excitable and non excitable cell types including skeletal muscle (for review see Latorre, Andres, Labarca and Alvarez,1989; Meech,1978 and Petersen and Maruyama,1984). Initial studies showed that in metabolically exhausted frog skeletal muscle fibres (fibres that are exhausted by treatment with cyanide and iodoacetate followed by repetitive stimulation) an increased potassium conductance resulted (Fink and Luttgau,1976). Such fibres were presumed to have a high sacroplasmic free calcium concentration. The potassium current could be blocked by the electrophoretic injection of the calcium chelator H_2EGTA suggesting it was regulated by intracellular Ca^{+2} levels. Subsequently the injection of H_2EGTA buffered calcium ions (10^{-5} M, free calcium) into cells or the application of extracelluar caffeine (which releases Ca^{+2} from the sacroplasmic reticulum)

have both been shown to activate outward potassium conductances on frog skeletal muscle fibres (Fink, Sigrid-Hase, Luttgau and Wettwer,1983) as well as in molluscan neurons (Meech,1974b) and Helix aspera neurons (Meech,1974c). Sanchez and Stefani (1978) later showed that a slow potassium conductance could be abolished completely when external calcium ions were replaced with Mg^{+2}. However this result has not been supported by others (see, Almers and Palade,1981; Traore, Cognard, Potreau and Raymond, 1986) who have reported an increase in the slow outward current upon replacement of external calcium with Mg^{+2}.

Stanfield (1983) first suggested that the slow outward potassium current on adult frog muscle fibres may be composed of two separate ionic conductances each regulated by the internal calcium ion concentration yet distinguishable on the basis of their pharmacological specificities. This has been supported by single channel recordings which have revealed the presence of two distinct classes of Ca^{+2} activated potassium currents.

The first class of calcium activated potassium channels to be identified at the single channel level had large conductances (200-300pS) and were called BK channels. These high conductance channels have since

been identified on most cell types including preparations of cultured rat skeletal muscle cells (Pallotta , Magleby, and Barrett, 1981), adult frog sympathetic neurons (Adams, Constanti, Brown and Clark, 1982) and chromaffin cells (Marty,1981). Activation of BK channels has been shown to be highly voltage dependent (Barrett, Magleby and Pallotta, 1982; Moczlydiowiski and Latorre,1983; Methfessel and Boheim, 1982) and sensitive to the internal concentration of free ionic calcium. Furthermore depolarization has been shown to increase the calcium sensitivity of these BK channels thereby increasing the channels conductance for any given internal calcium concentration (Barrett, Magleby, and Pallotta, 1982). However effective calcium concentrations ranged widely from one tissue type to the next.(Blatz and Magleby,1987). This calcium sensitivity was restricted to the internal side of the cell membrane (Barrett, Magleby, and Pallotta, 1982).

Tetraethylammonium was shown to be an effective blocker of BK channels when applied to the external membrane surfaces. Charybodotoxin, a scorpion venom protein has also been shown to be a potent blocker of BK channels in rat skeletal muscle (Miller,

Moczydlowski, Latorre, and Phillips, 1985). Apamin, which is effective at blocking the SK channels (described below) was ineffective at blocking the BK channels (Romey and Lazdunski,1984). The small TEA^+ sensitive, apamin insensitive component of the slow potassium current present on adult frog skeletal muscle fibres (Cognard, Traore, Potreau and Raymond, 1984) may reflect the contribution of BK potassium channels to this conductance.

A second class of calcium activated potassium channels identified on rat skeletal muscle have a much lower conductance (10-14pS) and have been called SK channels (Blatz and Magleby,1986). The activation of these channels did not appear to be membrane potential dependent but was very sensitive to internal calcium ion concentrations. At negative membrane potentials SK channels appeared to be ten times more sensitive to the internal calcium concentration than were BK channels (Blatz and Magleby,1987). Unlike the BK channels TEA did not affect the activity of SK channels. Apamin a polypeptide protein from bee venom (Habermann, 1972, 1984) has been found to block calcium dependent potassium currents in several cell types (Burgess, Claret and Jenkinson,1981; Hugues, Romey, Duval, Vincent and Lazdunski,1982; Hugues, Schmid, Romey,

Duval, Frelin and Lazdunski,1982; Maruyama, Gallacher and Petersen,1983; Seagar, Granier and Couraud,1984; Cook and Haylett,1985). External apamin blocked SK channels in the nanomolar concentration range (Baltz and Magleby,1986; for review see Lazdunski, Romey, Schmid-Antomarchi, Renaud, Mourre, Hugues and Fosset, 1988). The apamin sensitive, TEA^+ insensitive component of the slow potassium conductance described on preparations of adult frog skeletal muscle fibers (Cognard, Traore, Potreau and Raymond,1984) may be mediated by SK channels. These channels are thought to be localized in the tubular system membrane of muscle cells (Traore, Cognard, Potreau and Raymond,1986).

In summary, two types of calcium activated potassium currents have been identified on adult frog skeletal muscle fibres. This separation has been based upon the selective blocking actions of the bee venom peptide apamin and TEA. The first component was shown to be apamin sensitive but TEA insensitive and may be mediated through SK potassium channels. The SK channels are thought to be the major contributor to the slow potassium conductance (Cognard, Traore, Potreau and Raymond, 1984). A second, smaller component was insensitive to apamin, sensitive to external TEA and thought to be mediated through BK potassium channels.

Similar results have been obtained in preparations of rat skeletal muscle cells where the existence of two comparable classes of calcium dependent potassium channels have been described (Romey and Lazdunski,1984).

In addition to the BK and SK potassium channels described above there have been a number of reports of calcium activated potassium currents whose properties do not place them in either of these two categories (Blatz and Magleby 1987). Such channels have been referred to as other potassium channel types. For example in preparations of cultured rat skeletal muscle cells a potassium channel has been identified which is more sensitive to calcium than either BK or SK channels and has a very small (4pS) conductance (Blatz and Magleby, 1986).

I.3 INWARD POTASSIUM CURRENT

Katz (1949) described membrane properties of the frog skeletal muscle fibre immersed in an isotonic potassium sulfate solution, where K_2SO_4 (60mM) was the only electrolyte. In this solution the potassium concentrations on either side of the membrane were nearly equal and potassium was the sole permeant external ion.

In spite of symmetrical potassium conditions the conductance of frog muscle was high for inward current and low for outward current. This phenomena has been termed anomalous or inward rectification.

Since its original description, anomalous rectification has received the attention of many investigators. The electrophysiological properties of anomalous rectification have been best characterized in frog skeletal muscle fibres (Adrian and Freygang ,1962(a)(b); Almers,1971,1972(a)(b); Hestrin,1981; Hodgkin and Horowitz,1959; Gay and Stanfield,1977; Leech and Stanfield,1981; for review see, Stefani and Chiarandini,1982) and in starfish and tunicate egg cells (Hagiwara and Yoshi 1979, Hagiwara, Miyazaki and Rosenthal,1976; Hagiwara and Takahashi,1974; Ohmori, 1978,1980; Ciani, Kranse, Miyazaki, and Hagiwara,1978, for review see Hagiwara,1983). Hyperpolarization of the membrane to potentials more negative than the equilibrium potential for potassium (E_K) has been associated with a dual component inward current activation. The first part of the inward current activates immediately upon hyperpolarization. This component referred to as the instantaneous rectifying current has a conductance which is dependent upon both the electrochemical driving force for potassium ($V-E_K$)

and the concentration of potassium ions in the extracelluar solution ($[K]_o$). Conductance of this instantaneous current increased as $V-E_K$ became more negative. Following the initial instantaneous activation is a time dependent increase of inward conductance.

Early work on frog skeletal muscle demonstrated that unlike other voltage activated conductances the gating potential of inward potassium conductance was dependent on the external potassium concentration (Hodgkin and Horowitz,1959). For instance, the potential of half activation of inward conductance shifts along the voltage axis with a quantity close to the change in E_K, when $[K]_o$ was altered (Hagiwara and Yoshi,1979; Hestrin,1981). Because of an apparent relationship between $[K]_o$ and gating of inward conductance, current gating was said to be dependent on $V-E_K$ rather than being solely voltage dependent. However other experiments have shown that changes in $[K]_i$ do not produce a similar effect (Takahashi and Yoshi,1978; Hagiwara and Yoshi, 1979; Hestrin,1981; Leech and Stanfield, 1981).

In preparations of adult frog skeletal muscle fibres, the rise to peak inward current was followed

by an exponential decline to steady state levels (Adrian, Chandler and Hodgkin,1970b). This time dependent decline is thought to be the result of two distinct processes (Almers 1971, 1972a,b). For moderate hyperpolarizing voltage steps (to membrane potentials less negative than -125mV) the decline of inward current has been primarily attributed to a depletion of potassium ions from within the tubular system (Adrian and Freygang 1962; Almers 1971, 1972a). The electrophysiological experiments of Almers (1970, 1972a,b) suggested that the inward rectifier channels are associated with tubular system membrane. These conclusions were supported by experiments on single muscle fibres and on glycerol treated fibres (where the transverse tubule system is destroyed) which have shown that inward rectifier channels reside predominantly within the transverse tubules (Hodgkin and Horowitz, 1960; Nakajima, Nakajima, and Peachey,1969, Eisenberg and Gage,1969). Such findings have lead to the proposal of a depletion hypothesis which explains how under maintained depolarization the inward current appreciably diminishes the potassium concentration inside the tubular system thereby reducing the amount of available potassium ions mediating the inward current (Almers,1970,1972a). This depletion is thought to be responsible for the time dependent phase of

current decline seen at moderate hyperpolarizations. Finally these findings are supported by the absence of a time dependent decline in current amplitude measured on preparations which do not possess T-tubule structure (Hagiwara, Miyazaki and Rosenthal,1976; Hagiwara and Takahashi,1974; Ohmori, 1978, 1980).

With more extreme hyperpolarization (membrane potentials negative to -140mV), the decline in inward current is facilitated by a second process involving a voltage dependent decrease in potassium permeability (Adrian, Chandler, Hodgkin 1970b; Almers,1971,1972b). The decline in current amplitude at these extreme membrane potentials is such that the current at the end of a 100 m. pulse is less than the current observed at less negative potentials. This results in a region of negative slope on current-voltage curves at these higher potentials. The decrease in permeability has been attributed to a potential dependent block of inward rectifier channels by sodium ions in the external solution. In preparations of frog skeletal muscle and tunicate egg cells the decrease in potassium permeability can be reduced by replacing the external sodium with lithium and removed entirely by replacement of sodium with Tetraethylammonium, Choline^{+}, or Tris+ (Standen and Stanfield,1979; Ohmori,1978,1980;

Fukushima,1982). However these results have not been consistently seen across all preparations. For example in cultured neonatal rat ventricular myocytes, macrophage cells, and guinea pig heart cells (Sakman and Trube,1984; Mickinney and Gallin,1988; Josephson and Brown,1986) substitution of Tris or choline for external sodium did not abolish the negative slope conductance of the steady state current voltage curve. This result has been attributed to a sodium independent permeability change.

The pharmacology of anomalous rectification has been investigated. External application of cesium blocks inward rectification in a voltage and concentration dependent manner. These characteristics have been extensively described in preparations of frog skeletal muscle (Gay and Stanfield,1977; Oksana,1988), cat ventricular myocytes(Harvey and Ten Eick,1988), starfish egg cell (Hagiwara and Takahashi,1974; Hagiwara and Yoshi,1979), and tunicate egg cell (Ohmori, 1978,1980).

In addition to cesium other cations including Na^{+}, Rb^{+}, Li^{+}, Ba^{+2}, and Sr^{+2} have also been shown to produce voltage dependent block of anomalous rectification (Standen and Stanfield,1980; Ohmori, 1978,1980; Hagiwara,1983).

While the outward potassium currents are involved in the repolarization of the membrane following depolarization the role of the anomalous rectifier in skeletal muscle cells is not clear. What purpose could there be for a current whose conductance increases under hyperpolarization and decreases under depolarization ? One suggested role is that anomalous rectification may provide a pathway for potassium reentry from potassium loaded transverse tubules following action potentials (Hille, 1984). Another speculative role has been that anomalous rectification may act to hold the resting potential near the E_K when otherwise electrogenic Na^+ pumps would hyperpolarize the membrane (Hille,1984).

I.4 Aim of the Present Study

It has been hypothesized that potassium currents may play a role in the localization of acetylcholine receptors during synaptic development. Therefore a better understanding of the properties of potassium currents on developing embryonic tissue is essential. The aim of the present study was to identify and characterize some of the voltage sensitive potassium conductances on embryonic skeletal muscle cells from the frog Xenopus laevis growing in culture. This study

utilizes the tight seal, whole cell recording technique combined with pharmacological manipulations. Two potassium conductances have been identified on this preparation. The first conductance to be described is a hyperpolarization gated inward potassium current thought to be mediated through anomalous rectifier potassium channels. The second conductance to be described is a depolarization gated, outward potassium conductance. This current is believed to be mediated through delayed rectifier potassium channels. Although the aim of this thesis was predominantly to describe the anomalous and delayed rectifiers some experiments suggested the presence of a leak current near the resting membrane potential mediated by potassium and or chloride as well as a calcium current. These results are described briefly but the complete description of such currents would be more easily attained using single channel recording techniques.

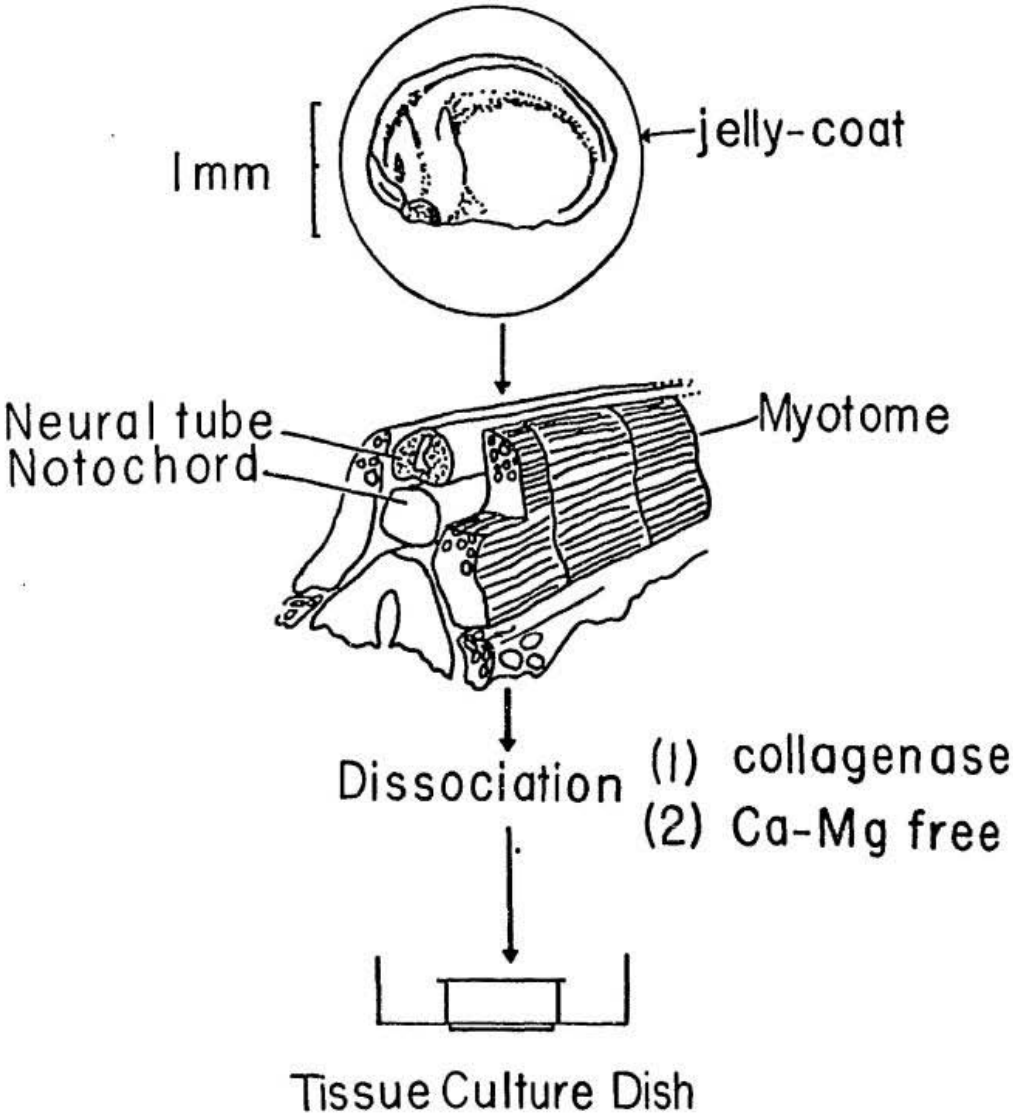
II METHODS AND MATERIALS

II.2 Cell Culture

Cultures of single myotomal muscle cells were prepared from stage 18-22 Xenopus laevis embryos (Nieuwkoop and Faber, 1956). Embryos were placed into a sterile dissecting medium which consisted of 66% Dulbecco's Modified Eagles Medium (DMEM), gentamicin (.01mg/ml), nystatin (5 U/ml), and 10mM HEPES buffer (pH of 7.4 with NaOH). Embryos were removed from their vitelline membrane and jelly coat. A portion of the embryo consisting of myotome, notochord, neural tube and skin (Fig. 1) was then separated from the rest of the organism and exposed to brief collagenase digestion (1mg/ml in DMEM, 20 minutes). Collagenase treatment facilitated the separation of the myotomal musculature from other tissue types. Myotomal tissue was then transferred into a sterile Ca^{2+}-Mg_{2+} free salt solution (67mM NaCl, 1.6mM KCl, 8mM HEPES, 2mM EDTA, pH 7.4). This treatment facilitated the dissociation of myotomes into single cells. Finally the single myotomal muscle cells were added to culture chambers containing plating medium (66% DMEM, penicillin (0.5 U/ml), streptomycin (0.5 g/ml), 5% dialysed horse serum, buffered with 10mM HEPES to a pH 7.4 with NaOH).

Fig. 1 Diagram illustrating the preparation of embryonic myotomal muscle cultures from Xenopus laevis. The drawings of the neurotube, myotome and notochord was adapted from Anderson, Cohen and Zorychta (1977). The drawing of the Xenopus embryo was adapted from Nieuwkoop and Faber (1956).

Preparation of Myotomal Muscle Cultures from Xenopus laevis



Culture chambers were constructed from 35mm Falcon petri dishes which had been modified to incorporate collagen-coated glass coverslip bottoms (Fig.1). The chambers were filled with plating medium, the muscle cells added to the bottom of the dish using a micropipette and the chamber was sealed with a sterile coverslip. Cultures were stored in the dark at room temperature (22°C).

DMEM, nystatin, gentamycin, collagenase, and dialyzed horse serum were all obtained from Gibco. Rat tail collagen was obtained from Sigma.

Muscle cells growing in culture were visualized with the 40X objective (400X magnification) of an IM35 Ziess microscope (Ziess, West Germany). A long working distance phase condenser allowed a working distance of 60-70mm. Cultures prepared in the above manner primarily consisted of muscle cells but as described by others (Anderson, Cohen and Zorychta,1977) fibroblasts and melanocytes were also observed. On rare occasions a few nerve cells were present.

II.2 Electrophysiology

Whole cell ionic currents were recorded using the

tight seal whole cell recording technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). An EPC-7 List Patch Clamp amplifier (List-Electronics) was used for this purpose. Currents were filtered at 10kHz using the 8 pole Bessel filter of the List Patch Clamp. The amplifier head stage was mounted on a motorized micro-manipulator (Ziess), which in turn was mounted on the mechanical stage of the inverted Microscope. The microscope was placed on an antivibration table (Technical Manufacturing Co., MA, USA).

Patch electrodes were fabricated from 100 µl glass micropipettes (Drummond Scientific Company, USA) using a two stage glass micro-electrode puller specifically designed for making patch electrodes (Narshige PP-83 electrode puller, Japan). Patch electrodes utilized in these experiments had resistances of 2-6 M ohms when measured in 5mM KCl recording solution (Table 1). Ground electrodes were constructed from 50µl micropipettes (Drummond Scientific Company, USA) which had been filled with a 2.0-2.5% agar - external recording solution mixture.

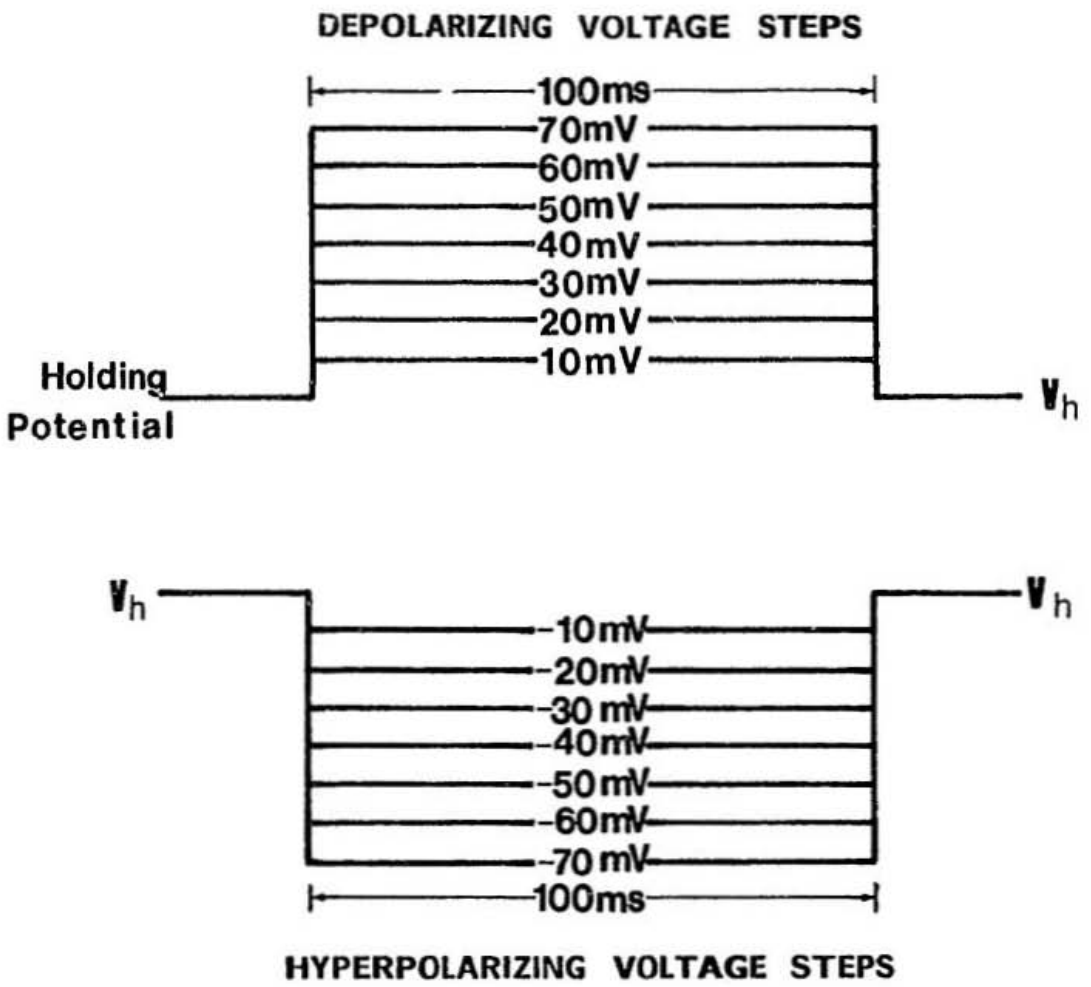
Voltage steps were provided to the patch clamp manually using an S95 stimulator (Medical Systems corp.). The voltage steps were scaled by a factor of

0.1 and the polarity of the pulses was adjusted using the stimulus scaling available on the patch clamp. Resting membrane potentials were measured in the current clamp mode of the patch clamp, with zero current applied. Unless otherwise stated muscle cells were voltage clamped at or near their resting membrane potentials. This potential will be referred to as the holding potential or V_H. Voltage steps (in 2mV, 5mV, or 10mV increments) were applied from V_H. The voltage clamp protocol shown in Fig. 2 demonstrates a typical voltage clamp experiment. In the majority of experiments hyperpolarizing and depolarizing voltage steps were 100 ms in duration and repeated every 9.1 seconds. The capacitance transient cancellation and series resistance compensation of the List Patch Clamp were not usually employed.

II.3 Data Storage and Analysis

Analogue current data generated during the course of these experiments was converted into digital form using a digital Pulse Code Modulator (Sony). Sampling frequency was 44.1kHz Digitized current data was stored on Beta video cassettes using an SL 2700 Betamax video recorder (Sony). An audio track of the VCR was utilized to facilitate the storage and retrieval of

Fig. 2 Voltage clamp protocol demonstrating a typical voltage clamping experiment. Individual muscle cells were voltage clamped at a constant membrane potential (V_H) and depolarizing and hyperpolarizing voltage steps applied in 10mV increments. The duration of the voltage steps was 100ms and the timing between successive steps was 9.1 seconds.


DEPOLARIZING VOLTAGE STEPS
100ms
70mV
60mV
50mV
40mV
30mV
20mV
10mV
Holding
Potential
Vh
Vh
Vh
-10mV
-20mV
-30 mV
-40mV
-50mV
-60mV
-70 mV
100ms
HYPERPOLARIZING VOLTAGE STEPS

current data and one channel was used to record the voltage steps and trigger pulses. Whole cell currents were transferred to computer disk and analyzed using a software package designed for this purpose (pClamp version 3.0, Axon Instruments, Inc. CA, USA) on either a Tatung or Tandy computer. Data were transferred and stored using the clampex program of pClamp.

The clampfit and clampan programs were used to analyze and plot current records. The leak subtraction command of the clampfit program was sometimes used. The resistance value required for this command was obtained in the following manner. Muscle cells were voltage clamped (V_h) at membrane potentials 10-15mV positive to the calculated Nernst potential for potassium (E_K) and hyperpolarizing voltage steps were applied in either 2 or 5mV increments (up to E_h). Membrane potential shifts were then divided by their resulting ionic currents thereby providing a value of membrane resistance. Resistance values were calculated over a small range of membrane potential where it was unlikely that voltage activated currents were present. These resistances were averaged, and their means were used as the leak subtraction parameter for the clampfit program. The pClamp software allowed measurements of current amplitude and exponential fits

of the time dependent events. Plots of the current traces were made from clampfit. Graphical representation of the data, for example, current-voltage plots, were constructed using Lotus (release 2.01). A Hewlett Packard plotter was used when plotting figures and current records.

II.4 Solutions and Drugs

Prior to whole cell recording sessions the plating medium in the culture chamber was replaced with an extracelluar recording solution. Patch electrodes were back filled with an intracellular recording solution. All solutions used in this study are listed in Table 1. Throughout the text of this thesis recording solutions will be referred to by the solution names given in Table 1. In preparations of adult frog skeletal muscle a significant chloride permeability has been reported (Hodgkin and Horowicz,1959; Hutter and Noble,1960). During the course of these experiments it was sometimes necessary to eliminate internal as well as external chloride ions in order to confirm the presence of a potassium current. Solutions in which chloride ions had been replaced with the impermeant anions L-Aspartic Acid and SO_4^{2-} are shown in Table 1. The pH of all solutions was adjusted to 7.4 using NaOH or KOH.

Table 1 Composition of electrophysiological recording solutions. Internal recording solution #I was used with all external recording solutions containing chloride ions while solution #II was used with all chloride free external recording solutions. pH of all solutions adjusted to 7.4 with either NaOH or KOH.
[1] KAsp - L-aspartic acid (monopotassium salt); NaAsp - L-Aspartic acid (monosodium salt).
[2] sodium free recording solution.

TABLE 1

External Recording Solutions (mM)

Solution Abbreviation	KCl	KAsp*	NaCl	NaAsp*	$CaCl_2$	$CaSO_4$	$MgCl_2$	$MgSO_4$	Tris-Cl	HEPES
2.5mM KCl	2.5	---	142.5	---	1.0	---	1.2	---	---	10
5.0mM KCl	5.0	---	140.0	---	1.0	---	1.2	---	---	10
5.0mM KAsp	---	5.0	---	140.0	---	1.0	---	1.2	---	10
5.0mM KClt!	5.0	---	---	---	1.0	---	1.2	---	140.0	10
10.0mM KCl	10	---	135.5	---	1.0	---	1.2	---	---	10
10.0mM KAsp	---	10.0	---	135.0	---	1.0	---	1.2	---	10
15.0mM KAsp	---	15.0	---	130.0	---	1.0	---	1.2	---	10
17.5mM KAsp	---	17.5	---	140.0	---	1.0	---	1.2	---	10
45.0mM KCl	45.0	---	100.0	---	1.0	---	1.2	---	---	10
100mM KCl	100	---	45.0	---	1.0	---	1.2	---	---	10

Internal Recording Solution (mM)

	KCl	KAsp*	EDTA	$MgCl_2$	$MgSO_4$	HEPES
I	140	---	1.0	5.0	---	10
II	---	140	1.0	---	5.0	10

! - sodium free solution
* - chloride free solution
pH adjusted to 7.4 with NaOH and KOH
Internal recording solution #II was used with all chloride free external recording solutions while internal recording solution I was used with all External recording solutions containing chloride.

Sodium channel activity was eliminated by adding 10^{-6} g/ml tetrodotoxin (TTX) to the extracelluar recording solution. This concentration of TTX has been shown to be effective in blocking sodium currents on embryonic Xenopus myotomal muscle cells (Decino and Kidokoro, 1985).

The following agents were used in an attempt to block potassium currents. Tetraethylammonium chloride, cesium chloride, apamin, quinine, 4-amino pyridine and 3,4 diaminopyridine (Sigma Chemical Co. St. Louis, MO). Unless otherwise indicated stock solutions of these drugs were prepared in extracelluar recording solutions and pH was adjusted to 7.4. In some cases calcium currents were blocked using nifedipine or (+)PN 200-110 (kindly provided by Dr. C. Triggle). Stock solutions of these were prepared in ethanol and stored in the dark at 4°C. The final ethanol concentration in all cases was less than 1%.

Drug applications were accomplished by the direct addition of small amounts (5ul) to the bath using a Gilson micropipette. This did not allow us to regain pre-drug levels for an individual muscle cell. However repeated cells were examined in a single dish after the culture was washed with 5-10 changes of extracelluar

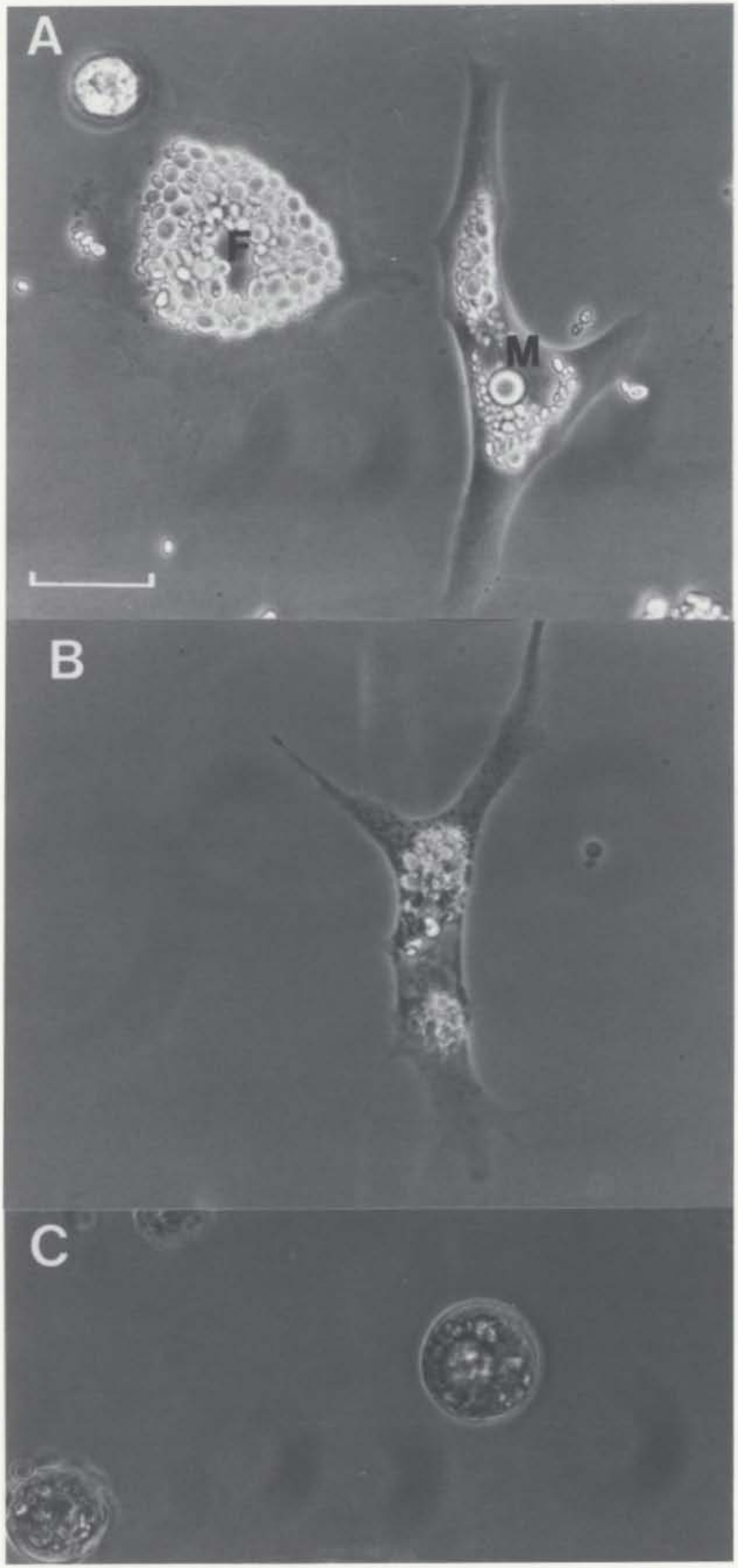
solution. Usually no more than 4 cells were recorded from a single culture. Experiments were conducted at room temperature (21-24 °C).

III RESULTS

The purpose of this study was to identify and characterize two of the voltage sensitive potassium currents in cultured Xenopus laevis embryonic myotomal muscle cells. Experiments were performed on individual muscle cells in 17-24h cultures. This age range was found to be the most suitable for voltage clamp recordings because most cells had yet to develop extensive visible cross striations and were less prone to contraction. At later times in culture (after 24h), striations were more visible and patch formation became increasingly difficult for two reasons. First the high concentration of potassium in the approaching patch electrode would often cause the cells to depolarize and contract. Secondly if it was possible to establish the whole cell recording configuration the applied depolarization caused contractions that dislodged the electrode.

Cell cultures contained primarily muscle cells but also fibroblasts, melanocytes and sometimes nerve. Fig. 3 shows a 40X view of an individual muscle cell at 24h in culture. Two morphological criteria were used to distinguish muscle cells from other cell types. (1)

Fig. 3 Phase contrast view of (A) single embryonic muscle cell (M) and fibroblast (F); (B) melanocyte and (C) a myoball in a 24 hour old culture. The culture was prepared from the myotomal musculature of a stage 21 Xenopus laevis embryo. Scale bar is 20 μm.


A
F
M
B
C

The main criteria, was based upon the cell density seen with phase contrast optics at 40X magnification during recording sessions (see Fig. 3). Under these conditions fibroblasts appeared faint with flattened cell bodies (Fig 3A). In contrast to the fibroblasts muscle cells had thicker cell bodies which appeared darker (Fig. 3A). Melanocytes were thin and contained numerous dark granules (Fig. 3B). Nerve cells (not shown) usually appeared as small round cell bodies usually with refractile material and very little visible cytoplasm. (2) The second criteria used to identify cultured muscle cells was the appearance of cross striations. As described by others and evident in this study, cross striations on non-innervated muscle cells become evident prior to 24 hours in culture (Kidokoro and Saito, 1988; Weldon and Cohen,1979). With time striations became increasingly evident. While the cross striations were faint prior to 24 hours they were evident on many of the muscle cells recorded from in this study. To confirm that these cells were muscle cells a few cultures were stained using rhodamine labelled alpha-bungarotixin (α-BuTX). Cultured muscle cells grown in the absence of nerve develop discrete patches of high acetylcholine receptor (AChR) densities (Anderson, Cohen and Zorychta, 1977) and are easily visualized using the

rhodamine α-BuTX staining technique (Anderson, Cohen and Zorychta,1977). A single muscle cell photographed with both phase contrast and fluorescence optics is shown in Fig. 4. Photographs were taken from a 24 hour old muscle cell which had been stained with the rhodamine α-BuTX (10^{-6}g/mL for 20 minutes). Bright patches on the fluorescent photograph (see arrows) represent areas of high acetylcholine receptor density. Fibroblasts and melanocytes did not show any evidence of rhodamine staining.

Resting membrane potentials (RMPs) were recorded from all cells prior to voltage clamping, and were repeatedly checked throughout the course of an experiment in order to confirm the viability of the cells. Table 2 shows mean RMP's recorded from individual muscle cells immersed in the various electrophysiological recording solutions described in Table 1. The mean RMP of muscle cells voltage clamped in 5mM KCl solution was -71.5mV +/- 4.0mV, n=177. The semilogarathmic plot shown in Fig. 5 demonstrates that the mean RMP's varied with changes in the external potassium ion concentration.

The large size of plated muscle cells used in these experiments left some question as to the effectiveness

Fig. 4 Phase contrast (A) and corresponding fluorescent (B) views of 24 hour old muscle cell growing in culture. A patch of ACh receptors can be seen in B (arrows). Muscle cells treated with 10^{-6} g/ml rhodamine alpha bungarotoxin. Scale is 30 μm.

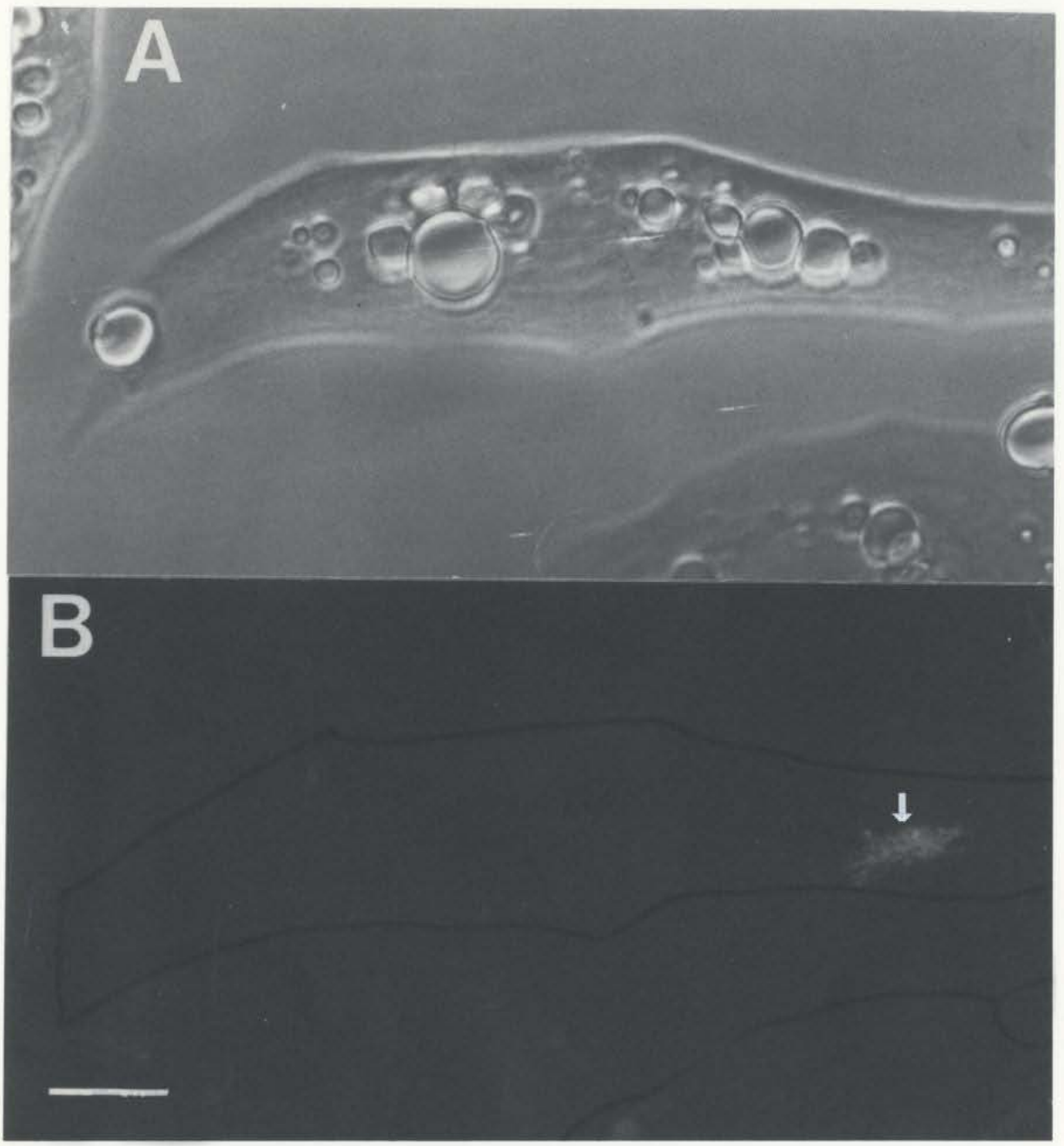

A
B

Table 2 Resting membrane potentials of individual embryonic skeletal muscle cells grown in culture.

[1] Equilibrium potentials (E_K) were calculated using the Nernst Equation for potassium at a temperature of 21°C

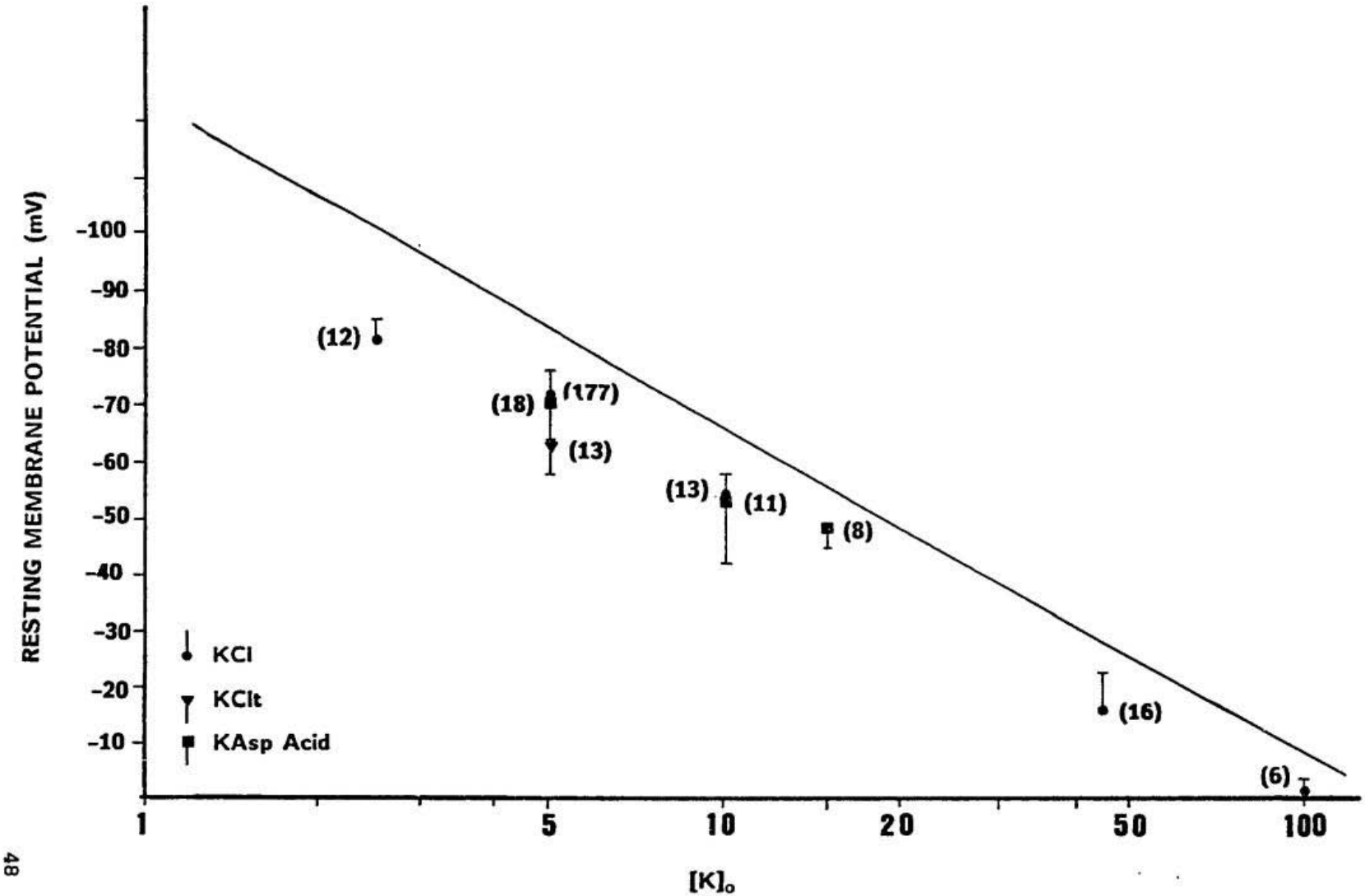
[2] Number in brackets represents number of cells examined.

TABLE 2

Resting Membrane Potentials (RMP) of Individual Embryonic Skeletal Muscle Cells Grown in Culture

External Recording Solution	RMP+/-(SD) (mV)	E_K[1] (mV)
2.5mM KCl (12)[2]	-80.9 (2.4)	-103
5mM KCl (177)	-71.5 (4.0)	-83.5
5mM KAsp (18)	-71.2 (6.5)	-83.5
5mM KClt (13)	-62.5 (5.2)	-83.5
10mM KCl (11)	-53.0 (3.7)	-66.4
10mM KAsp (13)	-54.3 (10.7)	-66.4
15mM KAsp (8)	-49.9 (3.9)	-56.0
45mM KCl (16)	-15.9 (6.6)	-28.5
100mM KCl (6)	-1.3 (2.0)	-8.5

Fig. 5 Dependence of the RMP on the external potassium ion concentration. Semilogarithmic plot of RMP as a function of external potassium ion concentration measured in both the presence (●) and absence (■) of chloride. Number in parenthesis represents the number of cells for which the RMP was determined. Vertical lines represent standard deviations. The straight line was calculated from the Nernst equation for potassium at 21°C.


RESTING MEMBRANE POTENTIAL (mV)
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
(12)
(18)
(177)
(13)
(13)
(11)
(8)
(16)
(6)
KCl
KClt
KAsp Acid
1
5
10
20
50
100
[K]o

of the spatial clamp. To address this concern a number of myoballs (spherical cells 15-30 μ m in diameter, Fig. 3C) were voltage clamped. Analysis of currents and membrane potential data obtained revealed no difference between voltage clamped myoballs and plated cells. Therefore these data have been included in the results of this study.

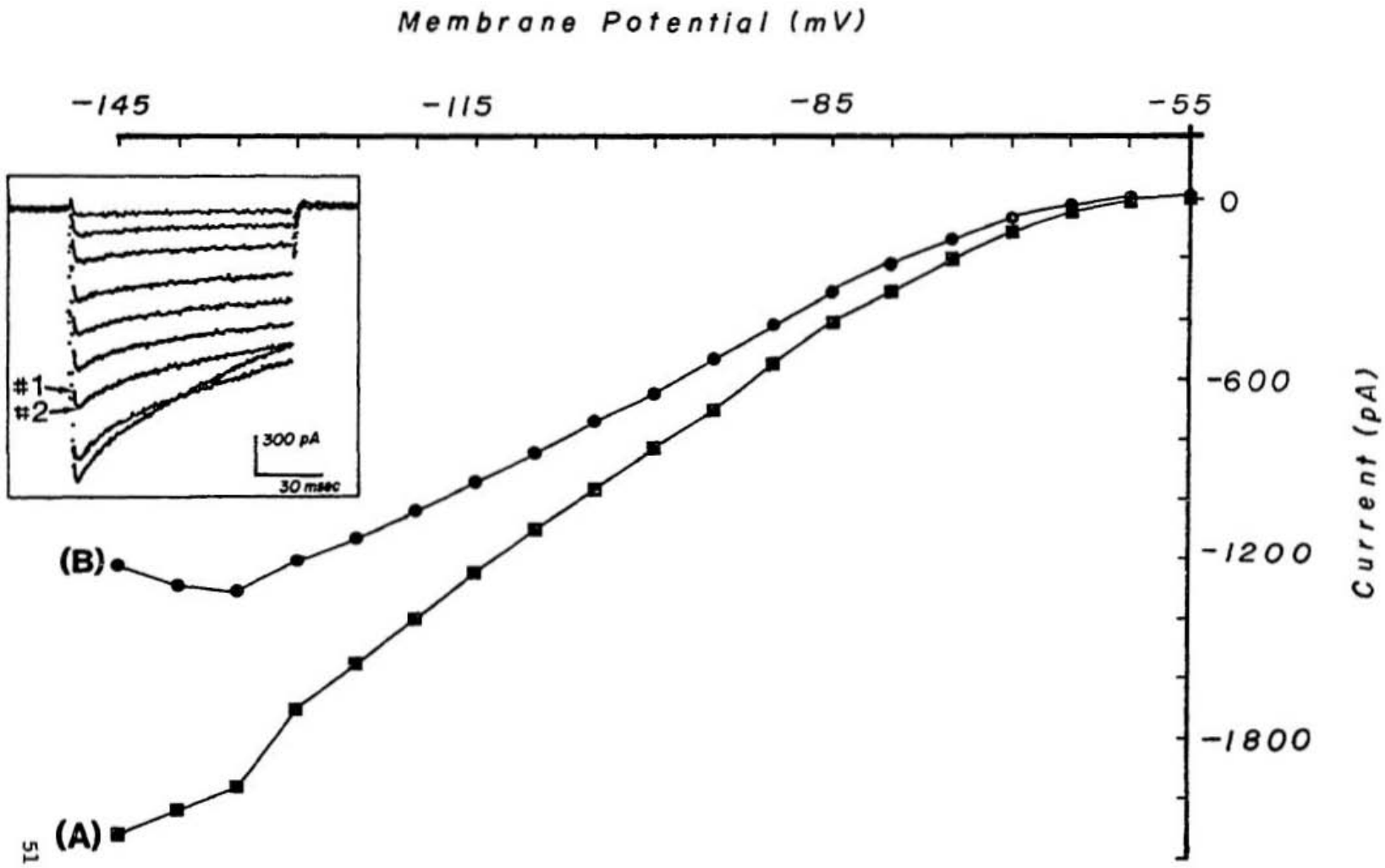
III.1 Whole Cell Inward Currents Activated by Hyperpolarization

Individual muscle cells were voltage clamped at membrane potentials 10-20mV positive to the calculated equilibrium potentials for potassium, and 100 ms hyperpolarizing voltage steps were applied in 5 or 10mV increments. The resulting whole cell inward currents were thought to be mediated by potassium ions flowing through anomalous rectifier potassium channels.

III.1a Activation Properties of the Inward Current

The set of current records shown in Fig. 6 (inset) typifies whole cell inward currents observed in response to hyperpolarizing voltage steps. Current records shown in this figure were recorded from a single muscle cell which was bathed in 10mM KAsp. The

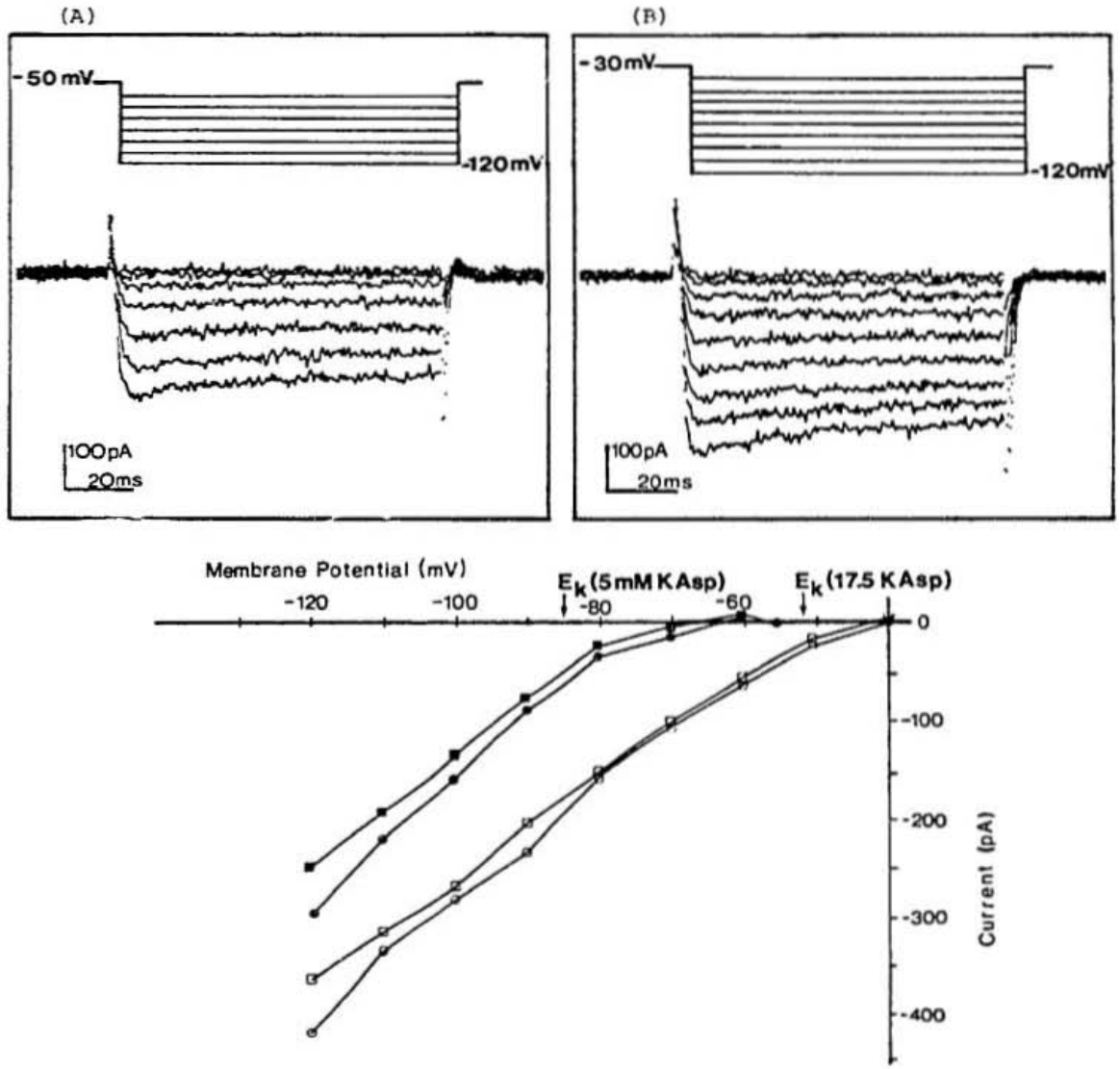
Fig. 6 Hyperpolarization gated whole cell inward currents recorded from a single muscle cell bathed in 10mM KAsp. Current-voltage relationship of peak (A) and steady state (B) currents from a single muscle cell in 10mM KAsp. Shown in the inset are whole cell membrane currents resulting from voltage steps to -140mV, applied in 10mV increments from a holding potential of -50mV. The muscle cell had a resting membrane potential of -40mV. Leak subtraction employed.


Membrane Potential (mV)
-145
-115
-85
-55
0
-600
-1200
-1800
Current (pA)
#1
#2
300 pA
30 msec
(B)
(A)

activation of this current was best described as an instantaneous jump (appearing in less than 1 ms, see arrow #1) followed by a time dependent increase to peak amplitudes (within 2-5 ms, see arrow #2). Amplitudes of the peak inward current showed wide variation from one muscle cell to the next. For example a hyperpolarizing step to -120mV, measured in 5mM KCl and 5mM KAsp resulted in peak current amplitudes ranging from 192pA to 2000pA (752pA+/- 373pA, n=72). After a brief period at peak amplitudes the inward current began a time dependent phase of current decline. Current at the end of the hyperpolarizing step (referred to as the steady state current) also showed a wide range of amplitudes. For example, hyperpolarizing voltage steps to -120mV (measured in 5mM KCl and 5mM KAsp) resulted in steady state inward currents ranging from 180pA to 1567pA (577 +/- 325.5, n=83).

The potential of activation of the inward current appeared to be related to the external potassium ion concentration ($[K]_o$). The two sets of current records shown in Fig. 7 were recorded from a single muscle cell bathed in 5mM KAsp (A) and after the addition of 5ul of 500mM KAsp acid to the external solution to bring the concentration to 17.5mM KAsp (B). The corresponding current-voltage curves presented in Fig. 7C show peak

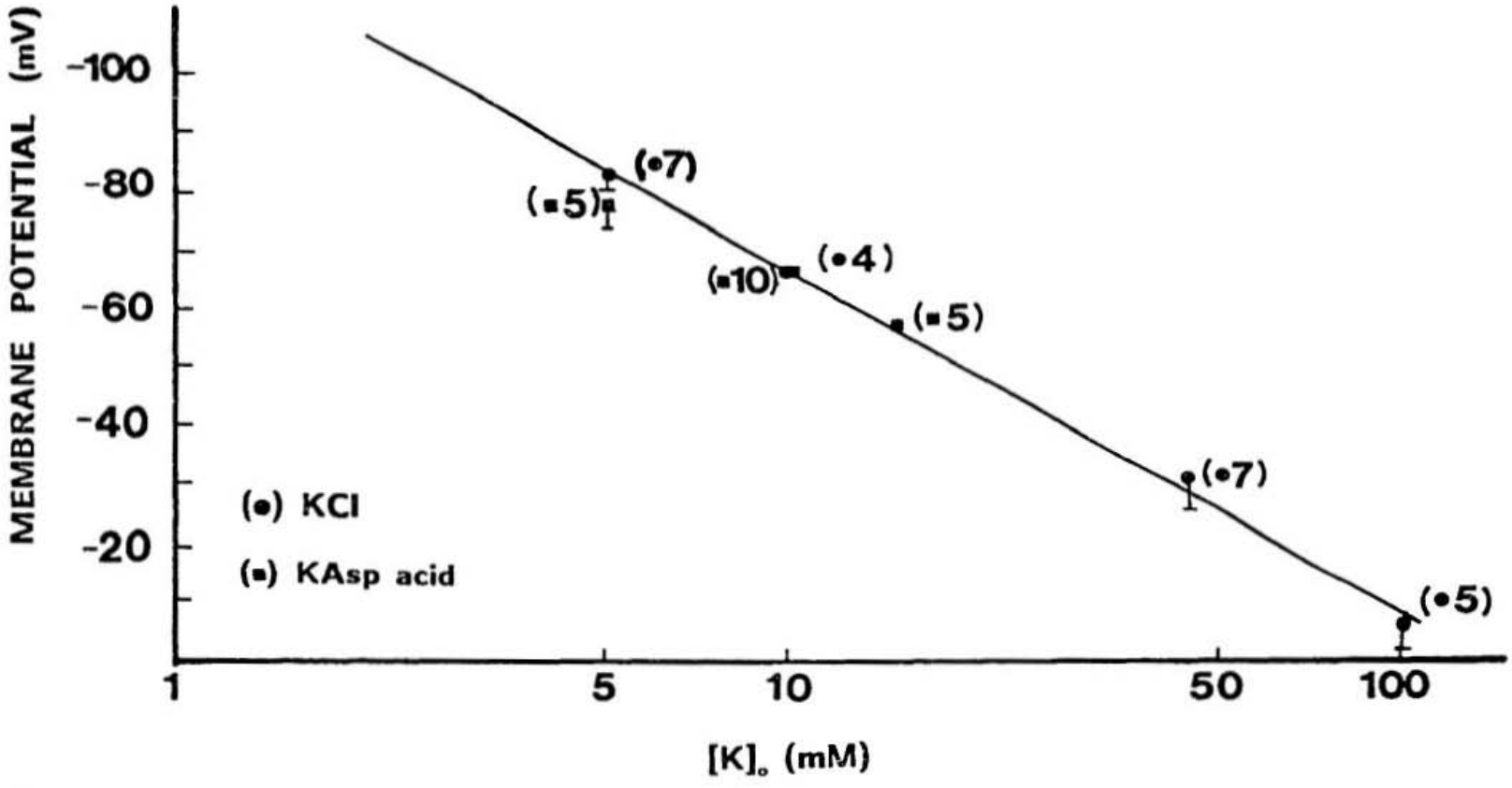
Fig. 7 Whole cell current records for a single muscle cell examined in the presence of (A) 5mM KAsp and (B) 17.5mM KAsp. Record B was obtained from the same cell after adding 5 µl of 500mM K Asp to the external recording solution. The cell was held at -50mV in (A) -30mV in (B). (C) The corresponding current-voltage relations for the peak (●) and steady state (■) currents for A (filled symbols) and B (open symbols) are shown. The values of E_K in these two solutions are indicated on the graph. Leak subtraction employed.


(A)
-50 mV
-120mV
100pA
20ms
(B)
-30mV
-120mV
100pA
20ms
Membrane Potential (mV)
-120
-100
E_k (5mM KAsp)
-80
-60
E_k (17.5 KAsp)
0
-100
-200
-300
-400
Current (pA)

and steady state values measured in both 5mM KAsp and 17.5mM KAsp. These current voltage records illustrate two points. (1) The potential of activation of the inward current lies close to the calculated E_K (E_K=-84mV in 5mM KAsp and -52mV in 17.5mM KAsp). (2) Increasing the $[K]_0$ from 5mM KAsp to 17.5mM KAsp produced a +25mV shift in the potential of activation which is close to the predicted +32mV shift in E_K.

The semilogarithmic plot shown in Fig. 8 demonstrates the relationship between the potential of activation for the steady state current records verses the $[K]_0$. Activation potentials were estimated for each cell by drawing a straight line through the current voltage curve and the point at which the straight line intercepted the membrane potential axis was taken as the value. The solid line shown in Fig. 8 was constructed from E_K values calculated from the Nernst equation for potassium (21°C) for external potassium concentrations ranging from 1 to 100mM. Activation potentials of the inward current corresponded closely to values predicted for pure potassium currents. The presence of chloride ions in the recording solutions did not change the activation potential values. These results illustrate the selective nature of this current towards potassium

Fig. 8 A semilogarithmic plot of the membrane potential of activation for the steady state currents versus the potassium concentration of the external recording solution. The numbers in parenthesis represent the number of cells at each potassium concentration and the vertical lines represent standard deviations. The straight line was calculated from the Nernst equation for potassium at 21°C.


MEMBRANE POTENTIAL (mV)
-100
-80
-60
-40
-20
(•7)
(▪5)
(•4)
(▪10)
(▪5)
(•7)
(•5)
(•) KCl
(▪) KAsp acid
1
5
10
50
100
[K]ₒ (mM)

ions. Similar results were obtained from the peak current-voltage curves.

III.1b Decline of the Inward Current During Hyperpolarization

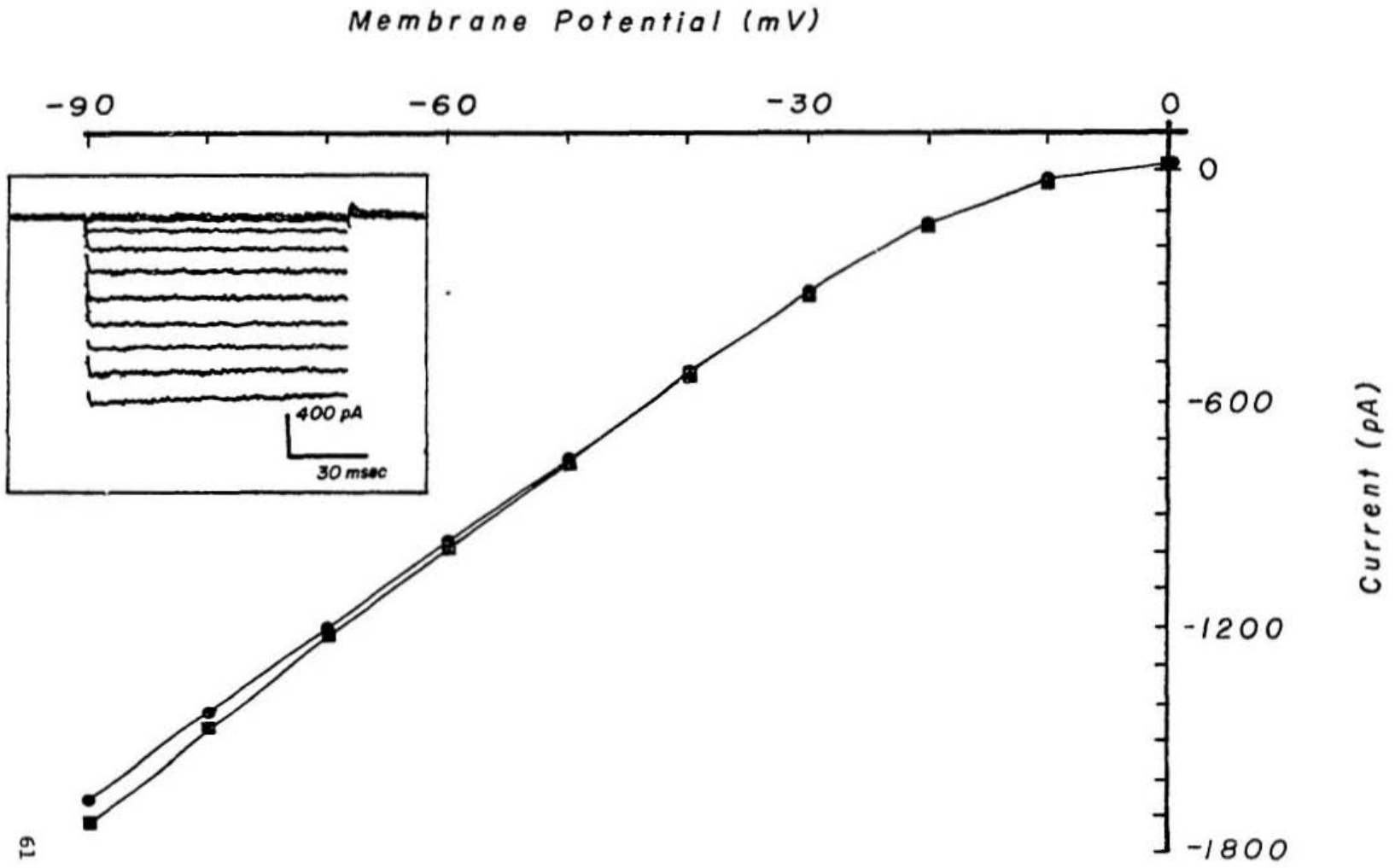
When embryonic skeletal muscle cells were hyperpolarized the resulting inward currents (Fig. 6, inset) showed a rapid rise to peak values followed by a time-dependent decline. The decline in the current amplitude is evident from both the current traces and examination of the current-voltage curves of Fig. 6. Curve A is the peak current-voltage curve measured at those points of maximum inward current for each voltage step. Curve B shows current-voltage curves measured at the end of the voltage steps (the current amplitude between 80 and 100 ms was averaged). Curve B was lower than curve A at all potentials. A similar time dependent decline was apparent on 95% of all muscle cells examined (n=125, in 5mM KCl, 5mM KAsp, 10mM KCl, 10mM KAsp, and 15mM KAsp). At higher hyperpolarizing potentials a second type of time-dependent decline became apparent. In these cases the decline was such that the steady-state current amplitude was less than the amplitude on preceding voltage steps. These two types of time dependent declines in current amplitude

are mediated by different processes and will be described separately below.

The time dependent current decline seen during moderate hyperpolarizations was reduced when high concentrations of potassium ions were used in the external recording solution. The set of current records shown in Fig. 9 were recorded from a single muscle cell bathed in 100mM KCl. This figure shows that the time dependent decline was much slower and smaller when compared to cells bathed in 10mM external potassium (Figs. 6 and 7). The corresponding current-voltage curves shown in Fig. 9 further illustrate the elimination of the time dependent current decline with high external potassium concentrations. Similar results were observed on 5 other muscle cells voltage clamped in 100mM KCl.

In addition to the time dependent decline seen at moderate hyperpolarizing potentials (membrane potentials less negative than -130mV), extreme hyperpolarizations (to membrane potential more negative than -130mV) resulted in a region of negative current slope (curve B, Fig.6). This region of negative slope was evident on 85% of all muscle cells examined (n=52) at these higher potentials. The region

Fig. 9 The peak inward current (■) and steady state current (●) versus membrane potential recorded from a muscle cell with 100mM KCl in the external recording solution. The current records are shown in the inset. The cell was held at +10mV and voltage steps applied in -10mV increments to -90mV. The resting membrane potential was 0mV. Leak subtraction employed.


Membrane Potential (mV)
-90
-60
-30
0
0
-600
-1200
-1800
Current (pA)
400 pA
30 msec

of negative slope and decline of the current amplitude at higher negative potentials could be reduced by removing sodium ions from the external recording solution (n=6) as shown in Fig. 10. This did not abolish the time dependent decline in inward current seen at moderately hyperpolarized potentials. The steady state current-voltage curve (Fig. 10) further illustrates the absence of negative slope conductance in the absence of sodium even upon hyperpolarizations to -185mV. Similar results were recorded from all muscle cells voltage clamped when sodium was replaced with tris (n=6, 5mM KCl-t).

III.1c Blocking Effects of Cesium on the Inward Current

Cesium chloride has been described as a potent blocker of anomalous rectification on adult frog skeletal muscle fibers (Gay and Stanfield,1977; Oksana, 1986). The effects of cesium on the hyperpolarization activated inward current in the present study was therefore investigated. The two sets of current records shown in Fig. 11 typifies the whole cell inward currents which were observed before (A) and after (B) the addition of 3mM cesium. In the presence of cesium both the peak and steady state currents were smaller

Fig. 10 The peak inward current (■) and steady state current (●) versus membrane potential recorded from a muscle cell with 5mM KClt in the external recording solution. The current records are shown in the inset. The cell was held at -60mV and voltage steps were applied in -10mV increments to -185mV. The resting membrane potential was -61mV.

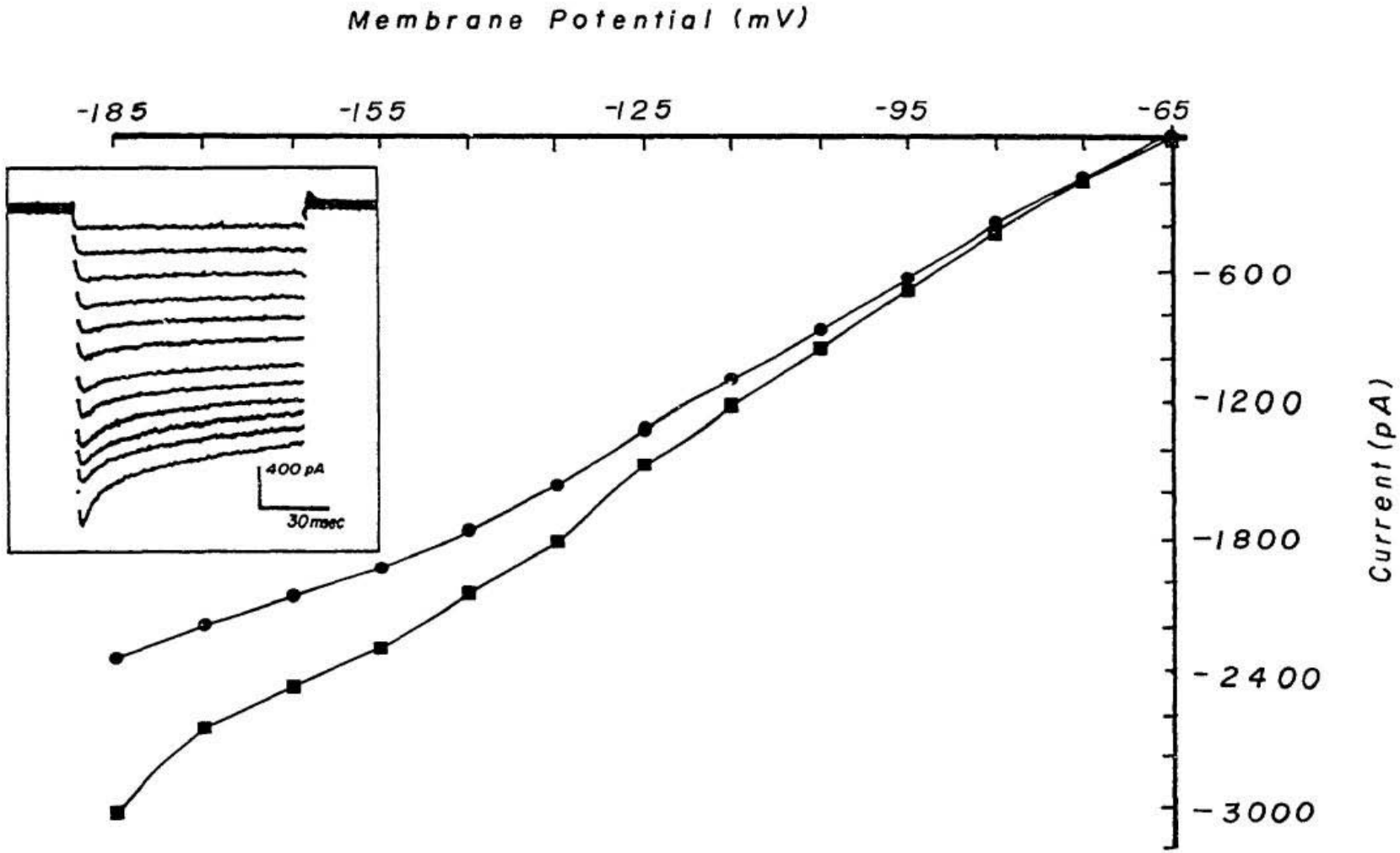
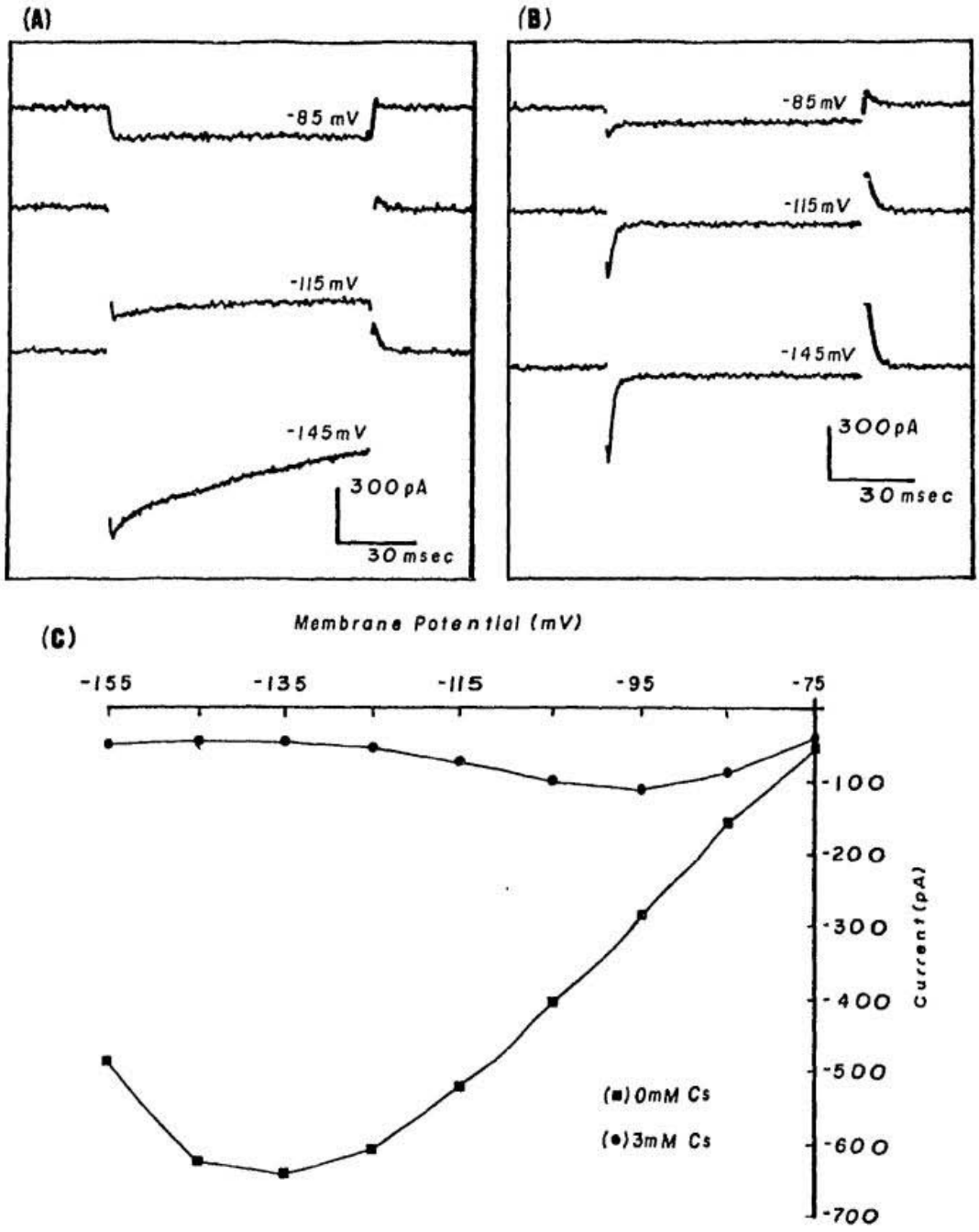

Membrane Potential (mV)
-185
-155
-125
-95
-65
-600
-1200
-1800
-2400
-3000
Current (pA)
400 pA
30msec

Fig. 11 Block of the hyperpolarization gated inward current by external cesium. Whole cell current records from a single muscle cell examined before (A) and after (B) the addition of cesium chloride to the external recording solution (final concentration of 3mM). (C) Current-voltage relationship of steady state currents before and after the addition of 3mM cesium. The resting membrane potential was -66mV, 5mM KCl.


(A)
-85 mV
-115 mV
-145 mV
300 pA
30 msec
(B)
-85 mV
-115 mV
-145 mV
300 pA
30 msec
(C)
Membrane Potential (mV)
-155
-135
-115
-95
-75
-100
-200
-300
-400
-500
-600
-700
Current (pA)
(■) 0mM Cs
(●) 3mM Cs

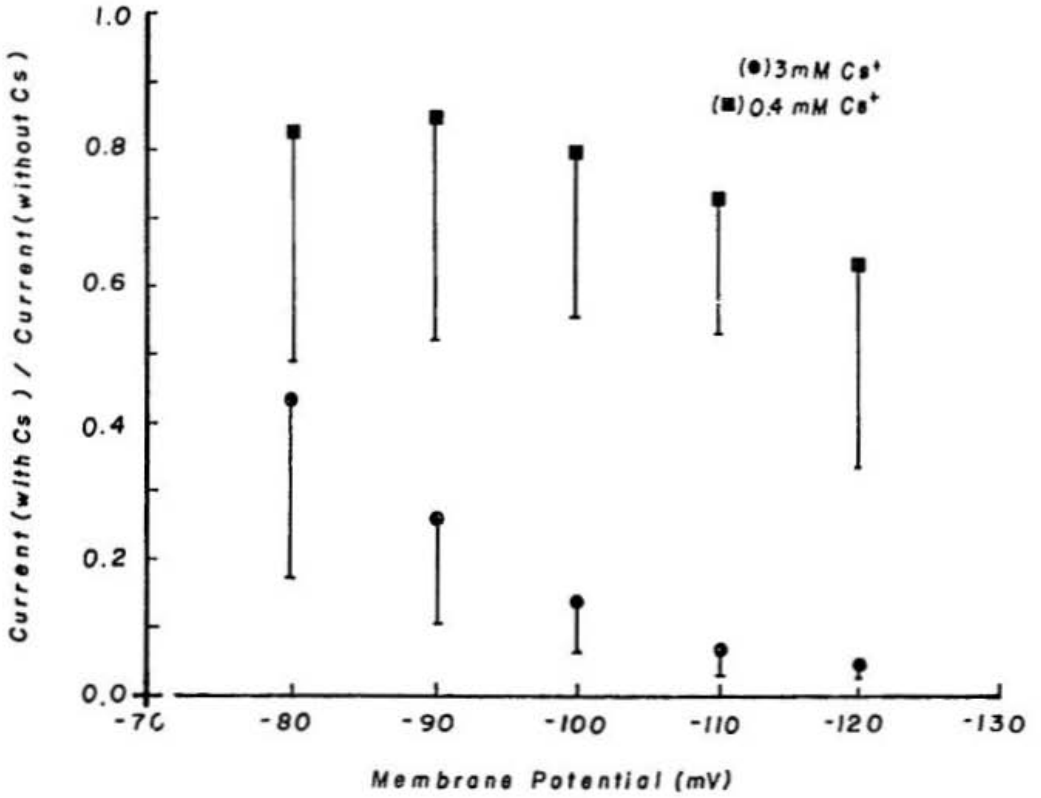
than in its absence. The voltage dependence of the cesium block was evident from the corresponding steady state current voltage curves shown in Fig 11C. With increasing hyperpolarization the block was more complete. For example, in the presence of cesium the steady state inward current was smaller at a membrane potential of -135mV than it was at a membrane potential of -95mV. This difference suggests that the ability of cesium to block the inward current increases with increasing hyperpolarizations.

The blocking effects of cesium were examined at two concentrations. Figure 12 shows the fraction of steady state current remaining in the presence of cesium plotted as a function of membrane potential for two concentrations of cesium. These results would suggest that the suppressing effects of cesium are concentration dependent. Furthermore for a given external concentration (3mM n=5 and 0.4mM n=4) the suppressing effect increased as the degree of hyperpolarization increased.

III.2 Whole Cell Outward Currents Activated by Depolarization

Voltage gated whole cell outward currents have been

Fig. 12 Cesium causes a potential dependent block of the hyperpolarization gated inward current. The fraction of current remaining in the presence of cesium is plotted as a function of membrane potential. Each symbol represents means of the ratios from a number of muscle cells (n=6 with 3mM cesium and n=4 with 0.4mM cesium). Vertical lines represent standard deviations. Holding potentials in all cases were -70mV, 5mM KCl external recording solution.


1.0
0.8
0.6
0.4
0.2
0.0
Current (with Cs) / Current (without Cs)
(●) 3mM Cs+
(■) 0.4 mM Cs+
-70
-80
-90
-100
-110
-120
-130
Membrane Potential (mV)

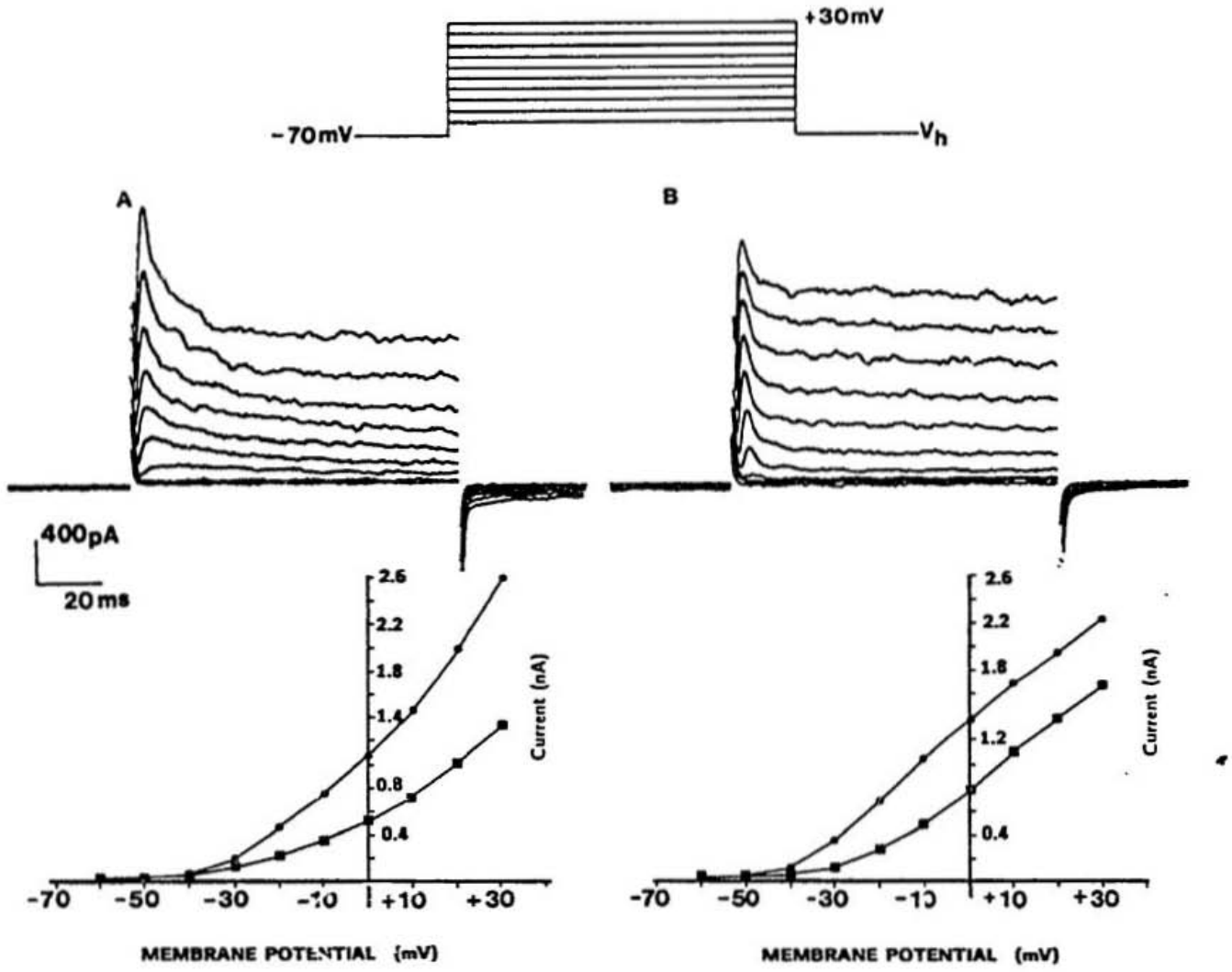
investigated on single embryonic muscle cells growing in culture. Individual muscle cells were voltage clamped at or near their mean resting membrane potentials (Table 2) and 100 ms depolarizing voltage steps were applied in 5 or 10mV increments. The resulting whole cell current records may represent a combination of at least 3 distinct ionic conductances acting together to produce the composite outward currents seen. The largest conductance, the topic of section III.2 moves in the outward direction and appears to be predominantly mediated by potassium ions flowing through delayed rectifier channels. Two additional conductances are present. We have identified an inward calcium current, which infrequently appears at one day in culture and if present usually has a small amplitude (see Table 1, Moody-Corbett, Gilbert, Akrabrali and Hall, 1989). A second conductance mediated by potassium and possibly chloride is present near resting membrane potentials and corresponds with the membrane leak current. This conductance is not linear over the depolarizing voltage ranges used in this study and decreases as the membrane is depolarized. It is apparent on the current traces and current voltage plots as a decrease in outward current when the membrane is depolarized from -60mv to -50mV. Because of the non-linearity of this resting

leak current, a leak subtraction routine was not used in analyzing the outward current data. While these two conductances were not examined in detail it is recognized that their presence may influence the characteristics of the outward potassium currents understudy. The contribution these additional currents make towards the outward potassium current will be considered in the discussion.

III.2a Delayed Outward Potassium Currents

The two sets of ionic current records shown in Fig. 13 typify the whole cell currents which were observed in response to 100 ms depolarizing voltage steps. Current records shown in Fig. 13(A) were recorded from a single muscle cell bathed in 5mM KCl while the records shown in Fig. 13(B) were obtained from a different muscle cell bathed in 5mM KAsp. Very little difference was apparent when chloride was substituted by an impermeant anion. As apparent in this figure and typical of most cells (96%, n=79) the current increased in amplitude within the first 5-10 ms and then declined. The amplitude of the current taken in the initial phase will be referred to as the peak amplitude and the current at the end of the 100 ms pulse will be called the late current. The time course of these

Fig. 13 Depolarization gated outward currents. Outward currents were recorded in (A) 5mM KCl and (B) 5mM K Asp. Depolarizing voltage steps were applied from a V_h of -70mV to potentials between -60mV and +30mV in 10mV increments. The corresponding current-voltage curves are shown below each set of current traces (●) peak outward , (■) and late currents. Values for peak curves were measured from the current records 7 ms following the onset of the voltage step while the late curves represent current amplitudes measured at 95 ms. The resting membrane potentials were -75mV (KCl) and -73mV (KAsp).


+30mV
-70mV
V_h
A
B
400pA
20ms
2.6
2.2
1.8
1.4
0.8
0.4
Current (nA)
-70
-50
-30
-10
+10
+30
MEMBRANE POTENTIAL (mV)
2.6
2.2
1.8
1.2
0.4
Current (nA)
-70
-50
-30
-10
+10
+30
MEMBRANE POTENTIAL (mV)

events is described in the next section. As with the activation of the inward current the amplitudes of these outward currents measured at peak and late levels showed wide variation from one muscle cell to the next (Table 3). The wide range of current amplitudes measured from one muscle cell to the next presumably reflects differences in potassium channel density.

III.2b Time Course of the Outward Current Activation

The outward current reached peak amplitude within 7ms of the beginning of the voltage step. Time constants (τ_R) describing the rate of rise to the peak were obtained by fitting the outward current record which lay between the end of the capacitance transient surge and the point of recorded peak amplitude as shown in Fig.14. The two arrows shown indicate the portion of that current which was fit to obtain τ_R. In this example the rate of rise was best fit by a single exponential with a τ_R of 1.22 ms., R=.9970. τ_R measured on this and 14 other muscle cells are plotted as a function of membrane potential in Fig. 15. These results indicate that the values of τ_R become briefer as the degree of membrane depolarization increases. A summary of these results is given in the Appendix (Table 4).

Table 3 The mean range in amplitudes of the outward current measured during a 100 ms depolarizing pulse to -10mV.

TABLE 3

The mean range in amplitudes of the outward current measured during a 100 ms depolarizing pulse to -10mV

	Peak Current(pA) Mean(SD), range	Late Current(pA) Mean(SD), range
5mM KCl (n=79)	671(304), 143-1750	416(196), 83-1167
5mM KAsp (n=11)	687(216), 404-1050	492(133), 342-792

Fig. 14 The measurement of the time constant τ_f for the peak outward current. The muscle cell was held at -70mV and the potential stepped to +10mV. That area of current record which lay betw en the two arrows was fit by a single exponential showing a τ_f of 1.22 ms, R=.9970. The resting membrane potential was -73mV. The external recording solution was 5mM KCl.

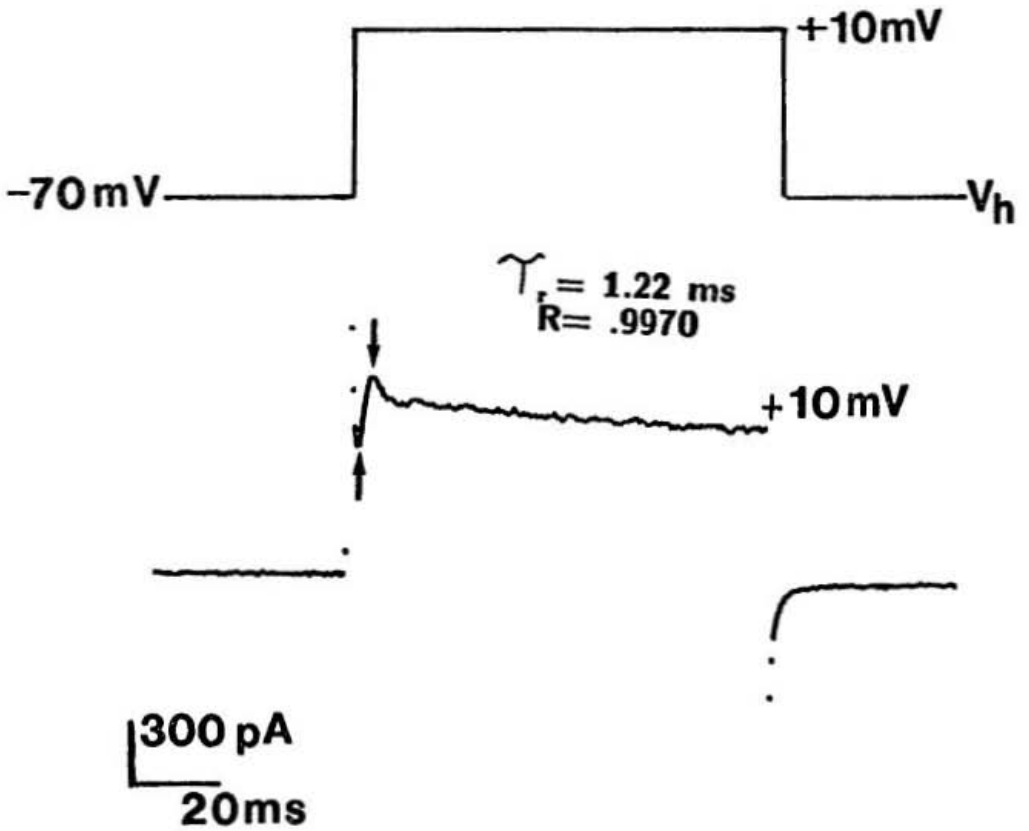
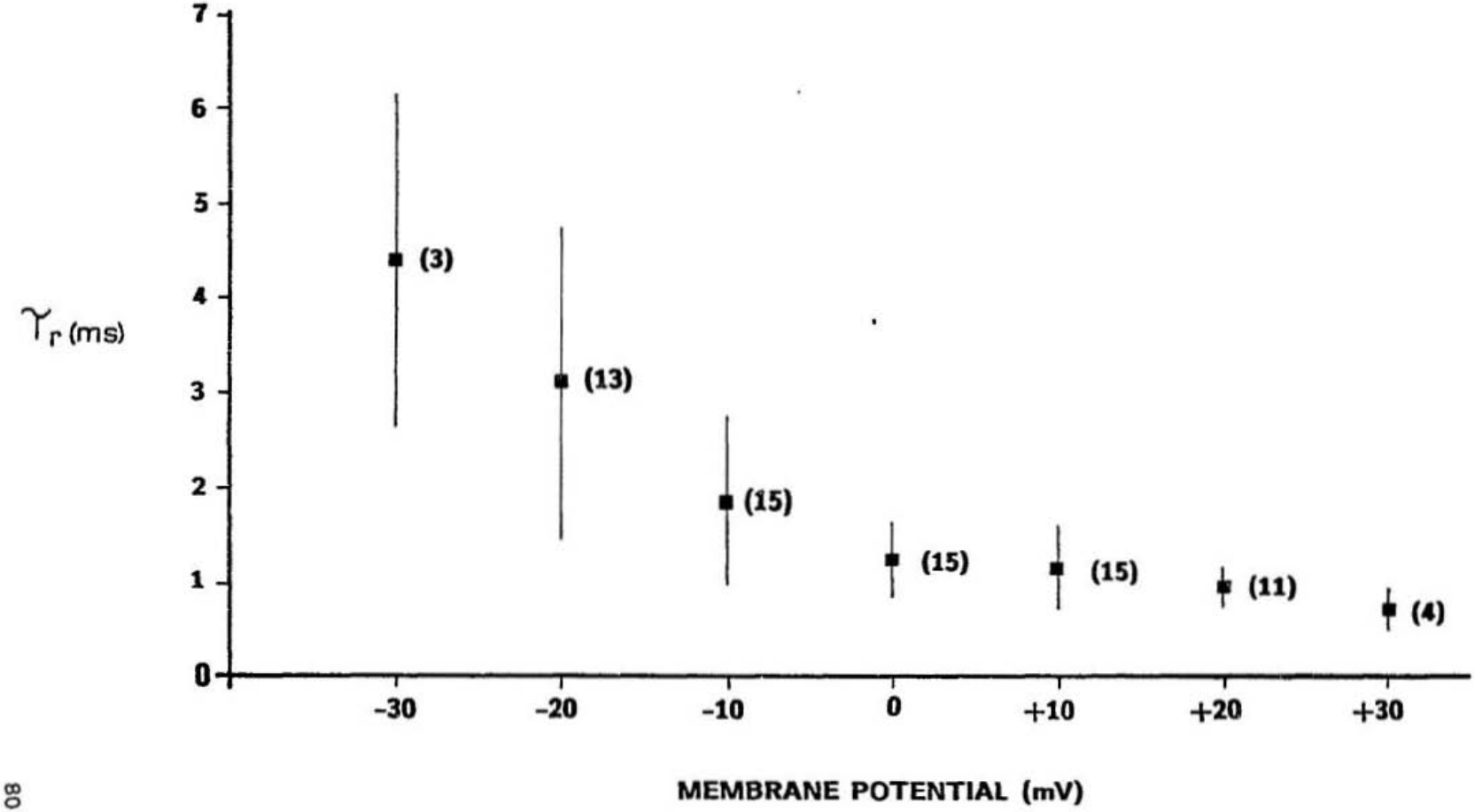

+10mV
-70mV
V_h
τ_r = 1.22 ms
R= .9970
+10mV
300 pA
20ms

Fig. 15 Mean time constants measuring the rise to peak outward current (τ_r) versus membrane potential. Time constants for the rise to peak amplitude were measured as described in Fig. 14 for 15 cells. The standard deviations are indicated in brackets. In all cases the external recording solution contained 5mM KCl.


τ_r (ms)
7
6
5
4
3
2
1
0
(3)
(13)
(15)
(15)
(15)
(11)
(4)
-30
-20
-10
0
+10
+20
+30
MEMBRANE POTENTIAL (mV)

III.2c Declining Phase of the Outward Current

Peak outward currents were generally transient in nature. Within 10 ms following the onset of a voltage step the typical current had risen to a peak amplitude and then declined to a lower current level. The phase of current decline which followed peak amplitudes varied from one muscle cell to the next. However two predominant types of time dependent changes occurred.

In 79% of muscle cells examined (n=89, 5mM KCl) the change in outward current which followed the peak could be described as consisting of a phase of rapid decline followed by a slower decline. Current records shown in Fig.13 typify the above described decline. In cells such as this the rate of decline was measured as shown in Fig. 16. In this example the decline was best fit by two exponentials with time constants of 3.0 ms and 106.2 ms. This type of decline in current amplitude was examined on a total of 12 muscle cells. Ten of these cells had a decline in current amplitude which could be best fit by a double exponential function. Time constants of these exponentials are shown in Table 5. Two cells had a decline in current amplitude best fit by single exponential functions.

Fig. 16 Time constant measurements of the rate of decline (τ_d) from peak outward amplitude. τ_d was measured during the current decline between the two arrows. The decline was best fit by a double exponential with τ_{d1} =3.0 ms and τ_{d2} = 106.2 ms. This is the same cell as shown in Fig. 14.

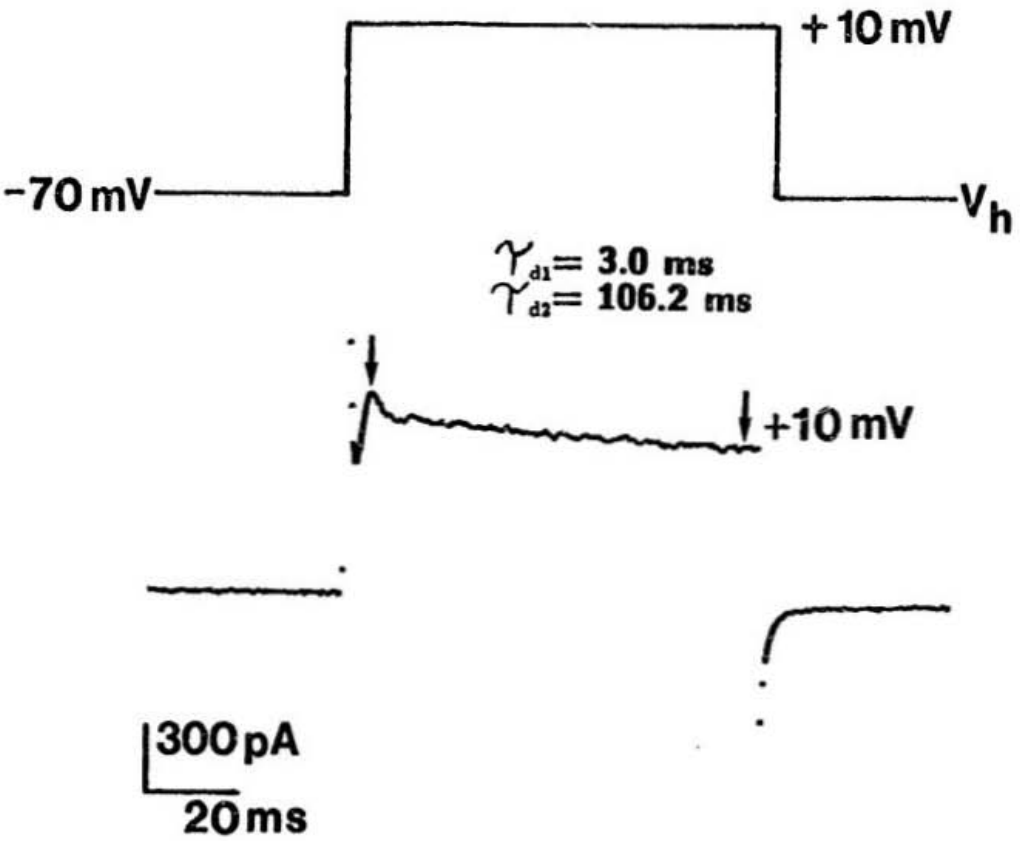

+10 mV
-70 mV
V_h
τ_{d1}= 3.0 ms
τ_{d2}= 106.2 ms
+10 mV
300 pA
20 ms

Table 5 Time constants for the decline from peak outward current amplitude measured during voltage steps to the indicated potentials. Current records were fit with double exponentials as described in Fig. 16.

TABLE 5

Time constants (ms) for the decline from peak outward current amplitude measured at various depolarizing voltage steps

(n)	Membrane Potential (mV)	τ_1(fast) Mean(SD),range (ms)	τ_2(slow) Mean(SD),range (ms)
8	-20	3.4(1.9),2-7	117.6(194.6),29-595
10	-10	3.3(2.4),2-9	53.8(29.3), 29-105
9	0	4.8(3.7),1-13	81.2(55.7), 12-189
10	+10	3.3(1.1),2-5	96.5(112.6),26-406
7	+20	2.9(0.8),2-4	158.5(262.8),16-705
2	+30	1.9(0.3),2-2	42.4(43.0), 12-73

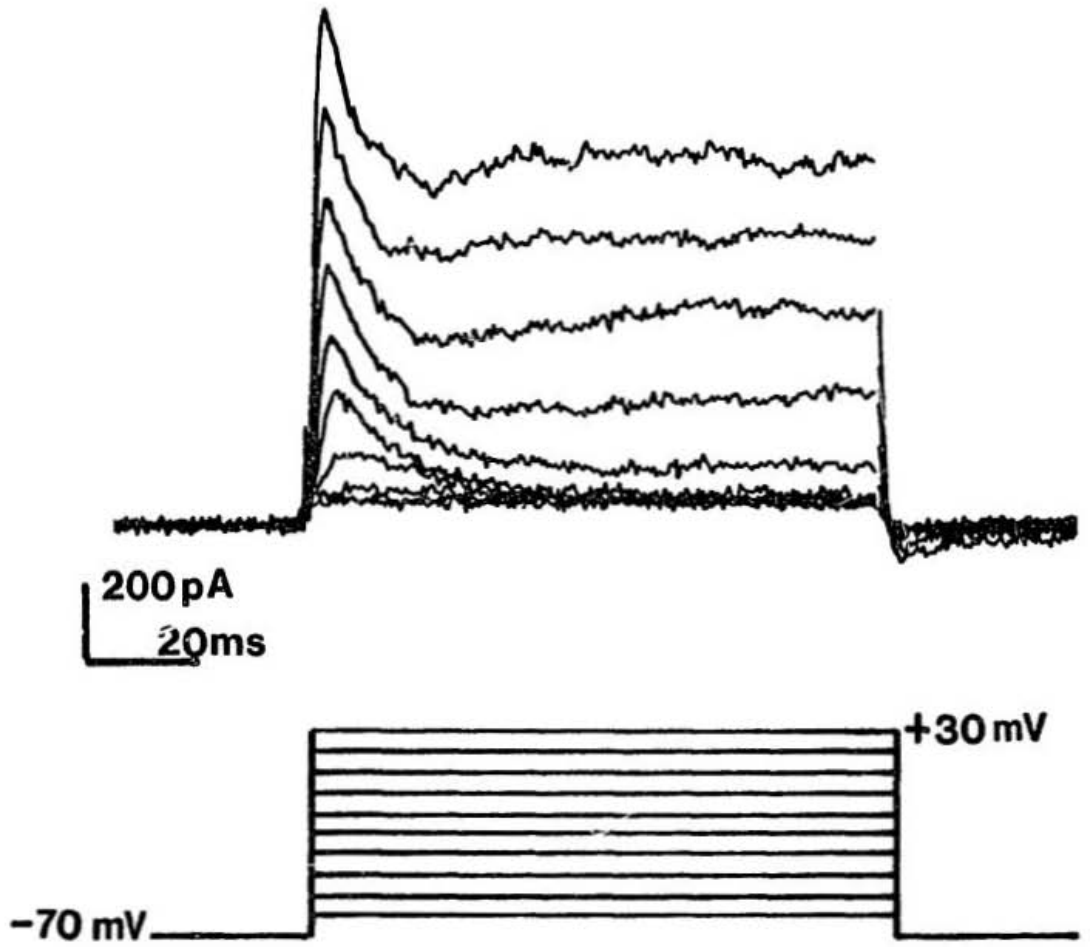
Unlike the values for τ_R, time constants of the declining phase of this current did not appear to be influenced by the degree of depolarization. Table 5 shows time constants measured for membrane potential steps from -20mV to +30mV.

In a small proportion of muscle cells, 21%, the current amplitude declined rapidly and this was followed by a slow rise in outward current. The whole cell current records shown in Fig. 17 were obtained from one such muscle cell. Whole cell current records of this type were identified in both the presence and the absence of chloride ions. This slow rise in outward current, following the rapid decline from peak levels may reflect the development of a slow activating potassium conductance which is characteristic of adult frog and rat skeletal muscle (Adrian, Chandler and Hodgkin,1970b; Stanfield,1970a; Barrett, Barrett and Dribin,1981; Hugues, Schmid, Romey, Duval, Frelin and Lazdunski,'982). Outward currents with this type of time dependent change were not included in this study.

III.2d Reversal Potentials of Depolarization Gated Outward Currents

Reversal potentials (V_{REV}) of the peak and late

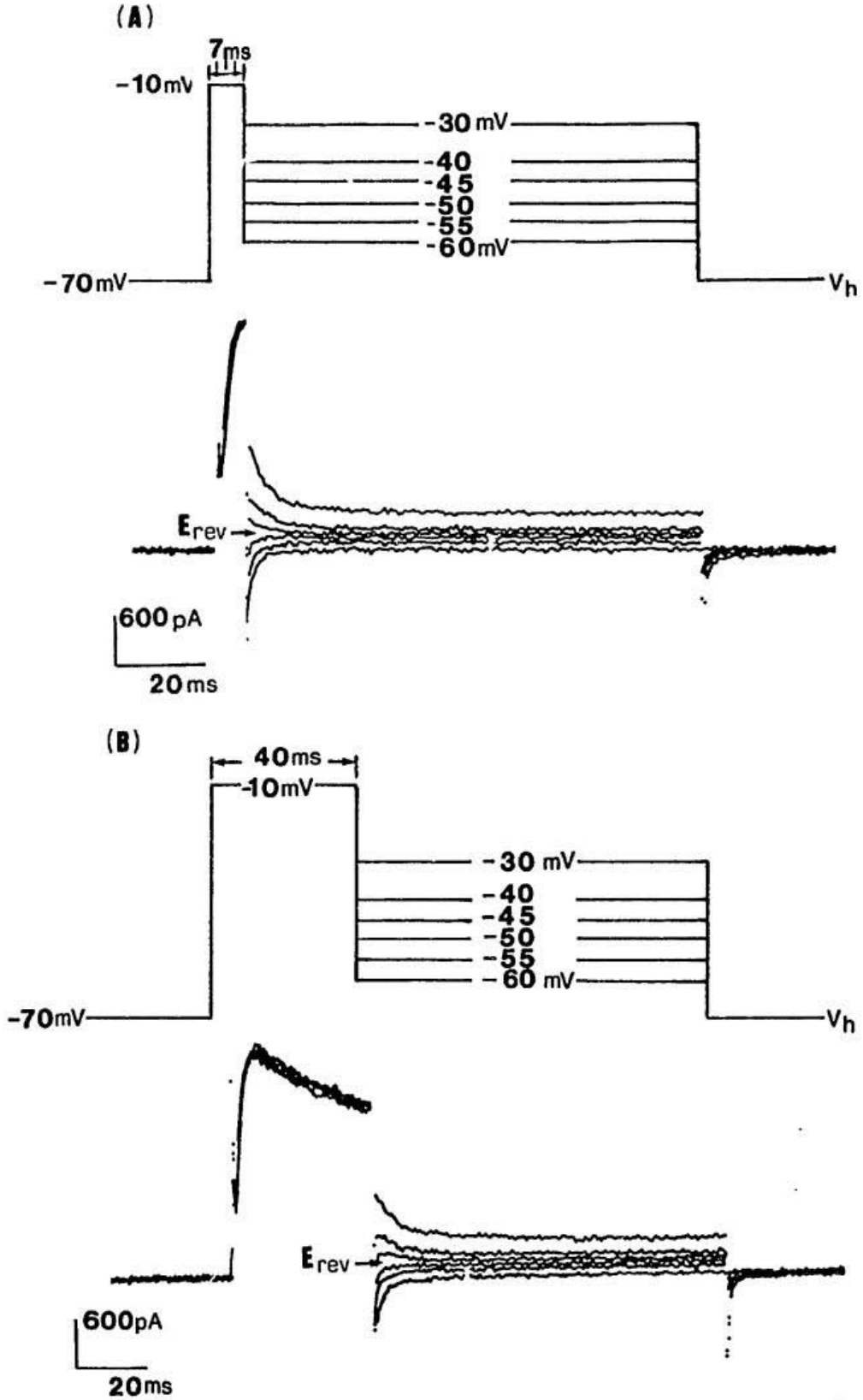
Fig. 17 Time-dependent change in the whole cell outward current observed during 100ms depolarizing voltage steps. Depolarizing steps applied from a V_h of -70mV to potentials between -60mV and +30mV. Resting membrane potential -74mV, 5mM KCl.


200 pA
20ms
+30 mV
-70 mV

components of the outward currents were determined using a conventional two step voltage clamp protocol as shown in Fig. 18. For the peak outward currents the initial voltage step was to a membrane potential known to elicit a substantial peak outward current (-10mV for 7 ms). This initial voltage step was immediately followed by repolarizing voltage steps to varying membrane potentials (-60mV to -30mV). Near the V_{REV} the membrane potential was incremented in 2 or 5mV steps. At the end of the initial voltage step there is a tail of current which may be either outward or inward depending on whether the membrane potential is above or below V_{REV}. Tail currents were typically quite small and showed rapid decline to steady state levels. In the example shown in Fig. 18A the current reversed between -45 and -50mV. The V_{REV} was taken as the midpoint between these values, ie. -47.5mV. Reversal potentials for the late component were measured in a similar way except in these cases the initial depolarizing step was to a membrane potential of -10mV or 0mV for 40-80 ms. Figure 18B shows the V_{REV} for the late component of the same cell shown in Fig. 18A. The late component, like the peak, had a V_{REV} between -45mV and -50mV and was taken as -47.5mV.

Reversal potentials of both the peak and late

Fig. 18 Reversal potentials (V_{REV}) of the peak (A) and late (B) outward currents on a single muscle cell bathed in 10mM KCl. The outward current was activated by a voltage step to -10mV and tail currents were examined when the membrane potential was stepped to the values indicated. The E_{REV} for the peak current was measured using an initial voltage step of 7 ms duration (A) and the late current was measured using an initial voltage step of 40 ms (B). In both (A) and (B) E_{REV} was between -50mV and -45mV.

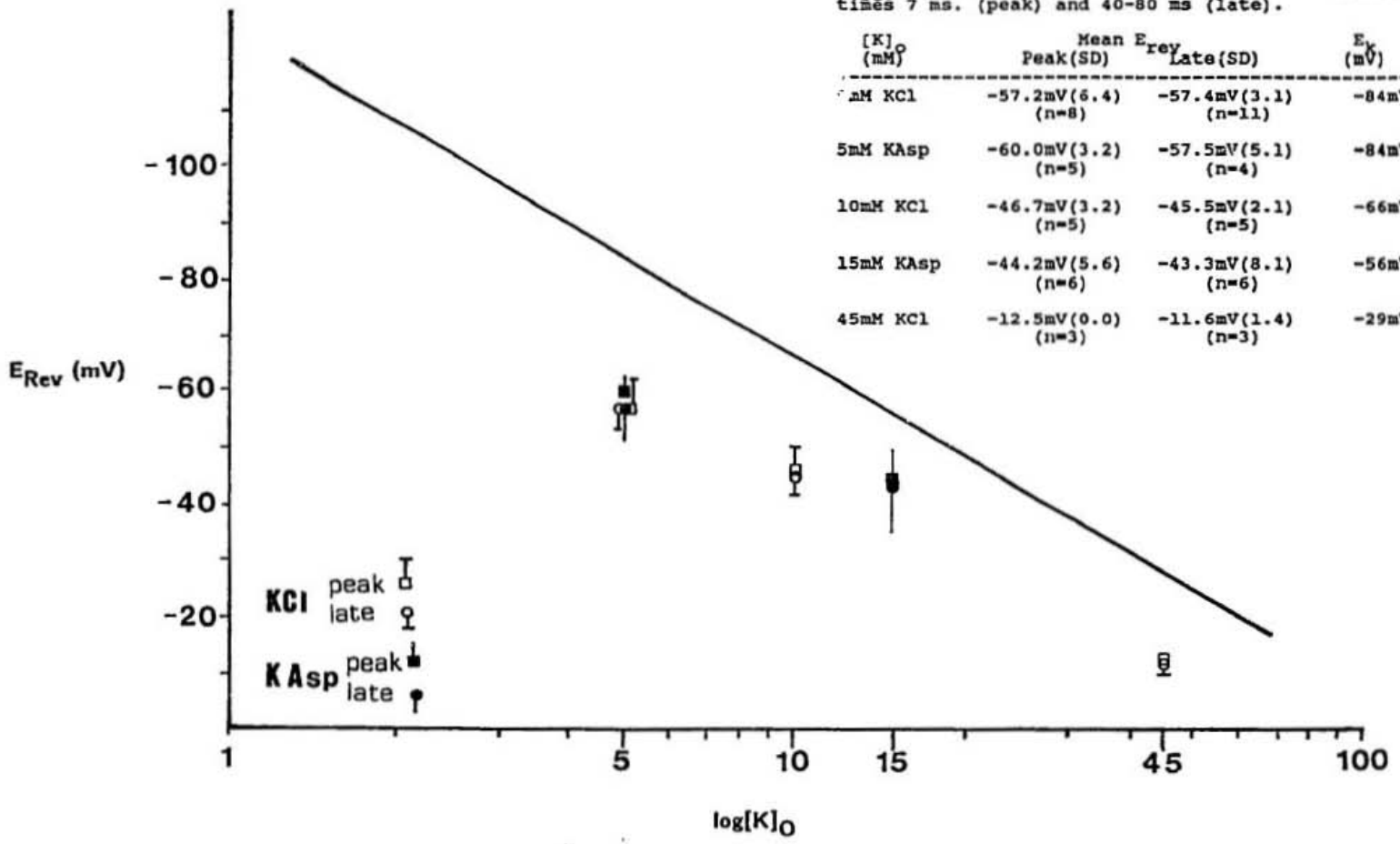

(A)
7ms
-10mV
-30 mV
-40
-45
-50
-55
-60mV
-70mV
Vh
Erev
600pA
20ms
(B)
40ms
-10mV
-30 mV
-40
-45
-50
-55
-60 mV
-70mV
Vh
Erev
600pA
20ms

components of the outward current were measured in three different external potassium chloride concentrations, 5mM KCl, 10mM KCl, and 45mM KCl and in 5mM KAsp and 15mM KAsp. The semilogarithmic plot in Fig. 19 illustrates the relationship between the mean reversal potential values for both the peak and late currents measured in these solutions and their predicted potassium equilibrium potentials. A number of points are illustrated by this figure. (1) The reversal potentials for both the peak and late components appear to be influenced by shifts in the external potassium ion concentration moving along the membrane potential axis in a manner which paralleled shifts in the calculated equilibrium potentials for potassium. (2) Reversal potentials of the outward currents did not appear to be altered by the substitution of chloride with L-Aspartic acid in the electrophysiological recording solutions suggesting that chloride did not play a major role in mediating the depolarization produced outward current. (3) The mean reversal potentials of the cells did not correspond exactly to the potentials predicted for an exclusively potassium selective channel suggesting that the depolarization produced outward currents were not perfectly selective for potassium ions and may involve other ionic species. (4) The

Fig. 19 Semilogarithmic plot of V_{REV} versus the external potassium concentration for the peak and late current components. The means and standard deviations are indicated on the graph and the actual values are shown in the table (inset). The number of cells for each concentration are indicated in the Table. The straight line was calculated from the Nernst equation for a pure potassium current at 21°C.

Effects of external $[K]_o$ on the reversal potential (E_{rev}) of the delayed outward current, measured at times 7 ms. (peak) and 40-80 ms (late).

$[K]_o$ (mM)	Mean E_{rev} Peak(SD)	Late(SD)	E_K (mV)
5mM KCl	-57.2mV(6.4) (n=8)	-57.4mV(3.1) (n=11)	-84mV
5mM KAsp	-60.0mV(3.2) (n=5)	-57.5mV(5.1) (n=4)	-84mV
10mM KCl	-46.7mV(3.2) (n=5)	-45.5mV(2.1) (n=5)	-66mV
15mM KAsp	-44.2mV(5.6) (n=6)	-43.3mV(8.1) (n=6)	-56mV
45mM KCl	-12.5mV(0.0) (n=3)	-11.6mV(1.4) (n=3)	-29mV



calculated reversal potentials for the peak and late component were very similar suggesting both components were mediated through the same ionic channel.

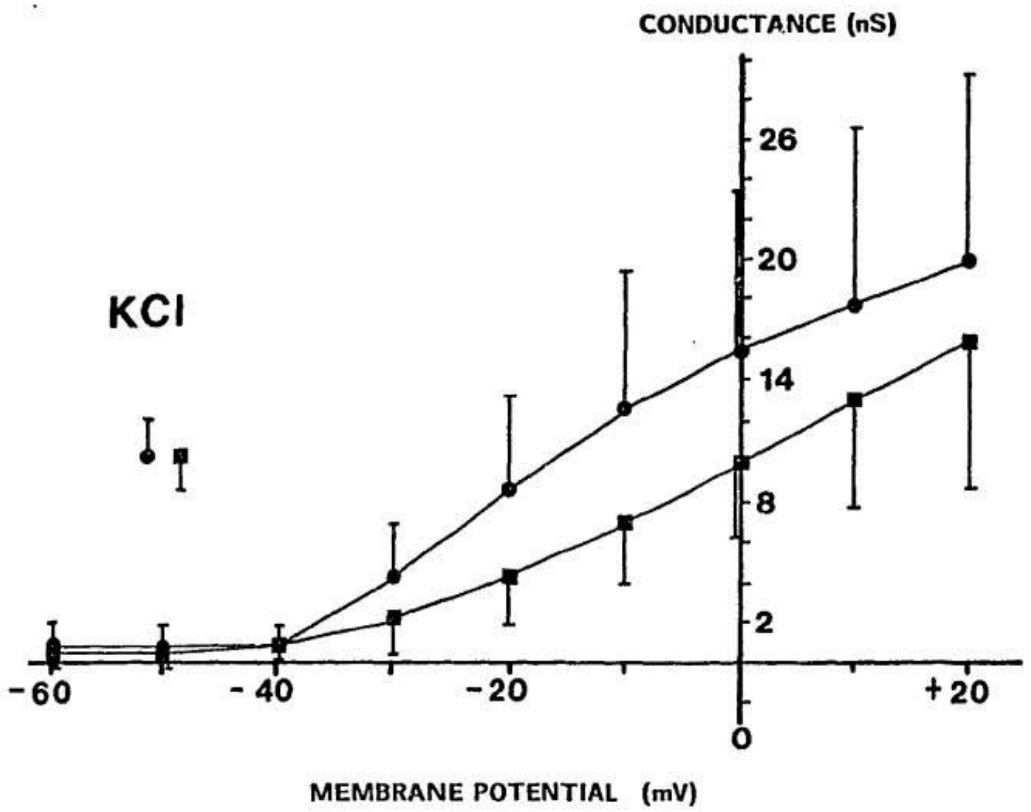
III.2e Conductance-Voltage Relations

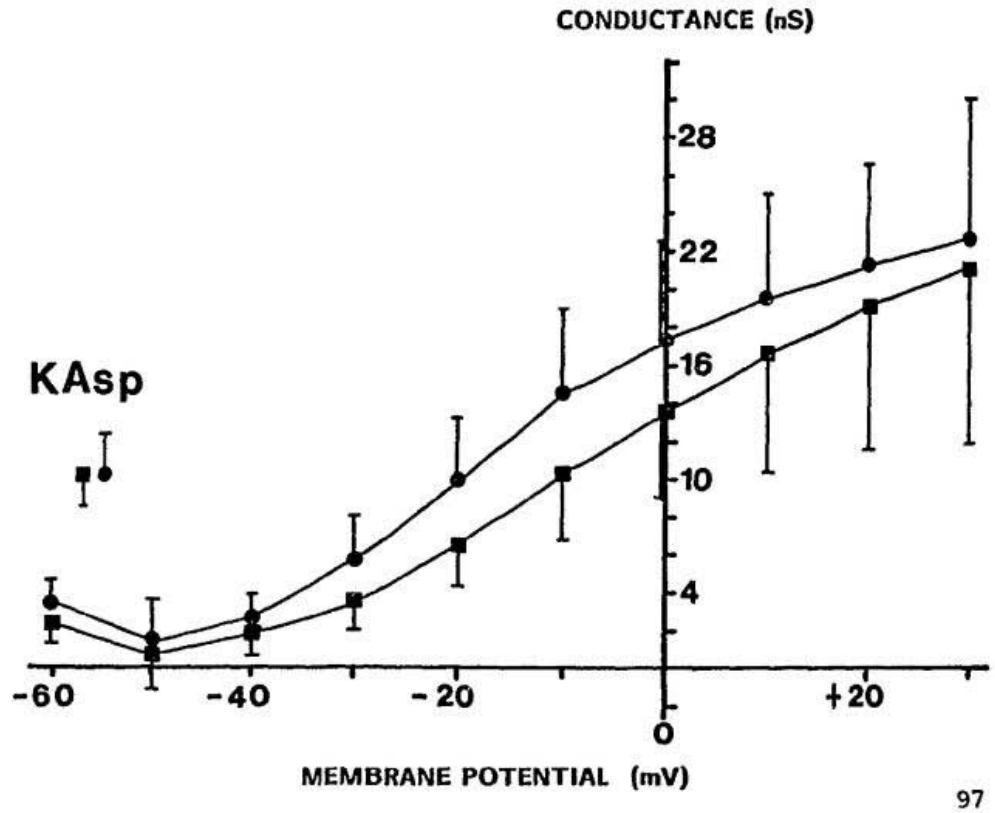
Conductance values were calculated from equation (1). V_{REV} was the mean reversal potential of the outward current (described above, section III.2e), and V_m was the membrane potential to which the cell was voltage stepped. I_{REV} the current measured at the mean reversal potential was zero and I_m was the current measured during the membrane potential step to V_m. The conductance of the peak and late components were determined at different membrane potentials and used to estimate the voltage of activation.

$$G = \frac{I_{REV} - I_M}{V_{REV} - V_m} \qquad (1)$$

Conductance values were determined for 13 cells bathed in 5mM KCl and 13 muscle cells bathed in 5mM KAsp and plotted against the membrane potential step (Fig. 20). As indicated in Fig. 20 the conductance for both components increased between -40mV and -30mV and

Fig. 20 Conductance voltage relations of the outward currents for muscle cells bathed in 5mM KCl (n=9) and 5mM KAsp (n=9). Points represent means, +/- standard deviations. (●) conductance measured at peak outward current amplitude, (■) late conductance values measured between times 80-90 ms. Conductance values measured using equation 1 (see text).


CONDUCTANCE (nS)
KCl
26
20
14
8
2
-60
-40
-20
0
+20
MEMBRANE POTENTIAL (mV)


CONDUCTANCE (nS)
KAsp
28
22
16
10
4
-60
-40
-20
0
+20
MEMBRANE POTENTIAL (mV)

continued to increase with further depolarization. These conductance-membrane potential curves demonstrate a sigmoidal relationship.

III.2f Voltage Dependence of Outward Current Inactivation

As shown in Fig. 20 conductance of the whole cell outward currents was dependent on the membrane potential to which the cell had been voltage stepped. However, the outward conductance also depended on the holding potential. In order to determine the inactivation potential a voltage clamp protocol similar to that described by Hodgkin and Huxley (1952c) for inactivation of the sodium current was used. Muscle cells were initially voltage clamped at various conditioning potentials (-70mV to -30mV) long enough to allow the inactivation process to reach steady state levels (1-2 minutes). A voltage step was then applied taking the cell to a membrane potential known to elicit a substantial outward current (test steps to 0mV). For each muscle cell the current amplitudes measured during test steps to 0mV were compared with the current amplitude evoked from the normal holding potential of -70mV. The fraction of current not inactivated at a given conditioning potential (h) was then plotted

against the conditioning potential and are presented in Fig. 21. These values represent the fraction of the outward current available to carry current when the membrane is depolarized to a given step potential. The results shown indicate that inactivation of both outward components is nearly complete (h=0.1) at holding potentials near -30mV and is almost entirely absent (h=1.0) when the holding potential was near the mean RMP (see Table 2).

III.2h Pharmacology

A variety of pharmacological agents were used to investigate the depolarization activated whole cell currents.

(1) Tetraethylammonium (TEA). External application of 50mM TEA readily reduced the depolarization produced outward currents in these muscle cells. Figure 22 shows outward currents recorded before (A) and after (B) the application of 50mM TEA. The corresponding current-voltage curves are shown in Fig 22(C). TEA (50mM) reduced but did not eliminate the two components of outward current. A variety of TEA concentrations were examined and the proportion of current remaining during a voltage step to 0mV was determined (Fig. 23).

Fig. 21 Inactivation curves for the depolarization gated whole cell outward currents of 6 muscle cells recorded in 5mM KCl. As shown in the upper right the membrane potential was taken from the holding potential (V_H) to a conditioning potential (V_C) for 1-2 minutes and then stepped to the test potential (V_T =0mV). The ordinate is the fraction of the outward current not inactivated at a given conditioning membrane potential (h). The abscissa is the conditioning membrane potential. (●) current inactivation measured at peak outward amplitudes and (■) late current values measured between time 80-90 ms.

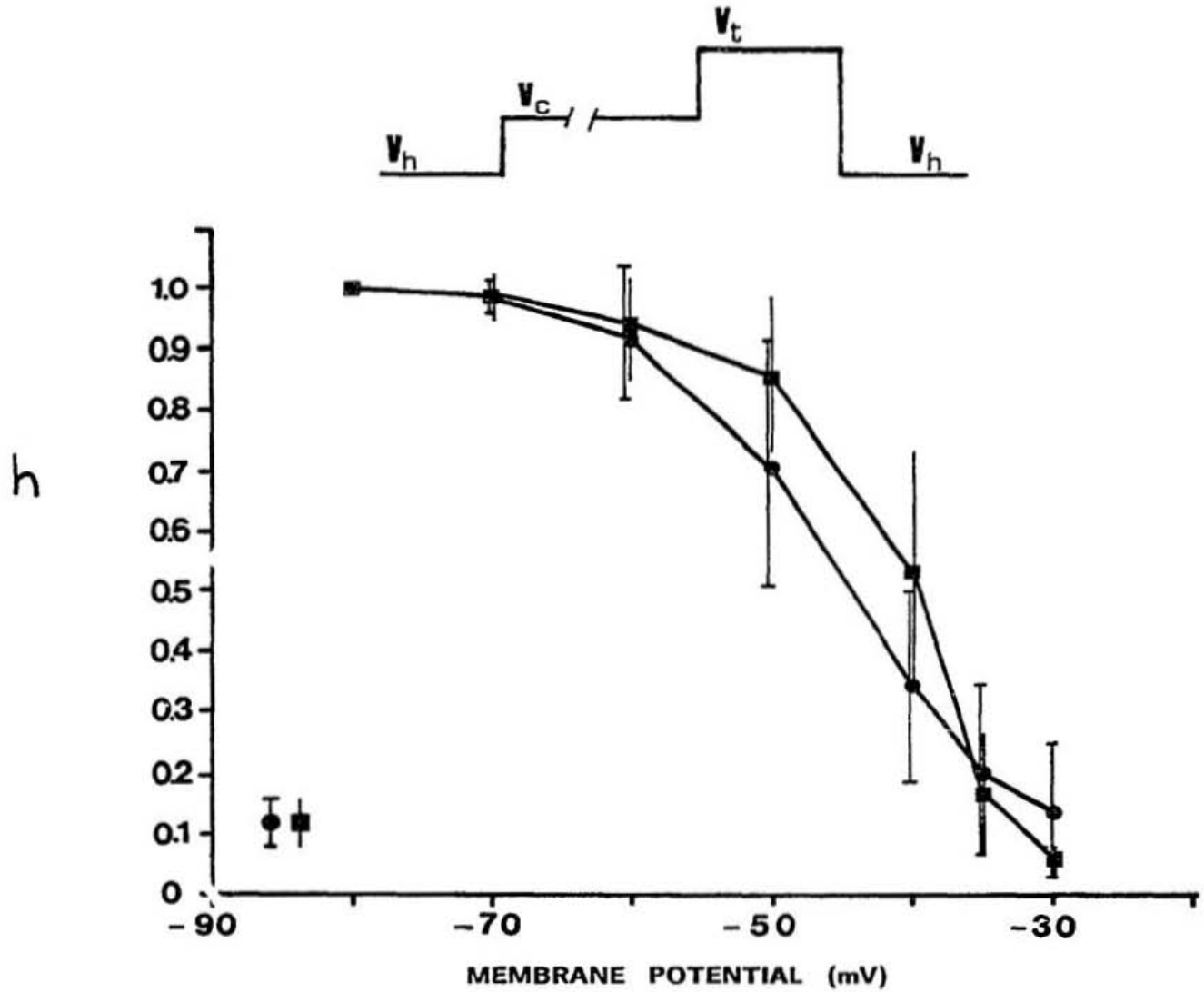

V_t
V_c
V_h
V_h
h
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-90
-70
-50
-30
MEMBRANE POTENTIAL (mV)

Fig. 22 Effect of TEA^+ on whole cell outward currents. Currents recorded before (A) and after (B) the addition of 50mM TEA^+ to the bath. Depolarizing voltage steps were applied in 10mV increments from a V_h of -80mV to potentials between -60mV and +20mV. (C) Corresponding current voltage curves of both the peak and late current components measured before and after the addition of 50mM TEA^+ to the bath. The resting membrane potential was -72mV, 5mM KCl.

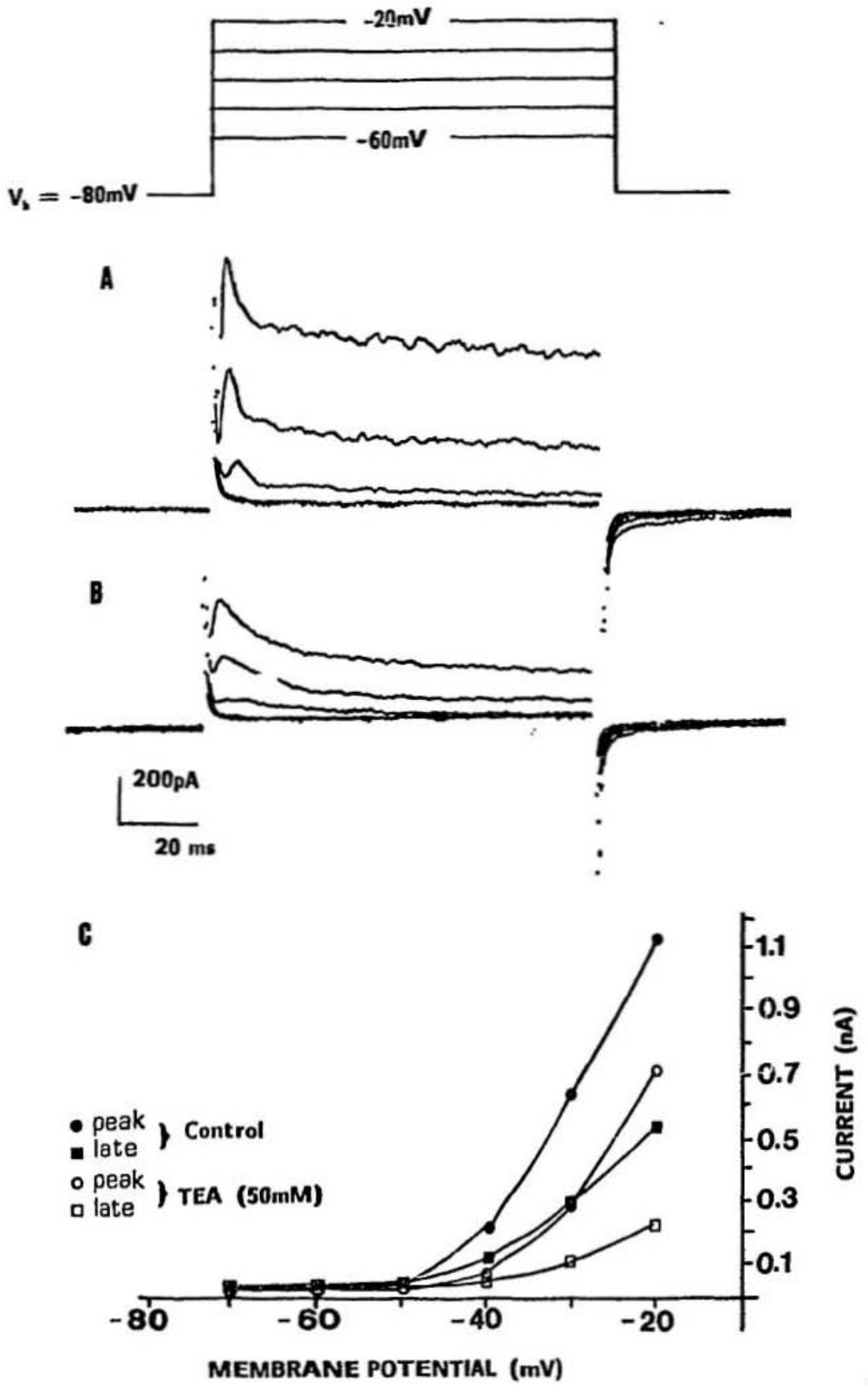

-20mV
-60mV
V_h = -80mV
A
B
200pA
20 ms
C
peak
late
Control
peak
late
TEA (50mM)
1.1
0.9
0.7
0.5
0.3
0.1
CURRENT (nA)
-80
-60
-40
-20
MEMBRANE POTENTIAL (mV)

Fig. 23 The effects of TEA concentration on the depolarization gated whole cell outward currents. The potassium current measured in the presence of TEA^+ relative to that in the absence of external TEA^+. The currents were activated by a step to 0mV from a V_H of -70mV. Points represent mean values, vertical lines represent standard deviation. The numbers in parentheses represent the number of cells comprising the means. The external recording solution was 5mM KCl.

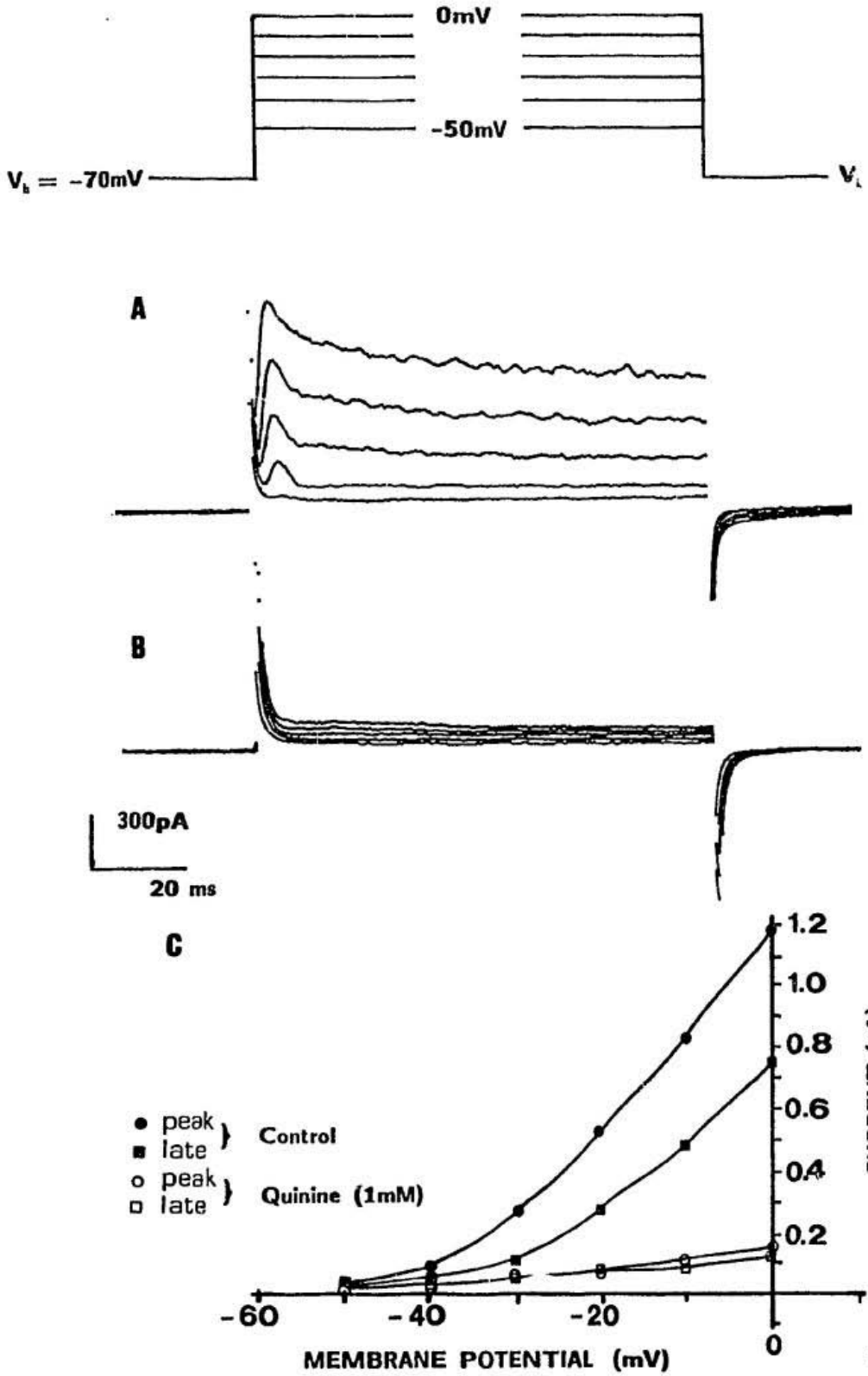
peak
late
(3)
(6)
(7)
(2)
(3)
(1)
(3)
1.4
1
0.5
0.1
0
10
20
30
40
50
60
TEA+ (mM)
Current (with TEA)/Current (without TEA)

The minimum concentration at which TEA was effective at reducing outward currents was 10mM. At concentrations less than 10mM, TEA appeared to produce an increase in outward current. Concentrations of TEA above 40mM did not continue to reduce the outward current. However in experiments where potassium was completely removed from the external and internal recording solutions no outward current is apparent (Moody-Corbett, Gilbert, Akrabrali and Hall, 1989).

(2) Quinine. Quinine was found to be more effective at blocking both components of the outward current. Bath application of quinine (0.5 - 2mM) strongly reduced the depolarization produced outward currents on these muscle cells. Figure 24 shows typical current records both before (A) and after (B) the external application of 1mM quinine. Corresponding peak and late current voltage curves are shown in Fig. 24(C). In this example quinine reduced both the peak and late currents equally. Similar results were obtained in three experiments.

(3) 4-aminopyridine (4AP). External application of 2-16mM 4AP (pH=7.0, n=4 and pH=10 n=6) generally reduced both components of the depolarization activated outward currents on these muscle cells. However the results

Fig. 24 The effects of quinine on the whole cell outward currents. Membrane ionic currents recorded before (A) and after (B) the application of 1mM quinine to the bath. Depolarizing voltage steps were applied from a V_h of -70mV to potentials between -50mV and -10mV. (C) Corresponding current-voltage curves of the peak and late current components in both the presence and absence of 1mM quinine. Resting membrane potential -60mV, 5mM KCl.


0mV
-50mV
V_h = -70mV
V_t
A
B
300pA
20 ms
C
peak
late
Control
peak
late
Quinine (1mM)
1.2
1.0
0.8
0.6
0.4
0.2
CURRENT (nA)
-60
-40
-20
0
MEMBRANE POTENTIAL (mV)

were not consistent from one muscle cell to the next. For example in one muscle cell 4AP (4mM) reduced the peak component by 69% while reducing the late component by 49%. However in another muscle cell 4AP (4mM) reduced the later component by 25% while increasing the peak current by 11%. The results presented in Table 6 illustrate the variability encountered when using 4AP. It was noted during these applications of 4AP that the cells showed evidence of numerous invaginations suggesting a toxic effect on these muscle cells. Such morphological changes were often accompanied by shifts in RMP's of +50 to +60mV.

(4) Calcium Current Antagonists. Previous investigations have identified a depolarization activated inward calcium current on this muscle cell preparation (Moody-Corbett, Gilbert, Akrabrali and Hall, 1989). A number of calcium current antagonists were found to be effective blockers of this inward calcium current including Nifedipine and (+)PN 200-110+. These agents were used to examine the possibility that the depolarization activated outward currents were dependent on calcium influx.

(a) Nifedipine. The effects of nifedipine (65 μM) on the depolarization activated outward currents on a cell are shown in Fig. 25. At this concentration nifedipine

Table 6 Effects of 4-Aminopyridine on the whole cell outward currents. All muscle cells bathed in 5mM KCl. pH adjustments made with HCl.

TABLE 6

EFFECTS OF 4-AMINOPYRIDINE ON THE DEPOLARIZATION ACTIVATED POTASSIUM CURRENTS

CELL#	[4AP] (mM)	pH	% CURRENT CHANGE PEAK* ↑(%)	PEAK* ↓(%)	LATE* ↑(%)	LATE* ↓(%)	INWARD (1) (%)
R80202C06	2.0	10	--	6*	--	35	---
R80324C04	3.0	10	--	35	--	69	---
R80202C06	4.0	10	--	69	--	49	---
R70709C01	4.0	10	--	37	--	21	2 (-120mV)↓
R80203C01	4.0	10	11	--	--	25	---
R71008C01	4.0	10	6	--	2	--	---
R80203C03	8.0	10	--	1	--	63	---
R80203C03	10.0	10	1	--	17	--	---
R80203C01	12.0	10	--	54	--	41	49 (-100mV)↓
R80203C03	14.0	10	--	30	--	5	21 (-100mV)↓
R80527C04	2.0	7	--	4	--	3	8 (-120mV)↓
R80406C01	2.0	7	8	--	--	6	--
R80325C01	3.2	7	--	6	--	45	--
R80406C01	4.0	7	5	--	--	55	17 (-110mV)↓
R80325C02	6.2	7	2	--	--	6	--
R80325C01	7.0	7	--	36	--	62	--
R80325C01	10.0	7	--	63	--	37	--
R80325C02	12.5	7	--	8	--	10	--
R80325C02	16.0	7	--	22	1	--	3.5(-110mV)↓

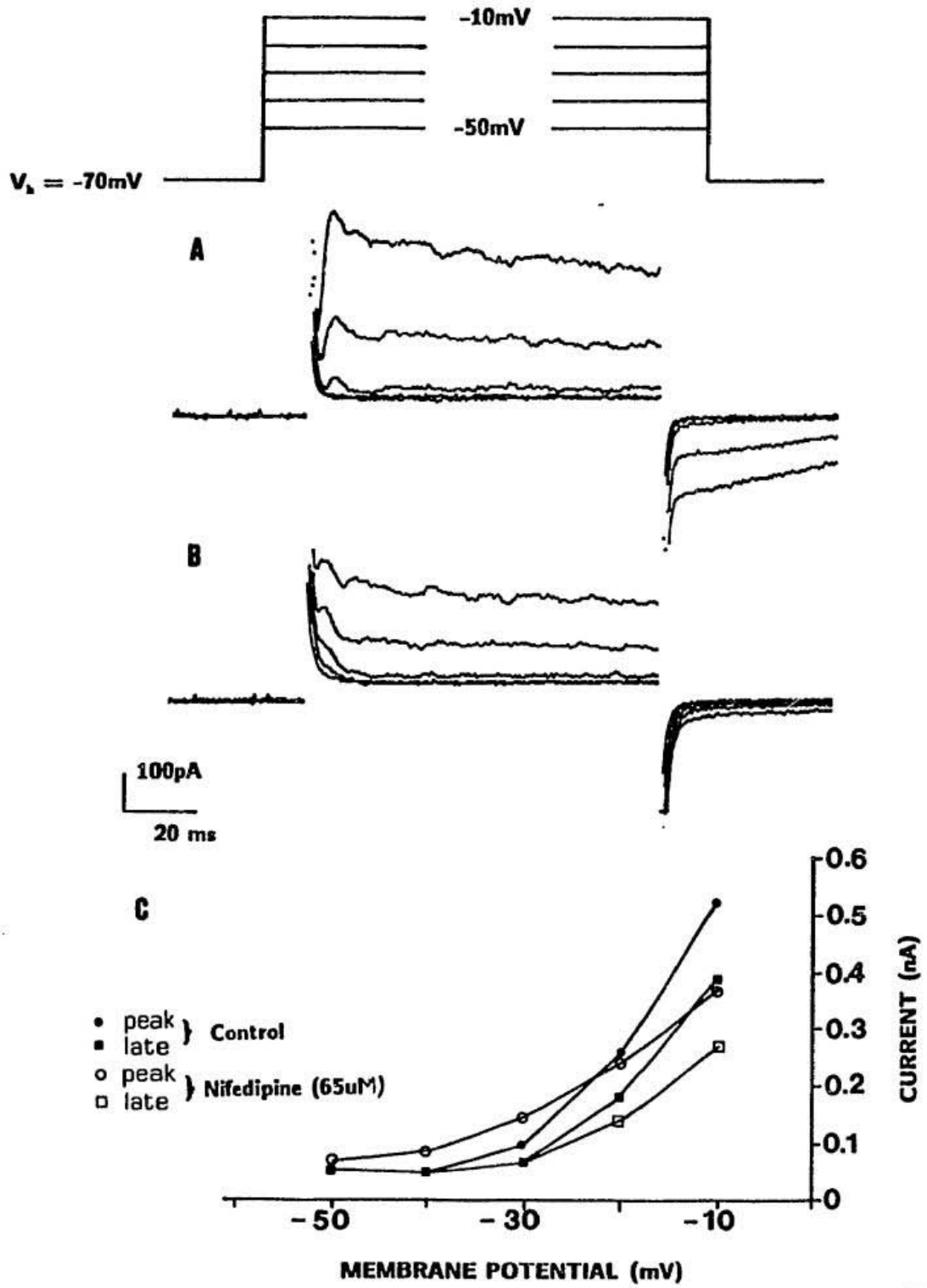
* Peak and Late current changes measured on membrane potential steps to -10mV

1 Number in brackets refers to the membrane potential at which current change was measured.

(↓) Refers to a reduction in current amplitude following external application of 4AP.

(↑) Refers to an increase in current amplitude following external application of 4AP.

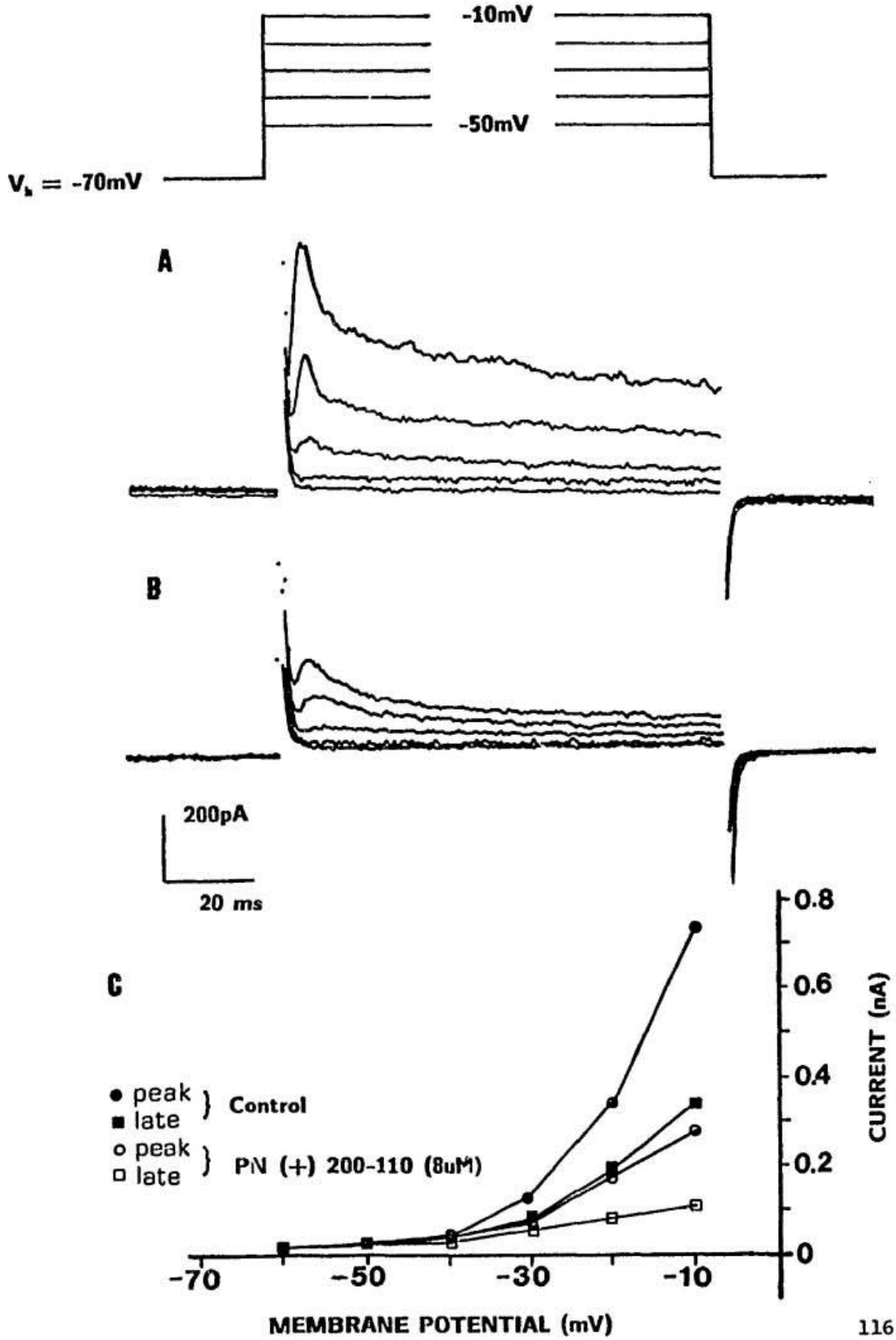
Fig. 25 Effects of nifedipine on the whole cell outward currents. Whole cell membrane currents recorded before (A) and after (B) the application of 65uM nifedipine to the bath. Depolarizing voltage steps were applied from a V_h of -70mV to potentials between -50mV and -10mV. (C) Corresponding current-voltage curves for the peak and late current components measured both before and after the addition of 65uM nifedipine. The resting membrane potentials were -70mV (control) and -73mV (after application of nifedipine), 5mM KCL.


-10mV
-50mV
V_h = -70mV
A
B
100pA
20 ms
C
peak
late
Control
peak
late
Nifedipine (65uM)
0.6
0.5
0.4
0.3
0.2
0.1
0
CURRENT (nA)
-50
-30
-10
MEMBRANE POTENTIAL (mV)

reduced both components of outward current by approximately 30%. Note the concurrent reduction in tail current in this example. Similar results were observed over concentrations ranging from 35-85μM nifedipine (n=4). Inward currents were not affected to the same degree. For example, 3 of 4 muscle cells showed a 20% decrease of inward current with the application of nifedipine (>35μM). A fourth muscle cell showed a 10% increase. External application of nifedipine was associated with 2-3mV negative shifts in the mean RMPs in 71% of the muscle cells examined (n=7). At concentrations less than 35μM, nifedipine reduced the outward currents in only one of four muscle cells examined and the other three were unchanged. However these concentrations are believed below the effective concentrations required for block of inward calcium currents.

(b) (+)PN 200-110. Bath application of (+)PN 200-110 (4-10μM , n=4) produced reductions in outward currents similar to that of nifedipine, (Fig. 26). At a concentration of 8μM (+)PN 200-110 both components of outward current were reduced by 65%. Hyperpolarization activated inward currents were also affected by external application of this drug. The inward currents were not affected to the same degree. For example, 2 of 3 muscle cells examined showed a 32% (averaged for 2

Fig. 26 Effects of PN(+)200-110 on the whole cell outward currents. Whole cell membrane currents recorded before (A) and after (B) the application of 8uM PN(+) 200-110 to the bath. Depolarizing voltage steps were applied from a V_h of -70mV to potentials between -50mV and -10mV. (C) Corresponding current-voltage curves for peak and later current components measured before and after the addition of 8uM PN(+) 200-110. The resting membrane potentials were - 50mV (control) and -57mV (after PN(+)). Muscle cells immersed in 5mM KCl.

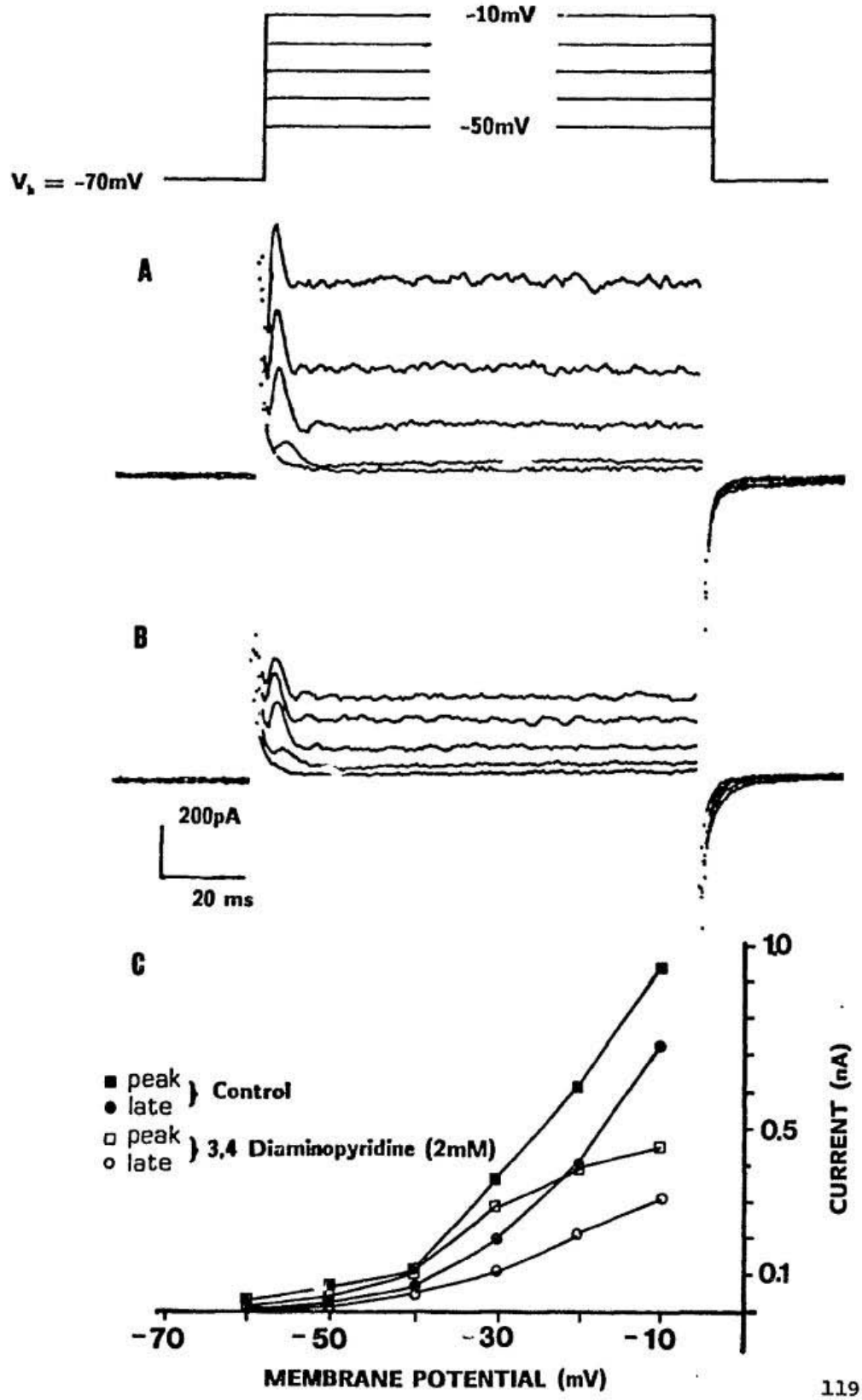

-10mV
-50mV
V_h = -70mV
A
B
200pA
20 ms
C
peak
late
Control
peak
late
PN (+) 200-110 (8uM)
0.8
0.6
0.4
0.2
0
CURRENT (nA)
-70
-50
-30
-10
MEMBRANE POTENTIAL (mV)

cells) reductions of inward current amplitudes. A third cell showed an increase of inward current amplitude.

(5) Apamin. Apamin is a polypeptide protein obtained from bee venom (Habermann,1972, 1984). This protein has been shown to be a selective blocker of a specific class of calcium sensitive slow potassium currents on frog skeletal muscle fibres in the 50-100nM concentration range (Cognard, Traore, Potreau and Raymond,1984). External application of apamin (50-80nM, n=5) did not reduce any part of the 100 ms depolarization activated outward current in this preparation.

(6) 3,4 Diaminopyridine (3,4DAP). The modulatory effects of 3,4DAP on the outward currents were also investigated. 3,4 diaminopridine (2-3mM pH=7.0) reduced both components of outward current when applied externally. The whole cell current records shown in Fig. 27 were recorded from a muscle cell before (A) and after (B) the addition of 2mM 3,4DAP. At this concentration both components of the outward currents were reduced approximately equally.

Fig. 27 The effects of 3,4-Diaminopyridine (3,4DAP) on the whole cell outward currents. Membrane ionic currents recorded before (A) and after (B) the application of 2mM 3,4DAP to the bath. Depolarizing steps were applied from a V_h of -70mV to potentials between -50mV and -10mV. (C) Corresponding current-voltage curves for the peak and late current components measured both before and after the addition of 2mM 3,4DAP. The resting membrane potential was -62mV, 5mM KCl.


-10mV
-50mV
V_h = -70mV
A
B
200pA
20 ms
C
peak
late
Control
peak
late
3,4 Diaminopyridine (2mM)
1.0
0.5
0.1
CURRENT (nA)
-70
-50
-30
-10
MEMBRANE POTENTIAL (mV)

IV Discussion

IV.1 Inward Currents Activated by Hyperpolarization

The inward current recorded on these embryonic Xenopus muscle cells in culture is similar to the anomalous rectification of adult frog skeletal muscle fibres (Adrian and Freygang,1962; Almers, 1971,1972a,1972b). In preparations of adult frog skeletal muscle fibres, activation of anomalous rectification has been described as consisting of two components, an instantaneous current followed by a time dependent increase to peak current amplitude (Adrian and Freygang,1962(a); Almers,1971,1972(a)). The activation properties of the hyperpolarization produced inward current on this preparation of cultured embryonic muscle cells are consistent with those described for adult tissue. Immediately upon hyperpolarization an inward current appeared followed by a time dependent rise to peak values.

In preparations of adult frog skeletal muscle fibres anomalous rectification has characteristic inactivation properties. This inactivation has been attributed to both a time dependent change in conductance as well as a voltage dependent change in

permeability (Adrian and Freygang,1962b, Almers, 1971,1972a,b). Similar conductance changes have been observed on this preparation of embryonic frog skeletal muscle cells. In adult muscle fibre preparations the time dependent decline in current amplitude measured at moderate hyperpolarizations has been attributed to depletion of potassium ions in the unstirred extracelluar space of the tubular system (Almers,1971; Adrian and Freygang,1962b). This hypothesis is supported by reports that in skeletal muscle fibres anomalous rectifying potassium channels are primarily located within the tubular system membrane (Eisenberg and Gage,1969) and the decline can be reduced by increasing the external potassium concentration. We have shown that increasing the $[K]_o$ to 100mM decreased the declining phase of inward current observed during moderate membrane hyperpolarizations. This suggests that the effect was due to increased potassium available. This finding is consistent with reports of Almers (1971) who demonstrated an absence of current decline for adult frog skeletal muscle fibres immersed in 117.5 K^+-glucuronate.

In preparations which lack tubular systems such as starfish and tunicate egg cells, and cat ventricular myocytes this depletion phenomenon is dramatically smaller (Hagiwara, Miyazaki and Rosenthal,1976;

Hagiwara and Takahashi,1974, Ohmori,1978,1980; Harvey and Ten Eick,1988).

In adult frog skeletal muscle fibres as well as tunicate egg cells the decline in current amplitude seen upon extreme hyperpolarizations (to membrane potentials above -140mV) has been attributed to a block of anomalous rectifier channels by external sodium ions (Standen and Stanfield,1980; Ohmori, 1978,1980). However the existence of a sodium independent permeability change has been reported in certain non-skeletal muscle preparations. In guinea pig heart cells Sackmann and Trube (1984) have demonstrated an inactivation of single anomalous rectifier channels, even in the absence of extracelluar sodium. Similar reports have been made for rat ventricular myocytes (Josephson and Brown,1986) and macrophage cells (Mickinney and Gallin,1988). In the present study, substitution of external sodium chloride by tris chloride resulted in a decrease in the current decline and the absence of negative slope in the steady-state current-voltage curve. This finding suggests that as in adult skeletal muscle, at extreme hyperpolarizations sodium plays a role in the inactivation of the inward current.

In adult frog skeletal muscle fibres the activation of anomalous rectification has been shown to depend on both membrane potential and the $[K]_o$ (Leech and Stanfield,1981; Hestrin,1981). When the membrane potential of these fibres was stepped to values hyperpolarized to the E_K, the anomalous rectifying current appeared. Furthermore, when the E_K was altered by changes in the $[K]_o$ the potential of activation of this current shifted in a manner which paralleled the changes in E_K. A similar relationship was observed for the hyperpolarization activated potassium current identified on Xenopus embryonic skeletal muscle cell in the present study. This inward current activates at membrane potentials close to the calculated E_K. Furthermore shifting the E_K by changing $[K]_o$ at a constant $[K]_i$ produced a parallel shift in the membrane potential of activation. This characteristic was found to be consistent over a wide range of $[K]_o$ and in both the presence and absence of chloride. These results show that the activation properties of the hyperpolarization activated inward current correspond well with the properties of anomalous rectification described in other preparations.

External application of cesium has been reported to

block anomalous rectifier potassium currents in such preparations as adult frog skeletal muscle fibres (Gay and Stanfield,1977; Oksana, 1986), starfish egg cells (Hagiwara and Takahashi,1974; Hagiwara, Miyazaki and Rosenthal,1976), and cat and rat ventricular myocytes (Josephson and Brown,1986; Harvey and Ten Eick,1988). Block by external cesium is a function of both membrane potential and the $[Cs]_o$. Similar results have been found in the present study. For a given $[Cs]_o$, block of the inward current was enhanced by increasing the degree of membrane hyperpolarization. Furthermore for a given hyperpolarizing value, block of the inward current increased as $[Cs]_o$ was increased. The effective concentration of cesium (3mM) in this preparation was found to be slightly higher than that required for block of adult frog skeletal muscle fibres (1.25mM) and starfish egg cells (0.3mM), but was less than that required for cat ventricular myocytes (5mM).

In summary, the results of the present study indicate that Xenopus muscle cells grown in culture develop an inward current with properties similar to the inward or anomalous rectifier found on adult frog muscle (Almers 1971, 1972a,b; Hestrin,1981).

IV.2 Outward Currents Activated by Depolarization

The depolarization gated outward currents on these embryonic Xenopus muscle cells rose to peak amplitude within 10 ms (peak component) and then declined to lesser current values (late component). A number of features suggest that both components of this current are mediated by potassium ions flowing through delayed rectifier potassium channels.

Evidence suggesting this current as being predominantly mediated by potassium ions is summarized as follows. (1) The direction of the outward current is along the electrochemical gradient for potassium ions. Under the present experimental conditions, driving forces on sodium, alcium and chloride ions could only result in inward currents. (2) Reversal potentials of the outward currents did not appear to be altered by the substitution of chloride with L-aspartic acid in the electrophysiological recording solutions. This finding suggested that chloride ions do not play a major role in mediating the outward current. (3) The mean reversal potentials of the outward currents lie closest to the calculated equilibrium potential for potassium suggesting that this ion was the major contributor. Furthermore for a given change in the external potassium concentration, mean reversal

potentials shifted along the membrane potential axis in a manner which paralleled shifts in the E_K. However the fact that calculated reversal potentials fall positive to E_K indicates that the current is not perfectly selective for potassium ions. It is possible that either sodium or calcium ions may also contribute to the whole cell outward current records. Both sodium and calcium have equilibrium potentials positive to +50mV. (4) External application of TEA (10-60mM) and quinine (0.5 - 2.0 mM) two antagonists of delayed rectifier potassium currents (Stanfield,1983; Fishmann and Spector, 1981) produced reductions in these whole cell outward currents.

The outward potassium current described in this thesis appeared to be mediated through delayed rectifier potassium channels similar to that described on adult frog skeletal muscle fibres (Adrian, Chandler and Hodgkin,1970; Stanfield,1970a). Evidence supporting this conclusion is discussed below.

(1) The delayed outward potassium current identified on adult frog skeletal muscle fibres is characterized by a rapid rise to maximum outward conductance followed by an inactivation to lesser conductance values. The depolarization gated outward

conductance identified on these embryonic Xenopus muscle cells possessed similar activation and inactivation properties. In the present study rise times to maximum outward conductances ranges from 0.8 to 1.93 ms (step to 0mV , room temperature). Although these values appear fast compared with time constants reported for adult muscle fibres (5-8 ms, Adrian, Chandler and Hodgkin,1970), the experiments on adult muscle were conducted at 3°C.

Following the rise to peak amplitude the outward current showed a decline during the 100 ms depolarizing steps used in this study. This decline in current amplitude was best fit by a double exponential function having both a slow and a fast component. For membrane depolarizations to +10mV the fast components ranged from 1.6 to 4.9 ms while the slow components had time constants ranging from 25.6 to 406.3 ms. In adult muscle fibres, Adrian, Chandler and Hodgkin (1970) also report a decline in current amplitude in the delayed rectifier potassium current. However the inactivation is fit by a single exponential function with much slower time constants (400-1200 ms, +10mV, 20°C). Stanfield (1970) reported for a single muscle fibre a time constant of 270 ms (10mV, 20°C). The faster kinetics seen on these embryonic muscle cells may

reflect differing current fitting parameters. When determining the time constants of inactivation of the delayed current, Adrian, Chandler and Hodgkin (1970) fit a portion of current nearly 4 seconds long. Under maintained depolarization of this length an additional slow calcium dependent outward potassium conductance activates. Activation of this slow potassium current may have prevented accurate measurement of the delayed current inactivation. Adrian, Chandler and Hodgkin (1970) estimate that their rates of inactivation may have been increased by as much as 70%. In the present study the portion of current fit when determining time constants of inactivation lay within the first 100 ms of membrane depolarization. Within this brief time frame the slow outward potassium current may not have had time to become apparent. The inactivation of the delayed rectifier has been described on numerous preparations (for a review see, Rudy, 1988)

(2) Inactivation curves describing the relationship between holding potentials and the ability of a membrane conductance to undergo permeability change were examined using embryonic muscle cell preparation. Both the peak and late current components of the delayed outward current showed inactivation which was almost complete (h=0.1) at holding potentials positive

to -30mV and entirely absent (h=1.0) for holding potentials negative to -70mV. Adrian, Chandler and Hodgkin (1970) reported similar values of h=1.0 at -80mV and h=0.1 at -20mV for the delayed potassium current on adult muscle fibres. The apparent similarities between peak and late current components suggests both components of the outward current may be mediated through the same channel type.

(3) On adult frog muscle fibres, reversal potentials of the delayed rectifier current were shown to be mainly dependent on potassium ions but did not follow potassium completely, having a reversal potential which was less negative than E_K. Similar results were found in the presence study. Furthermore in this preparation of embryonic muscle cells mean reversal potentials of both the peak and late components were approximately the same. These results suggest that both the peak and late current components may be mediated through the same channel type.

In summary, the kinetic properties of the outward current described in this thesis indicate this current is similar to the delayed rectifier potassium currents described on adult frog skeletal muscle fibers.

A variety of pharmacological agents have been described as blockers of the delayed rectifier current but not of the slow potassium current on adult frog skeletal muscle fibres. While many of these agents including TEA^+, 3,4 DAP, and 4AP also appear to be blockers of the delayed outward current on these embryonic Xenopus muscle cells they did not appear perfectly selective and were not particurally useful diagnostic tools, at least within this preparation. Tetraethylammonium (50mM) reduced both peak and late components of the outward current by approximately 50%. Stanfield (1970) observed similar effects of TEA^+ on the delayed potassium currents of adult frog skeletal muscle fibres. The inhibition of the delayed outward current by the external application of 3,4 DAP is consistent with the results of Cognard, Traore, Potreau and Raymond (1984) who showed that the delayed potassium current in adult muscle fibre is blocked by 5mM 3,4DAP. 4-Aminopyridine has also been described as a selective blocker of delayed potassium currents on adult muscle fibres. Gillispie (1977) reported a 50% reduction in maximal delayed conductance with the external application of 1mM 4AP. The results, although less clear, indicate a reduction in both components of outward current in the presence of 4AP. However this

agent rapidly killed these muscle cells.

There have been no reports of the effects of quinine on the delayed rectifier of skeletal muscle preparations. However in neuroblastoma cells, quinine has been shown to be a potent blocker of delayed rectification (Fishman and Spector,1981). In the present study quinine (1mM) blocked the delayed outward current on embryonic skeletal muscle cells. In addition to the effects of quinine on the delayed rectifier it has also been described as a blocker of sodium currents, calcium activated potassium currents on guinea pig myenteric and submucosa plexus neurons (Cherubini, North and Surprenant,1984) and bovine chromaffin cells (Glavinovic and Trifaso,1988) as well as inward rectifying potassium currents (single channel studies) on pancreatic islet cells (Findlay, Dunne, Ullrich, Wollheim and Petersen,1985). These results suggest that quinine is far from being a specific blocker of a particular channel type.

Apamin, a bee venom toxin has been shown to reduce the major portion of the slow calcium dependent potassium conductance on adult frog muscle fibres (Cognard, Traore, Potreau and Raymond,1984) and on cultured rat muscle cells (Blatz and Magleby,1986).

The external application of 50-80mM apamin did not affect the delayed outward current of this embryonic muscle cell preparation. This finding suggests that a slow calcium dependent potassium conductance similar to that described in adult muscle does not activate within the 100 ms depolarizing voltage steps employed in this study.

As discussed above (section III.2d), previous investigations have identified a depolarization-gated inward calcium current on this embryonic muscle cell preparation (Moody-Corbett, Gilbert, Akrabrali and Hall, 1989). This conductance appears to activate at membrane potentials positive to -30mV and was very small when present at one day in culture (see Table 1, Moody-Corbett, Gilbert, Akrabrali and Hall, 1989). The delayed rectifier current on this preparation also activates at -30mV. The effects of this simultaneously activating inward calcium current on the delayed outward current would be a reduced net whole cell outward conductance. External application of calcium current blocking agents known to reduce the inward calcium current on these muscle cells were therefore expected to produce an increase in net outward currents. Contrary to these expectations a reduction was observed. Whether these results reflect a calcium

dependence of the delayed outward current (resulting from a voltage gated influx of calcium ions) or a direct effect of dihydropyridines on the delayed current channels is not clear. An apamin insensitive calcium dependent potassium current has been identified on embryonic muscle (Romey and Lazdunski, 1984) which is sensitive to charybdotoxin. Such a current may be present on these embryonic muscle cells as well. The identification of a calcium sensitive potassium current would be best achieved using single channel recording techniques.

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IV APPENDIX

Table 4 Time constants for the rise to peak outward current (τ_R) measured across various depolarizing voltage steps. Rising phase of all current records fit with a single exponential.

TABLE 4

Time constants for the rise to peak outward current (τ_1) measured at various depolarizing voltage steps

Membrane Potential (mV)	Mean(SD) , Range (ms)	n=
-30	4.4(1.8) , 5.8-2.4	3
-20	3.1(1.6) , 5.8-1.0	13
-10	1.9(0.9) , 3.7-0.8	15
0	1.3(0.4) , 1.9-0.8	15
+10	1.2(0.5) , 2.0-0.4	15
+20	1.0(0.2) , 1.4-0.8	11
+30	0.7(0.2) , 1.0-0.5	4