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# Genetic and Physiological Evidence Concerning the Development of Chemically Sensitive Voltage-Dependent Ionophores in $L_6$ Cells

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SUMMARY

The electrophysiological properties of a tissue culture muscle line, $L_6$, and a $K^+$ resistant mutant ($MK_1$) derived from $L_6$ were determined to elucidate certain aspects of membrane differentiation and function. $MK_1$ was selected as a clone of myoblasts resistant to the toxic effects of 55 m*M* $K^+$. The resting potentials of $L_6$ and $MK_1$ myoblasts and myotubes were $K^+$ dependent and equal. The amplitudes of the action potentials were equal in normal medium, but 27.7 m*M* $K^+$ interfered with or eliminated the ability of $L_6$ myotubes to produce action potentials. $MK_1$ myotubes produced nearly normal action potentials under these conditions. Thus, the $K^+$ resistant myoblasts differentiate into myotubes which have an action potential generating mechanism much less sensitive to $K^+$ than the normal mechanism. Also, both *d*-tubocurarine and $\alpha$-bungarotoxin enhance the amplitude of the action potentials produced by $L_6$ myotubes in the presence of 27.7 m*M* $K^+$; these compounds do not enhance the amplitude of the action potentials produced by $MK_1$ myotubes under the same conditions. It is proposed that as a consequence of differentiation a type of ionophore present in myoblasts becomes a voltage-dependent ionophore in myotubes. Furthermore, these voltage-dependent ionophores can be chemically sensitive.

## INTRODUCTION

The rat skeletal muscle cell line described by Yaffe (1968) has been characterized in some detail. $L_6$ myoblasts typically are neither electrically active nor have nicotinic cholinergic receptors (Harris, Heinemann, Schubert, and Tarakis, 1971; Patrick, Heinemann, Lindstrom, Schubert, and Steinbach, 1972; Kidokoro, 1973, 1975; Land, Ravdin, Salpeter, and Podleski, 1974; Steinbach, 1975). Although electrical activity has been reported for $L_6$ myoblasts, the action potentials described were small ($\leq$20 mV) and their maximum rate of rise was approximately ten times smaller than that of the corresponding myotubes (Kidokoro, 1973, 1975). In our hands, less than 10% of the myoblasts are electrically

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active and the action potentials produced are small ($\leq$20 mV). The myoblasts fuse to form myotubes which can be electrically active, spontaneously twitching, sensitive to acetylcholine (AcCh), striated and capable of being innervated by rat spinal cord explants (Yaffe, 1968; Harris et al., 1971; Kidokoro and Heinemann, 1974). Myotubes typically are resistant to TTX, though totally sensitive myotubes develop among the TTX resistant myotubes (Kidokoro, 1973; Land, Sastre, and Podleski, 1973). Thus the cells of the $L_6$ line have many of the characteristics of embryonic rat skeletal muscle (Harris and Marshall, 1973). As part of an attempt to learn more about the excitability of membranes of $L_6$, we isolated and characterized a $K^+$ resistant mutant of $L_6$.

## METHODS

### *Growth conditions*

Myoblasts were grown at 37°C in Dulbecco's modified Eagles medium (Vogt and Dulbecco, 1963) supplemented with 10% fetal calf serum in an atmosphere containing 5% $CO_2$, 95% air, and 100% humidity on either 60 mm or 100 mm Falcon or Corning plastic tissue-culture dishes. Cells were removed from the dishes and dissociated for transfer or counting by incubation at 37°C in Eagles medium containing 0.25% (w/v) Viokase (Gibco). Cells were counted in a Bio/Physics Systems Inc. Cytograf 6301.

### *Mutant selection*

Approximately $2 \times 10^7$ cells were plated on each of four 100 mm Falcon tissue culture dishes. The cells were incubated in Dulbecco's modified Eagles medium containing a final concentration of 60 m*M* $K^+Cl^-$. The medium was removed and replaced with fresh medium of the same composition once every 2 days. The cells were exposed to these conditions for 30 days. Then the cells from one selective plate were removed using Viokase and aliquots distributed to ten 100 mm plastic tissue culture dishes. These cells were grown in Dulbecco's modified Eagles medium containing a 55 m*M* final concentration of $K^+Cl^-$. The medium was changed once every 2 days. Probable clones of healthy cells appeared on a background of deformed cells. Approximately 20 clones developed on each of the ten recipient plates. Assuming that between 20 and 100 cells existed in each clone on the original selective plate, mutants appeared with an approximate frequency of from $1/10^7$ cells to $5/10^7$ cells. A number of clones were isolated using a glass clone ring. The bottom surface of the clone ring was coated with sterile silicone grease. The clone ring was placed over a presumptive clone. Viokase in Dulbecco's modified Eagles medium was added to the inside of the ring. The cells were transferred to a 35 mm tissue culture dish containing growth medium. This process was repeated three times. One purified clone, $MK_1$, was characterized in detail.

### *Electrophysiological techniques*

Intracellular electrical measurements were made in buffered Tyrode's solution of the following composition in millimoles/liter: NaCl 138, KCl 2.7, $MgCl_2$ 1.8, $CaCl_2$ 1.8, $NaH_2PO_4$ 0.42, glucose 5.5, HEPES (*N*-2-hydroxy ethylpiperazine-*N'*-2-ethanesulfonic acid) buffer 18.0. The pH was adjusted to 7.3–7.4. All measurements were done at room temperature, 21–23°C. Solution was changed every 30–40 min to minimize the effects of evaporation.

Test solutions were introduced into the culture dish by perfusion in such a way as to hold the volume in the dish constant at 2–3 ml. Routinely, during solution changes, 15 ml were flushed through the dish. Electrical properties were measured when they reached steady values after $\simeq$1–3 min.

Microelectrodes filled with 3*M* KCl and having a dc resistance between 40 and 80 MΩ were used. Stimulating current was passed through the recording electrode. Cells were stimulated by hyperpolarizing current. Normally 25 nA for 40 msec was sufficient to stimulate action potentials at the anodal break. Each action potential was measured after stimulus was terminated, therefore bridge balance did not affect measurement of action potentials. Inexcitability was defined as the inability to excite an action potential with up to 50 nA for 300 msec. When input impedance of cells was measured, the stimulation method of Brennecke and Lindemann (1971) was used. This method consists of quickly alternating between injecting current and measuring voltage through one electrode. The cell membrane acts as an integrator for short pulses so that a steady voltage builds up. Since no current is flowing from the electrode while the voltage is measured, the effects of electrode resistance nonlinearities and spreading resistance are eliminated. To obtain accurate voltage readings, the rise time of the electrode must be much shorter than the rise time of the cell. Typical rise times for the myotubes were 6 msec and for electrodes approximately 50 to 100 μsec. The myotube input resistances measured ranged from 3 to 44 MΩ. Input resistances measured ranged from 3 to 6 MΩ using a two electrode stimulus-recording method. Specific membrane resistances calculated from one and two electrode input resistance determinations agreed well with each other, implying that the ranges of input resistance seen were due to myotube geometry, and that the one electrode method was producing reasonable results.

## RESULTS

The selection of mutants resistant to $K^+$ is based on the toxicity of $K^+$ to normal cells. Dulbecco's modified Eagle medium is 5.3 m*M* in $K^+$ (Vogt and Dulbecco, 1963). $L_6$ cells require $K^+$, and grow well in the presence of this concentration of $K^+$. The concentration of $K^+$ in the medium can be increased to more than 25 m*M* without it having a striking effect on the rate of growth. However, 25 m*M* $K^+$ alters the electrophysiological properties of both $L_6$ myoblasts and myotubes (Steinbach, 1974). $K^+$ at this concentration depolarizes the resting potentials of myoblasts and myotubes; it also either eliminates the ability to normal myotubes to produce action potentials or it drastically reduces the amplitude of those action potentials. In contrast to 25 m*M* $K^+$, 55 m*M* $K^+$ not only alters the cells electrophysiologically, it also adversely affects the growth of myoblasts. This high concentration of $K^+$ eventually kills cells. Thus, 55 m*M* $K^+$ provides selective pressure for the enrichment of mutants which are specifically resistant to the toxic effect(s) of $K^+$. $K^+$ resistant mutants were selected as described in Methods.

Clones of cells presumed to be resistant to $K^+$ (MK mutants) were identified in the 55 m*M* $K^+$ medium by their normal appearance against a background of deformed cells. The presumed mutants were purified to obtain a homogeneous population. One purified isolate designated $MK_1$ was characterized in detail.

Preliminary characterization of the $MK_1$ strain revealed that myoblasts of this strain are different from myoblasts of normal $L_6$. Myoblasts of the $MK_1$ strain can grow exponentially in Eagles medium containing 55 m*M* $K^+Cl^-$ (see Fig. 1). In contrast, myoblasts of normal $L_6$ do not grow exponentially under these conditions, and the cells are eventually killed by $K^+$. The inability of myoblasts of $L_6$ to grow exponentially in medium supplemented with a high concentration of $K^+Cl^-$ is the result of the action of $K^+$ ions. This is demonstrated by the ability of normal myoblasts to grow at a nearly normal rate in medium supplemented with $Na^+Cl^-$ to increase the concentration from 157 m*M* to 212 m*M* (an increase of 55 m*M*). Thus the $MK_1$ strain has the specific ability

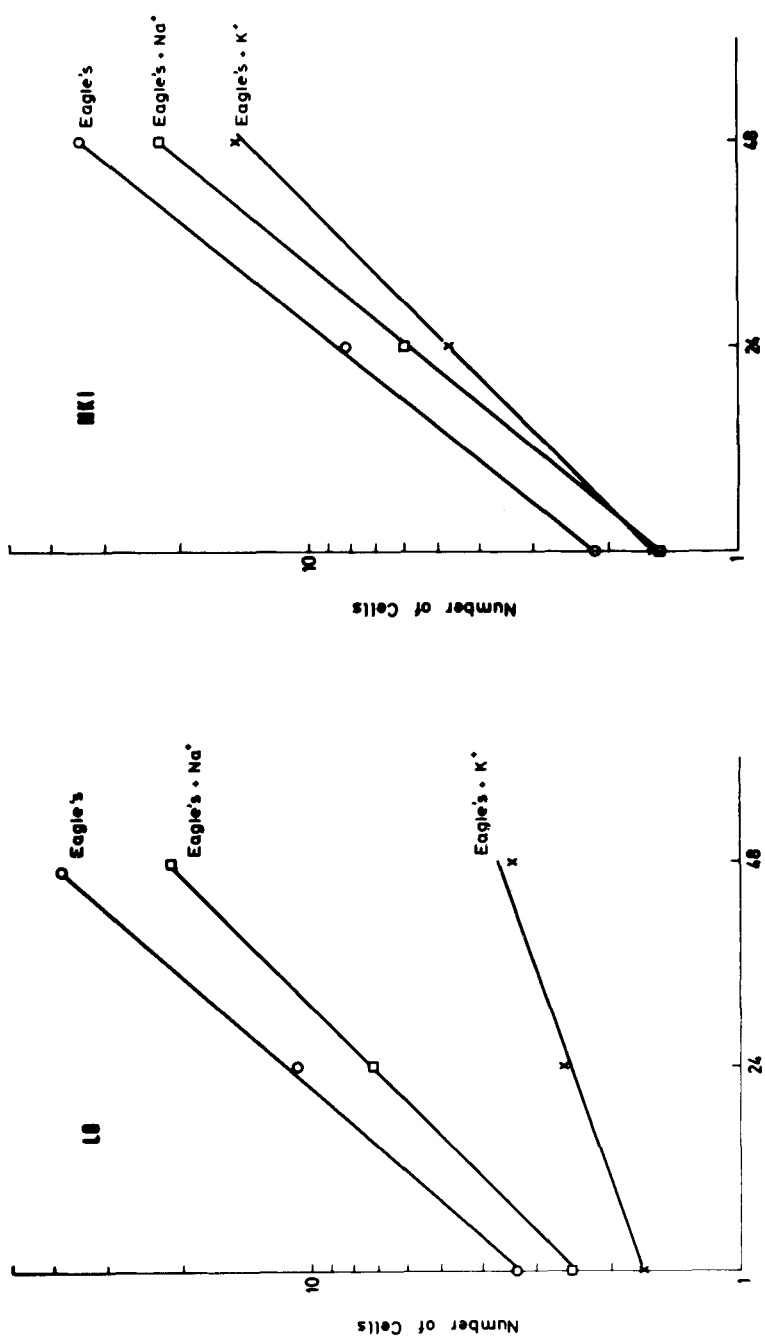


Fig. 1. $L_6$ and $MK_1$ myoblasts were grown in normal medium (157 m*M* $Na^+Cl^-$ and 5.3 m*M* $K^+Cl^-$), in medium which was 212 m*M* in $Na^+Cl^-$ (an increase of 55 m*M*) and had the normal concentration of $K^+Cl^-$ (5.3 m*M*) and in medium containing the normal concentration of $Na^+Cl^-$ (157 m*M*) and 30.3 m*M* $K^+Cl^-$ (an increase of 25 m*M* $K^+Cl^-$). These conditions demonstrate the resistance of the $MK_1$ mutant to the toxic effect(s) of $K^+$.

TABLE 1
Myotube Characterization[a]

| | $RP_{Na}$ | $RP_K$ | $AP_{Na}$ | $AP_K$ | $\Delta V$ $AP_{Na} - AP_K$ | |
|---|---|---|---|---|---|---|
| $L_6$ | 54 ± 4 | 34 ± 3 | 71 ± 3 | 18 ± 5 | 53 ± 5 | $*t = 11.2$[b] $p < .001$ |
| $MK_1$ | 53 ± 4 | 36 ± 2 | 82 ± 4 | 57 ± 4 | 25 ± 3 | $*t = 4.3$[b] $p < .005$ |
| | $t = .195$ $p < .5$ $df = 14$ | $t = .52$ $p < .5$ $df = 14$ | $t = 2.05$ $p < .1$ $df = 14$ | $t = 6.01$ $p < .001$ $df = 14$ | $t = 3.67$ $p < .005$ $df = 14$ | |

[a] The resting potentials and action potentials of $L_6$ and $MK_1$ myotubes were determined in normal Tyrode's solution supplemented with $2.5 \times 10^{-2}$ m*M*/ml of either $Na^+Cl^-$ or $K^+Cl^-$. First, the resting potential of a myotube was determined in $Na^+$ supplemented Tyrode's solution. Then the magnitude of the action potential was determined in the same solution. Next, the solution was changed to $K^+$ supplemented Tyrode's solution for the determination of the resting potential and action potential. Finally, the solution was replaced by $Na^+Cl^-$ supplemented Tyrode's solution. Data were not used unless a myotube went through all the media changes successfully. This procedure was followed for each myotube. All table entries are in mV ± the SEM. $RP_x$ means resting potential in a given solution where $x$ is $Na^+$, $K^+$, or $K^+$ + *d*TC.

[b] Statistical treatment of $\Delta V$ between $AP_{Na}$ and $AP_K$ within each cell type. The *t* values are the results of an unpaired student *t*-test evaluation with the exception of the *t* values marked with an asterick which were paired. The *p* values given are the two tailed probabilities using the calculated *t* values with *df* (degrees of freedom).

to grow and survive in the presence of a concentration of $K^+$ which is lethal to normal $L_6$ cells.

Mutation probably is the basis of the resistance to $K^+$ which is characteristic of the $MK_1$ strain. This conclusion is based on the facts that the change to resistance to $K^+$ is a permanent heritable trait which occurs with low frequency. The heritability and stability of resistance to $K^+$ was demonstrated by growing cells of the $MK_1$ strain for more than 15 generations in normal medium and then testing progeny of these cells for their ability to grow in the presence of 55 m*M* $K^+Cl^-$. The progeny of the cells grown in normal medium for more than 15 generations were still resistant to $K^+$. Resistance to $K^+$ occurs with low frequency, estimated to be about $1/10^7$ to $5/10^7$ cells. Base pair changes (Albrecht, Biedler, and Hutchison, 1972; Chan, Whitmore, Siminovitch, 1972; Beaudet, Roufa, and Caskey, 1973; Sharp, Capecchi, and Capecchi, 1973) and the gain and/or loss of chromosomes of a cell are alternative methods which are known to cause mutant phenotypes in mammalian cells (Terzi, 1974; Chasin and Urlanb, 1975).

The selective pressure which resulted in the selection of the $MK_1$ mutant was exerted on a population of myoblasts. However, since myotubes are the products of fusion of myoblasts, we characterized both myoblasts and myotubes of the $MK_1$ mutant.

Electrophysiological characterization of myotubes of the $MK_1$ mutant revealed both similarities and differences in comparison with myotubes of $L_6$ (see Table 1). Under normal conditions (see Methods), the resting potentials of both types of myotubes were nearly identical. Similarly, a concentration of 27.7 m*M* $K^+$

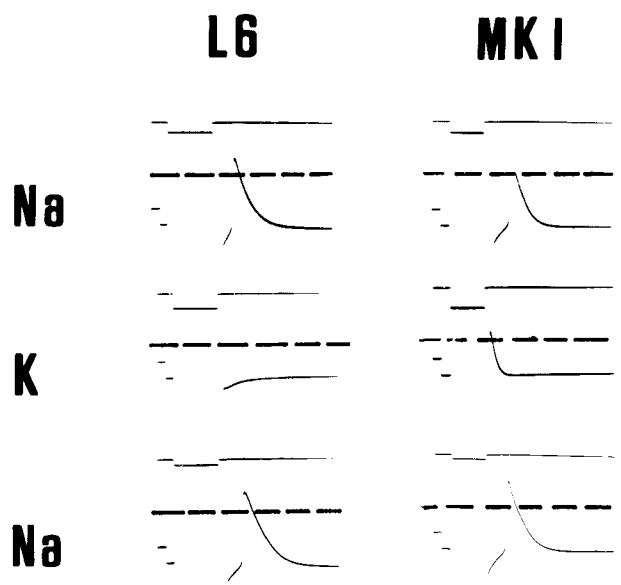


Fig. 2. Effects of 27.7 m$M$ $K^+$ on action potentials produced by $L_6$ and $MK_1$ myotubes. The top row shows action potentials produced in Tyrode's containing a 25 m$M$ higher concentration of $Na^+$ than normal Tyrode's. The second shows voltage-dependent responses in Tyrode's containing a 25 m$M$ higher concentration of $K^+$ than normal Tyrode's. The action potential produced by the $L_6$ myotube is much more attenuated by $K^+$ than the action potential produced by the $MK_1$ myotube. The third row shows recovery of the action potentials in the presence of $Na^+$ supplemented Tyrode's. The dotted lines are the voltage trace zero levels. The square pulse at the beginning of each voltage trace is 20 mV high and 10 msec long. The top trace on each frame is the intracellular stimulating current record. A downward deflection on the current trace denotes a current causing hyperpolarization. The current calibration is 25 nA per height of 20 mV voltage calibration pulse.

caused approximately the same extent of depolarization in both types of myotubes. A concentration of 27.7 m$M$ $K^+$ caused either the reversible loss of the ability of normal $L_6$ myotubes to produce action potentials or a drastic decrease of the magnitude of the action potentials. In contrast, $MK_1$ myotubes produced normal or nearly normal action potentials under these conditions. Figure 2 demonstrates an aspect of the fundamental difference between excitable membranes of $L_6$ myotubes and $MK_1$ myotubes. Notice that the $L_6$ myotube could not produce an action potential in the presence of 27 m$M$ $K^+$ whereas the $MK_1$ myotube could. Thus the data presented in Table 1 and Figure 2 reveal that the action potential generating mechanism of $MK_1$ myotubes is much less sensitive to $K^+$ than the normal mechanism. This difference in sensitivity to $K^+$ exists between these two types of myotubes which have otherwise quite similar electrophysiological characteristics.

A decreased membrane impedance which would have made the production of action potentials more difficult is not the basis of the effect of 27.7 m$M$ $K^+$ (an increase of 25 m$M$ over the normal concentration of 2.7 m$M$ of Tyrode's solution) on the action potential generating ability of $L_6$ myotubes. This is clear because a comparison of the impedances of $L_6$ myotubes in 163 m$M$ $Na^+$ ($ZNa^+$)

TABLE 2
Myotube Action Potentials ± $d$TC[a]

| | $AP_{Na}$ | $AP_K$ | $\Delta V$ $AP_{Na} - AP_K$ | $AP_K + d$TC | $\Delta V$ $AP_K + d$TC $- AP_K$ | |
|---|---|---|---|---|---|---|
| $L_6$ | 70 ± 6 | 18 ± 10 | 52 ± 8 | 41 ± 8 | 23 ± 3 | $*t = 6.99$[b] $p < .005$ |
| $MK_1$ | 80 ± 3 | 52 ± 6 | 28 ± 9 | 56 ± 1 | 5 ± 7 | $*t = .67$[b] $p > .75$ |
| | $t = 1.59$ $p < .2$ $df = 6$ | $t = 2.74$ $p < .05$ $df = 6$ | $t = 1.9$ $p < .1$ $df = 6$ | $t = 1.8$ $p < .2$ $df = 6$ | $t = 2.5$ $p < .05$ $df = 6$ | |

[a] Electrophysiological determinations made as described under Table 1 except that where indicated action potentials were also determined in the presence of $10^{-4}$ $M$ $d$TC.
[b] Statistical analysis of $\Delta V$ between $AP_K + d$TC $- AP_K$ within each cell type.

(an increase of 25 m$M$ $Na^+$ over the 138 m$M$ $Na^+$ of normal Tyrode's solution) with the impedance of the same myotubes in 27.7 m$M$ $K^+$ ($ZK^+$) reveals no significant difference ($ZNa^+/ZK^+ = 1.2 \pm 0.2$ (SEM), measurements were made on five myotubes). Thus 27.7 m$M$ $K^+$ affects the action potential generating mechanism of $L_6$ myotubes by a means other than decreasing membrane impedance.

The ability of $K^+$ to prevent the formation of action potentials by normal $L_6$ is not simply the result of depolarization. This conclusion is supported by the fact that the electrical reversal of $K^+$-caused depolarization did not eliminate the ability of $K^+$ to block action potentials in normal $L_6$. Furthermore, electrically caused depolarization even more extensive than that caused by 27.7 m$M$ $K^+$, to −18 mV, did not prevent action potential formation in response to hyperpolarization. Thus $K^+$ caused depolarization is not sufficient to block the formation of action potentials; therefore $K^+$ must have another mode of action. It may be that $K^+$ binds to the voltage-dependent ionophore (Henderson, Ritchie, and Strichartz, 1974) and thereby blocks the influx of $Na^+$ required for the generation of action potentials (Kidokoro, 1973; Land et al., 1973). Additional evidence supporting the fact that the ability of $K^+$ to prevent the formation of action potentials is not simply the result of depolarization is presented later.

In the course of characterizing $MK_1$ and $L_6$ myotubes, we discovered that $d$-tubocurarine ($d$TC) affects the response of normal myotubes to 27.7 m$M$ $K^+$. Both types of myotubes were exposed to $10^{-4}$ $M$ $d$TC in the absence and presence of 27.7 m$M$ $K^+$. In the absence of 27.7 m$M$ $K^+$, $d$TC had no effect on the action potentials produced by either type of myotube. In contrast, $d$TC either restored the ability of $L_6$ myotubes to produce action potentials in the presence of 27.7 m$M$ $K^+$ or it greatly enhanced the magnitude of the action potentials produced in the presence of 27.7 m$M$ $K^+$. This ability of $d$TC to facilitate the production of action potentials occurred even though the myotubes were depolarized to the usual extent by 27.7 m$M$ $K^+$ in the presence of $d$TC. $d$TC had very little effect on the action potentials produced by $MK_1$ myotubes in the presence of 27.7 m$M$ $K^+$ (see Table 2). This result was not surprising because 27.7 m$M$ $K^+$ had very

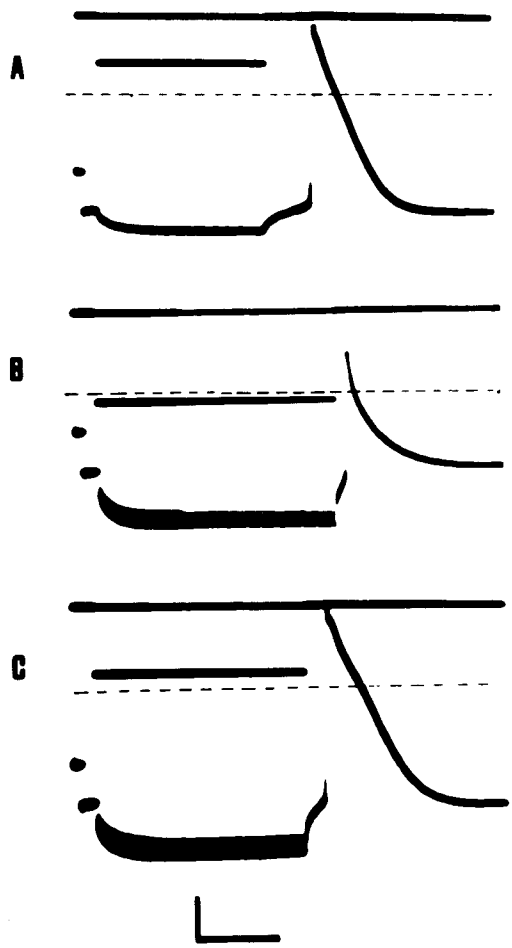


Fig. 3. $L_6$ myotubes were treated with $\alpha$-BuTX as described in the text. (A) $\alpha$-BuTX treated myotubes in Tyrode's solution which is 163 m$M$ in $Na^+Cl^-$ (an increase of 25 m$M$ over the normal concentration) and 2.7 m$M$ in $K^+Cl^-$ (the normal concentration). (B) The same myotube in Tyrode's solution which is 27.7 m$M$ in $K^+Cl^-$ (25 m$M$ above the normal concentration) and 138 m$M$ in $Na^+Cl^-$ (the normal concentration). (C) Recovery of the same myotube in Tyrode's solution which is 163 m$M$ in $Na^+Cl^-$ and 2.7 m$M$ in $K^-Cl^+$. The zero voltage level is marked by a dotted line. The voltage trace starts with a calibration pulse, then a short segment of the resting potential before the current induced hyperpolarization. During stimulation the membrane voltage is read from the bottom of the wide dark bands. The scale at the bottom is 20 mV high (or 2.5 nA for the current trace) and 100 msec long.

little effect on the action potentials produced by $MK_1$ myotubes. The resting potentials of $MK_1$ myotubes were consistently depolarized about 5 mV by $d$TC in the presence of 27.7 m$M$ $K^+$. The ability of $d$TC to enable $L_6$ myotubes to produce action potentials in the presence of 27.7 m$M$ $K^+$ may depend on the specific binding of $d$TC to AcCh receptors.

Because myoblasts have no external functional nicotinic cholinergic receptors

(Patrick et al., 1972; Land et al., 1974; Steinbach, 1975), myoblasts should not be affected by *d*TC if this antagonist exerts its effects through nicotinic receptors. Under normal conditions, the resting potentials of normal myoblasts and myoblasts of the $MK_1$ mutant are not significantly different. As is the case with the myotubes, $K^+$ causes approximately the same extent of depolarization in both types of myoblasts. As expected, *d*TC had no effect on resting potentials of myoblasts either in the presence or absence of a high concentration of $K^+$. Also, *d*TC did not enhance the ability of normal myoblasts to grow in medium containing 55 m*M* $K^+$. These results demonstrate that both types of myoblasts are indistinguishable electrophysiologically by these criteria and that *d*TC does not protect normal myoblasts from the physiologically deleterious effects of 55 m*M* $K^+$.

The possibility that antagonists bound to AcCh receptors confer the ability to produce action potentials in the presence of 27.7 m*M* $K^+$ on $L_6$ myotubes was investigated further by using the antagonist $\alpha$-bungarotoxin ($\alpha$BuTX). $L_6$ myotubes were exposed to $2 \times 10^{-7}$ *M* $\alpha$-BuTX for 2 hr at 37°C. This is both a sufficient concentration and period of time for the $\alpha$-BuTX to bind to nearly all of the AcCh receptors (Land et al., 1974). The myotubes with inactivated AcCh receptors were tested for their ability to produce action potentials in the absence and presence of 27.7 m*M* $K^+$. In the presence of the normal concentration of $K^+$ the myotubes had normal resting potentials and produced normal action potentials. Thus, the $\alpha$-BuTX did not affect the electrophysiological characteristics of the myotubes. In contrast, even though these myotubes were depolarized to the usual extent by 27.7 m*M* $K^+$, most but not all of these myotubes produced nearly normal action potentials (Fig. 3). Thus not just *d*TC, but another antagonist, $\alpha$-BuTX, can also protect the action potential generating mechanism of normal myotubes from $K^+$. The fact that either *d*TC or $\alpha$-BuTX can confer the ability to produce nearly normal action potentials in the presence of 27.7 m*M* $K^+$ on $L_6$ myotubes probably means that specific binding of antagonist to AcCh receptors is required to cause this effect.

The ability of the antagonists to enable $L_6$ myotubes to produce action potentials in the presence of 27.7 m*M* $K^+$ is not the result of an increased membrane impedance. This was revealed by a comparison of impedances of $L_6$ myotubes in 27.7 m*M* $K^+$ ($ZK^+$) with the impedances of the same myotubes in 27.7 m*M* $K^+$ plus $10^{-4}$ *M* *d*TC ($ZK^+$ plus *d*TC). This comparison reveals no significant difference under the two conditions ($ZK^+ + d\text{TC}/ZK^+ = 1.10 \pm .1$ (SEM), the measurements were made on three myotubes). Thus, the antagonists are enabling the $L_6$ myotubes to produce action potentials in the presence of 27.7 m*M* $K^+$ by a means other than simply increasing membrane impedance.

## DISCUSSION

Four different observations demonstrate that $K^+$ does not prevent the formation of action potentials by $L_6$ myotubes simply by causing depolarization. (*1*) The ability of $K^+$ to prevent action potentials is not reversed by hyperpolarization. (2) Electrically caused depolarization of the resting potentials of

myotubes did not prevent the production of action potentials. (*3*) $L_6$ myotubes depolarized by 27.7 m*M* $K^+$ in the presence of $10^{-4}$ *M* *d*TC also produced action potentials (Table 2). (*4*) Finally, $L_6$ myotubes whose receptors were inactivated by $\alpha$-BuTx produced action potentials in the presence of 27.7 m*M* $K^+$ (Fig. 2). Therefore, the ability of $K^+$ to block action potentials is due to some action other than depolarization.

A simple hypothesis for the effect of $K^+$ is that $K^+$ binds to the voltage-dependent ionophores (Henderson et al., 1974) and thereby blocks the influx of $Na^+$ which occurs during an action potential. The alternative explanation, consistent with the effects of the antagonists, that $K^+$ prevents action potentials by binding to AcCh receptors is unlikely because the binding of antagonists to receptors under normal conditions did not alter the electrophysiological properties of the cells. Thus, it is difficult to understand how $K^+$ binding to AcCh receptors could prevent the production of action potentials. Also, the properties of the mutant $MK_1$ make it seem unlikely that $K^+$ is preventing action potentials by binding to AcCh receptors. This point is discussed subsequently.

The assumption that $K^+$ prevents the formation of action potentials by binding to the voltage-dependent ionophores, is supported by the characteristics of the $K^+$ resistant mutant. The resting potentials of $L_6$ and $MK_1$ myoblasts were quite similar in 2.7 m*M* $K^+$ and both types of myoblasts were depolarized to the same extent by 27.7 m*M* $K^+$. Similarly, both types of myotubes had similar resting potentials in 2.7 m*M* $K^+$ and were depolarized to the same extent by 27.7 m*M* $K^+$ and 55 m*M* $K^+$. Consequently, the internal concentration of $K^+$ and the permeability to $K^+$ are about the same in both cell lines. Thus, the ionophore that is presumed to be modified in $MK_1$ cells probably is not a $K^+$ ionophore.

Furthermore, since the selective pressure which resulted in the enrichment of the $MK_1$ mutant acted on a population of myoblasts, not myotubes, any explanation of the physiological basis for the resistance to $K^+$ must be based on a change which expressed itself in a myoblast. Thus if the resistance to $K^+$ is the result of a modification of a type of ionophore, this modification must have affected a type of ionophore which is present in myoblasts. This constraint on the analysis of the effect of the mutation which conferred resistance to $K^+$ on the $MK_1$ cells provides information concerning the composition of the membranes of both undifferentiated and differentiated cells. Assuming that a particular type of ionophore was altered by the mutation which conferred $K^+$ resistance on the cells, it follows from the characteristics of the mutant, and the arguments stated above, that the altered ionophores must be present before and after differentiation. Furthermore, since there is no evidence that a significant number of nicotinic cholinergic receptors exist on the external membrane of $L_6$ myoblasts (Patrick et al., 1972; Land et al., 1974; Steinbach, 1975), it follows that the hypothetical altered ionophores which eventually can be protected by AcCh receptors are present and functional before active AcCh receptors are in the membrane. Thus, it seems reasonable to suggest that $K^+$ affects the cells by binding to ionophores rather than by binding to AcCh receptors.

Consideration of the overall characteristics of the $MK_1$ mutant and the response of $L_6$ myotubes to *d*TC and $\alpha$-BuTX leads to the development of an in-

teresting model. $MK_1$ myotubes produce voltage-dependent action potentials under conditions which either eliminate the ability of normal $L_6$ to produce action potentials or drastically reduce the amplitude of the action potentials produced. Thus assuming that a single mutation is responsible for the characteristics of the $MK_1$ mutant, the same mutation which conferred resistance to $K^+$ on the $MK_1$ cells produced a voltage-dependent action potential generating system much less sensitive to the effects of $K^+$ than the normal system. The simplest explanation for these characteristics of the $MK_1$ mutant is that they are caused by modification of a single type of ionophore. Furthermore, in view of the response of $L_6$ myotubes to *d*TC and $\alpha$-BuTX, it seems likely that this type of ionophore in myotubes can be both responsive to AcCh, via the receptors, and under the appropriate conditions participate directly in the production of voltage-dependent action potentials. According to this model, $L_6$ myoblasts possess a type of ionophore which as a consequence of differentiation becomes capable of producing a voltage-dependent response in myotubes. In noninnervated $L_6$ myotubes, these voltage-dependent ionophores can also be chemically sensitive if they are associated with AcCh receptors. Thus, these chemically sensitive ionophores would be capable of producing voltage-dependent responses. Consistent with this model, Nachmansohn (1974) has proposed that AcCh receptors are involved in action potential generation in nerve, and Thesleff (1973) has proposed an association between AcCh receptors and voltage-dependent ionophores in denervated muscle.

Our explanation for the characteristics of $MK_1$ is made less plausible by the fact that we cannot demonstrate that a single mutation is the cause of these characteristics. Furthermore, it can be argued that a single mutation occurred which affects the expression of more than one protein or that an altered polypeptide produced by $MK_1$ is a common component of two different types of ionophores. These arguments, however, are not critical because the behavior of normal $L_6$ myotubes supports our model.

The effects of *d*TC and $\alpha$-BuTX on the myotubes of normal $L_6$ are pertinent to the hypothesis that a single type of ionophore can both respond to AcCh and produce a voltage-dependent response. The summary of data presented in Table 2, and the data of Figure 3 reveal that *d*TC and $\alpha$-BuTX, presumably acting through AcCh receptors, greatly enhance the ability of $L_6$ myotubes to produce action potentials in the presence of 27.7 m*M* $K^+$. Both antagonists in the presence of 27.7 m*M* $K^+$ either enhanced the magnitude of the action potentials produced by $L_6$ myotubes or they restored the ability of myotubes to produce action potentials. These effects of the antagonists are particularly interesting because they occur in normal myotubes; they reflect normal cell function. They demonstrate effects of antagonists on the voltage-dependent action potential generating mechanism. Thus, the behavior of normal cells provides evidence that the same ionophore can both produce a voltage-dependent response and be chemically sensitive. This evidence coupled with the genetic argument concerning the overall characteristics of $MK_1$ provides strong support for the hypothesis that the same ionophore originally present in a myoblast can be responsive in myotubes to both a voltage change and AcCh.

The intriguing aspect of this work is that the deleterious effects of $K^+$ on $L_6$ myotubes which are described here may have resulted from an extension of a normal function of $K^+$. That is, $K^+$ may regulate the influx of $Na^+$ in certain types of electrically excitable cells.

## ADDENDUM

Recently Cohen, M., Palti, Y., and Adelman, W. J. Jr., [*J. Membrane Biol.* **24**: 201 (1975)], have shown that external potassium directly inhibits inward sodium current flow in voltage-clamped squid axons. Also, Adelman and Palti (Adelman, W. J. Jr., and Palti, Y. (1972). In: *Perspectives in Membrane Biophysics*, D. P. Agin, Ed., Gordon and Breach, New York) have previously shown that the shape of the sodium inactivation-membrane potential curve depends on the external potassium concentration and that in order to remove the inactivation, hyperpolarization to −120 mV was required. While we have not measured the membrane potential during the stimulus pulse, we estimate on the basis of circuit used for stimulation, that hyperpolarization to at least 120 mV was achieved in the experiments done on $L_6$ myotubes. Thus the results of Cohen, Palti, and Adelman referred to here are consistent with our explanation of the effects of external potassium on voltage-dependent $Na^+$ influx in $L_6$ myotubes.

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## REFERENCES

ALBRECHT, A. M., BIEDLER, J. L. and HUTCHISON, D. J. (1972). Two different species of dihydrofolate reductose in mammalian cells differentially resistant to amethopterin and methasquin. *Cancer Res.* **32:** 1539.

BEAUDET, A. L., ROUFA, D. J. and CASKEY, C. T. (1973). Mutations affecting the structure of hypoxanthine: guanine phosphoribosyltransferase in cultured chinese hamster cells. *Proc. Nat. Acad. Sci. USA* **70:** 320.

BRENNECKE, R and LINDEMANN, B. (1971). A chopped-current clamp for current injection and recording of membrane polarization with single electrodes of changing resistance. *T.I.T. J. Life Sciences* **1:** 53.

CHAN, V. L., WHITMORE, G. E. and SIMINOVITCH, L. (1972). Mammalian cells with altered forms of RNA polymerase II. *Proc. Nat. Acad. Sci. USA* **69:** 3119.

CHASIN, L. A. and URLAUB, G. (1975). Chromosome-wide event accompanies the expression of recessive mutations in tetraploid cells. *Science* **187:** 1091.

HARRIS, A. J., HEINEMANN, S., SCHUBERT, D. and TARAKIS, H. (1971). Trophic interaction between cloned tissue culture lines of nerve and muscle. *Nature* **231:** 296.

HARRIS, J. B. and MARSHALL, M. W. (1973). Maturing muscle fiber becomes progressively more sensitive to effect of tetrodotoxin. *Nature New Biol.* **243:** 191.

HENDERSON, R., RITCHIE, J. M. and STRICHARTZ, G. R. (1974). Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channels of nerve membrane. *Proc. Nat. Acad. Sci. USA* **71:** 3936.

KIDOKORO, Y. (1973). Development of action potentials in a clonal rat skeletal muscle cell line. *Nature* **241:** 158.

KIDOKORO, Y. (1975). Developmental changes of membrane electrical properties in a rat skeletal muscle cell line. *J. Physiol.* **244:** 129.

KIDOKORO, Y. and HEINEMANN, S. (1974). Synapse formation between clonal muscle cells and rat spinal cord explants. *Nature* **252:** 593.

LAND, B. R., SASTRE, A. and PODLESKI, T. R. (1973). Tetrodotoxin-sensitive and -insensitive action potentials in myotubes. *J. Cell Physiol.* **82:** 497.

LAND, B. R., RAVDIN, P., SALPETER, M. M. and PODLESKI, T. R. (1974). Correlation between acetylcholine sensitivity and density of receptor sites. *J. Cell Biol.* **63:** 183a.

NACHMANSOHN, D. (1974). Biochemical foundation of an integral model of nerve excitability. In: *Mosbacher Colloquium der Gesellschaft für Biologische Chemie*, L. Jaenicke, Ed., Springer-Verlag, Berlin.

PATRICK, J., HEINEMANN, S. F., LINDSTROM, J., SCHUBERT, D. and STEINBACH, J. H. (1972). Appearance of acetylcholine receptors during differentiation of a myogenic cell line. *Proc. Nat. Acad. Sci. USA* **69:** 2762.

SHARP, J. D., CAPECCHI, N. E., and CAPECCHI, M. R. (1973). Altered enzymes in drug-resistant variants of mammalian tissue culture cells. *Proc. Nat. Acad. Sci. USA* **70:** 3145.

STEINBACH, J. H. (1974). Role of muscle activity in nerve-muscle interaction *in vitro*. *Nature* **248:** 70.

STEINBACH, J. H. (1975). Acetylcholine responses in clonal myogenic cells *in vitro*. *J. Physiol.* **247:** 393.

TERZI, M. (1974). Chromosomal variation and the origin of drug resistant mutants in mammalian cell lines. *Proc. Nat. Acad. Sci. USA* **71:** 5027.

THESLEFF, S. (1973). Functional properties of receptors in striated muscle. In: *Drug Receptors*, H. P. Rang, Ed., Macmillan, London.

VOGT, M. and DULBECCO, R. (1963). Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. Nat. Acad. Sci. USA* **49:** 171.

YAFFE, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Nat. Acad. Sci. USA* **6P:** 477.

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