Tetrodotoxin-sensitive and -insensitive Action Potentials in Myotubes [1]

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ABSTRACT The study of long-term cultures of myogenic cells has proven that electrical excitability develops only after the development of electrical coupling between the cells. That is, neither surface contact in itself nor coupling in itself is sufficient to cause excitability to develop in these cells. Following the formation of multinucleated myotubes, several different types of electrical responses develop. Some of the action potentials are sodium-dependent and are blocked by tetrodotoxin (TTX). Others are dependent upon sodium and possibly calcium and they are not blocked by TTX. Furthermore, these two types of responses may exist in a myotube at the same time. Under some circumstances the kinetics of the two systems are sufficiently different to result in action potentials that have two peaks. Under these conditions the first peak is always of shorter duration and it is always blocked by TTX.

Considerable evidence has accumulated supporting the suggestion that tetrodotoxin (TTX) blocks specifically the voltage-dependent sodium conductance that is responsible for the excitability of many nerve and muscle fibers (Narashi et al., '64; Nakamura et al., '65; Hille, '68). Recently, however, it has been reported that adult skeletal muscle, following denervation, develops an action potential that is not blocked by TTX but is blocked by replacing sodium with sucrose (Redfern and Thesleff, '71). This observation contradicts the generalization that TTX blocks all sodium-dependent action potentials, and suggests that the sensitivity of the muscle membrane to TTX is controlled by the nerve fiber. Sensitivity of the muscle membrane to TTX could be then considered as one of the membrane properties that is regulated by innervation (Harris et al., '71).

Because of our interests in the molecular events of synaptogenesis, we have studied the development of TTX sensitivity in myotubes in tissue culture. In the course of our studies on the development of myotubes from myoblasts we observed several different types of electrical responses that vary in their wave form, duration and sensitivity to TTX. Action potentials in some myotubes were greatly attenuated by TTX, while others were either slightly affected or unaffected by concentrations of up to 10^{-5} M. Furthermore, we were able to identify myotubes in which both TTX-sensitive and TTX-insensitive action potentials occur simultaneously.

For the majority of our studies we have used cells derived from the L_6 line of myoblasts (Yaffe, '68). These cells have been in continuous tissue culture for about five years. In addition to these cells, we have made observations on primary mouse cultures. The myoblasts for the primary cultures were isolated from fetal or newborn mice within two weeks of the time electrical recordings were made. We have not found major differences between these two groups of cells, but all the illustrations presented in this report are taken from the myotubes derived from the L_6 line. It is important to realize, however, that the length of time the L_6 cells have been in culture does not alter in any obvious way the electrical behavior of the cells, at least when compared to mouse primary cultures.

This cellular system seems ideal for testing the possibility that TTX sensitivity in muscle depends upon the presence of innervation. It is unlikely that these cells were ever innervated since the myoblasts in both the primary cultures and L_6 were

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isolated as single cells. Furthermore, in the case of the L_6 cells, they are grown in the absence of any other cell type, i.e., all cells are myoblasts, there are no fibroblasts or nerve cells. In addition, the L_6 cells can be cloned and grown in large quantities for biochemical and physiological studies. This line of cells is, therefore, extremely useful for studying the molecular basis of the interactions that occur between nerve and muscle during the process of synaptogenesis (Harris et al., '71), as well as the molecular control of myogenesis.

There have been several studies made on embryonic muscle cells in tissue culture, and a variety of responses to TTX have been observed. It has been reported that heart myoblasts in tissue culture may be insensitive to TTX (Sperelakis and Shigenobu, '72), but chick skeletal muscle generates two types of action potentials: one is sensitive to TTX, whereas the other is not (Kano and Shimada, '72; Kano et al., '72). In the latter case it was shown that the action potential that was blocked by TTX was dependent upon sodium whereas the one that was not blocked was probably due to both sodium and calcium. Since the commonly encountered sodium dependent, TTX-sensitive responses in adult systems also have a small calcium component (Baker et al., '71) it would be interesting to study these two systems during the process of embryonic differentiation, as innervation occurs, as the system matures to its adult state.

MATERIALS AND METHODS

Myoblasts were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, at 37° C, in 5% CO_2, 95% air and 100% humidity. Cells were plated at a density of about 10^4 cells per dish. Falcon plastic petri dishes 60 mm in diameter were used. In about 7 to 12 days the myoblasts grew into dense colonies of confluent cells and fused to form myotubes.

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, NaH_2PO_4 0.42, glucose 5.5, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid) buffer 18, pH 7.4. All measurements were done at room temperature, 20–22° C. Sometimes the Tyrode's solution was covered with oil, but more often, particularly when the solutions were to be changed, the oil was not used. When oil was not used, the solution was changed every 20–30 minutes in order to minimize the effects of evaporation. TTX was applied either through a hydraulically driven micropipet filled with 3×10^{-5} M TTX, or added directly to the Tyrode's solution at the concentrations indicated.

Microelectrodes filled with 3M KCL and having a resistance of 50–100 MΩ were used. Stimulating current was applied through the recording microelectrode by the use of a bridge circuit. When membrane voltage was to be measured through the stimulating electrode during current injection, small currents, i.e., 2 nA were used, and the bridge was balanced inside the cell by the method suggested by Engel et al. ('72). This method eliminates the IR drop across the microelectrode by subtracting the voltage with the rise time characteristic of the microelectrode from the total voltage. The remaining voltage difference is due to the resistance of the cell.

We only recorded from cells that had steady resting potentials for the first two to three minutes following penetration. Occasionally a slight hyperpolarization (2–5 mV) was observed immediately after impalement. In the best cases we were able to hold the cell for 30–90 minutes.

Fig. 1 "A" and "B" are recordings made from a mutant cell derived from the normal L_6 line. The recordings show the small responses caused by two different stimulus intensities. The upper line is zero potential, and it also shows the stimulus current. The distance between the two lines is the resting membrane potential of the cell. The calibration is 20 mV in amplitude, 20 msec in duration and a 20 mV deflection on the current trace represents 19.5 nA. "C" shows recordings made simultaneously from the two confluent L_6 cells shown in "D". One electrode in each cell is used. The distance between the lines "a" and "b" represents the resting membrane potential in one cell. The resting potential in the other cell is identical. Current is passed through the electrode in one cell and the change in membrane potential is recorded in both cells, i.e., "b" and "c". The calibration for these recordings is shown on the left side of the voltage traces; it is 20 mV in amplitude and 10 msec in duration. The current calibration is 2.5 nA for 20 mV deflection on the current trace. The calibration bar in "D" is 10 μ long.

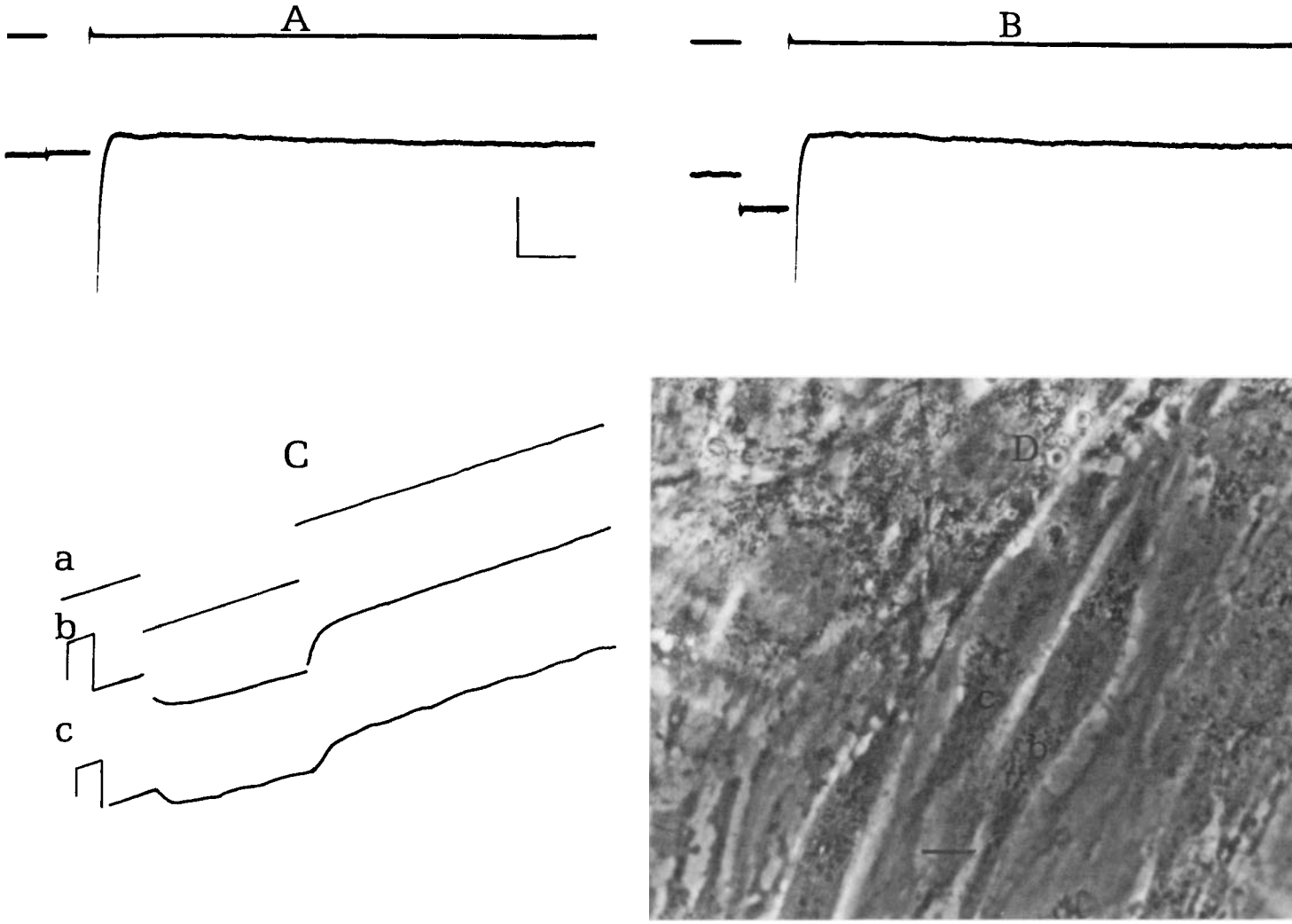

Figure 1

RESULTS

We have found that mononucleated cells are either not excitable or give small electrical responses on the order of 10 to 20 mV. We have studied three classes of mononucleated cells: (1) single myoblasts, (2) confluent myoblasts, and (3) a mutant cell line that does not fuse to form myotubes (Linden, Howell, Podleski, unpublished observations). An example of the electrical response of the mutant cell to two stimulus intensities is shown in figure 1A and 1B. Our observations on single myoblasts are similar to those made previously by Kidokoro ('73). We have found, however, that all three classes of cells respond in the same manner to electrical stimulation, and the cells were also found to be unresponsive to chemical stimulation by acetylcholine. We conclude, therefore, that excitability is a property the cells develop only after fusion begins to take place.

In order to determine when the cells develop excitability we studied confluent cells that were electrically coupled to one another. Inward directed current was passed through one cell and the resulting changes in membrane potentials were recorded in both cells. In the example shown, fig. 1b and c, the cells have a low resistance pathway between them, since the change in membrane potential caused by the current flow is almost identical in both cells. The degree of coupling that we observed was variable, and we never recorded regenerative responses from coupled mononucleated cells. In these cases it is not possible to determine whether the cells are completely fused or not. It is possible, for example, that they are joined by gap junctions (Shimada, '71; Rash and Fambrough, '73). We can conclude, however, that chemical and electrical excitability are properties that develop after the formation of electrical coupling between cells.

Figure 2 shows three major types of action potentials recorded from myotubes. Action potentials occurred either spontaneously (2C) or were evoked by passing current across the cell membrane (2A, 2B). The action potentials shown in figure 2A and B result from hyperpolarization only, but occasionally action potentials may arise from depolarization. The most likely interpretation of this result is that hyperpolarization removes the inactivation of an ionic conductance caused by the low resting potentials (Hodgkin and Huxley, '52). Voltage-clamp experiments are in progress to study this point.

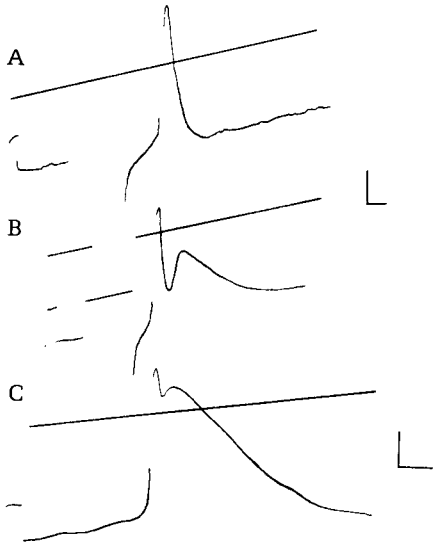

Fig. 2 Three types of action potentials recorded from myotubes. "A" and "B" resulted from external stimulation; "C" was spontaneous. "B" and "C" are composed of two components, an early fast component followed by a slow component. The age of the cultures, with the day of plating taken as day zero, is "A", 22 days; "B", 16 days; and "C", 29 days. The calibration for each recording is shown as a positive deflection on the voltage trace; the deflection is 20 mV in amplitude and 5 msec in duration; another calibration is provided to the right. This calibration is 20 mV in amplitude and 10 msec in duration. The upper calibration is for "A" and "B" and the lower for "C". The upper line in all cases is zero potential, but the stimulus current is also recorded on this trace. A downward deflection is inward current. A deflection of 20 mV on the current trace represents 3 nA. No current is shown for "A", but in subsequent figures the current is shown when external current was used as a stimulus. The potentials were recorded on moving film, and this causes the slight distortion seen on these recordings, especially with slow sweep speeds.

The most striking features about the action potentials are that they are of a long duration, i.e., greater than 10 msec, and in some cases they seem to be composed of two components (fig. 2B, 2C), namely, a rapid and a slow component. We will refer to the type with two com-

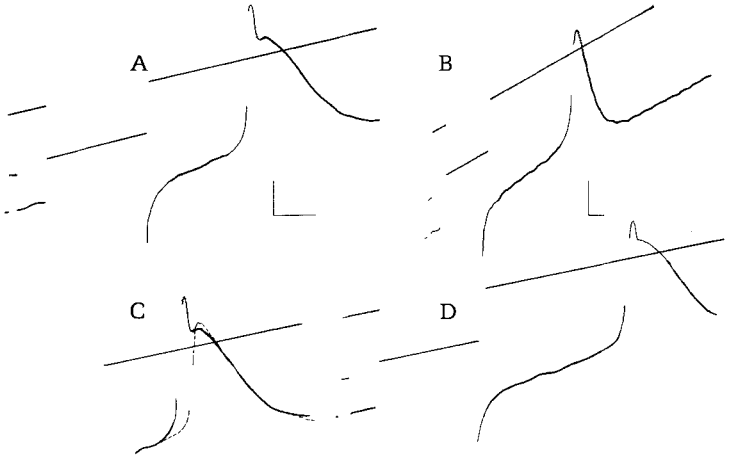

Fig. 3 The selective action of TTX on the fast component of a compound action potential. TTX blocks the early fast component of the action potential but not the second slow component. "A" is the control response before the addition of TTX. "B" was recorded during the application of TTX, "D" is the recovery. The calibration for each response is shown to the left on the potential record, and has the same values as for figure 2. Another calibration is provided; the one under "A" is the calibration for "A", "C", and "D" and is 20 mV in amplitude and 20 msec in duration. The one under "B" is for "B" only and has the same values as for "A", "C", and "D". Current calibration is the same as figure 2. TTX was applied through a micropipette that was positioned very close to the myotube. The age of the culture is 16 days.

ponents shown in figure 2B and 2C as a compound action potential, and the type with one component shown in figure 2A as a simple action potential. Since both components of the compound action potential are able to generate positive membrane potentials, it was anticipated that both would be blocked by TTX.

Experiments shown in figure 3A–C, however, illustrate that only the rapid component is blocked by TTX. The control response is shown in figure 3A; TTX was applied from a micropipet and within a few minutes the first rapid peak was blocked completely, figure 3B and 3C (also see table 2). Figure 3D shows that the effects of TTX are reversed within one to five minutes. For easier comparison of the effect of TTX, figure 3C shows the action potential in TTX superimposed upon the control response on the same time base. The major difference between the two responses shown in figure 2C is that the first rapid component is blocked in TTX whereas the changes in the slow component are a slight, but significant increase in amplitude, and a change in latency. This experiment proves that compound action potentials are composed of two independent responses: the first is blocked by TTX and is rapid in its time course, and the second is not blocked by TTX and it is slow in its time course. While a completely different mechanism seems to be responsible for the second component, it is sensitive to TTX in that it usually becomes larger. This increase in amplitude would be expected if two ionic conductances were responsible for the second component. For example, if the sodium equilibrium potential is less positive than the equilibrium potential for the second ion, blocking the sodium with TTX would cause the second component to increase in amplitude.

The experiments done in TTX suggest that possibly the simple action potentials may result from some cells being able to generate only one type of two classes of action potentials, i.e., TTX sensitive or TTX insensitive. If this were the case, we should be able to find simple action potentials that are sensitive to TTX and others that are not. Furthermore, because of the striking difference in duration between the two components of a compound action potential, it might be expected that simple fast action potentials, such as the one in figure 2A, would be similar to the fast component of the

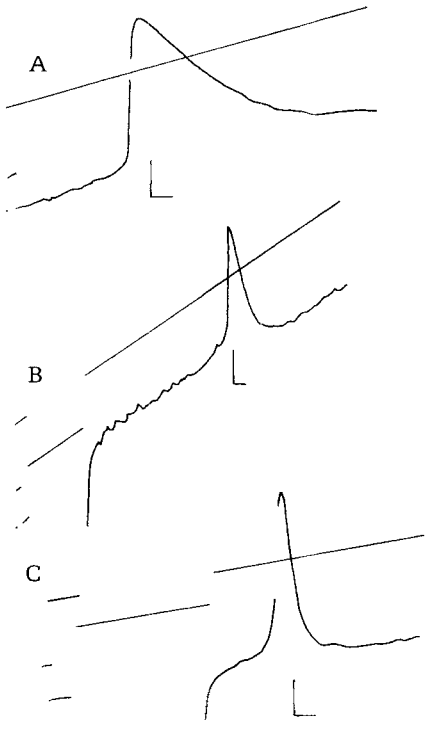

Fig. 4 The failure of TTX to block three different types of action potentials. "A" is a spontaneous potential recorded in 10^{-6} M TTX; "B" is an externally stimulated potential in 10^{-5} M TTX; "C" is an externally stimulated potential in 10^{-6} M TTX. Note the different time scale for "B". Calibration is the same as in figure 2. The age of the culture is, "A", 29 days; "B", 20 days; "C", 29 days.

compound action potential, and conversely the simple slow action potential such as the one in figure 4A would resemble the slow component. If this suggestion were true, we would expect that TTX would block only the fast action potentials, for example, those less than 10 msec in duration, whether they occurred as simple action potentials or as rapid components in the compound action potentials. We found, however, that, while many simple action potentials are sensitive to TTX (see table 1, fig. 5), we could not find a simple correlation between the sensitivity to TTX and the duration of the action potential. These data are summarized in table 1 and figure 5.

Since the concentration of TTX in the solution surrounding the myotubes is not known when TTX is applied from a micropipet, we decided to determine the effects of 10^{-5} M and 10^{-6} M TTX when added directly to the solution bathing the myotubes. Unfortunately, we were not always able to change the solution with the recording microelectrode inside the myotube. The procedure we followed for most of the experiments listed in table 1 was to apply the TTX 10 to 30 minutes before recordings were made from the myotubes. Under these circumstances we do not know what the control responses are, but, in both concentrations of TTX, action potentials could be recorded, figure 4. These action potentials must be considered insensitive to TTX. It should be emphasized that no complex action potentials were recorded in either concentration of TTX. We conclude, therefore, that the first component of the compound action potentials is probably blocked by 10^{-5} M and 10^{-6} M TTX. This was confirmed by several experiments in which the control response and the effect of 10^{-5} M and 10^{-6} M TTX was studied by changing the solution while the electrode remained in the myotube. If the action potential was a complex one, a reversible reduction in the first components was always observed. If the action potential was a simple one, often 10^{-6} M TTX reduced or blocked the action potential. If the action potential was reduced by 10^{-6} M TTX, 10^{-5} M TTX invariably blocked it completely.

In order to obtain a better understanding of these action potentials, we attempted to determine their ionic basis. We reduced the external sodium concentration and replaced the sodium with either choline chloride, tetraethylammonium bromide, or tris (hydroxymethyl) aminomethane·HCl (Tris). In 68 mM Na^+, a 50% reduction in sodium concentration, with either choline or tetraethylammonium as the replacement ion, there is no statistically significant change in either the resting potential or action potentials. In 14 mM sodium, there is a reduction in the amplitude of simple action potentials with no accompanying change in the resting potential. The change in amplitude represents about a 23 mV change for a ten-fold change in external sodium concentration, but we did not wait for a new steady-state to be established in the low sodium, therefore, these observations cannot be used to fit

TABLE 1

Experiment	No. of cells	Resting potential	Action potential	Duration [1]	No. of spontaneous action potentials
		$-mV$	mV	$msec$	
Control	42	45.2 ± 12.3	67.3 ± 19.0	20.0 ± 13.7	19
TTX (10^{-6} M)	6	42.8 ± 12.7	74.2 ± 17.0	19.4 ± 13.3	2
TTX (10^{-5} M)	3	50.3 ± 2.5	70.6 ± 3.0	17.3 ± 2.5	0
TTX (pipet)	17	37.6 ± 10.2	29.8 ± 30.4 [2]	13.9 ± 7.9 [3]	2 [13]
68 mM Na^+	7	39.4 ± 18.0	57.7 ± 6.8	19.4 ± 8.3	0
14 mM Na^+	7	47.8 ± 9.9	44.7 ± 18.3 [4]	24.1 ± 13.7	1
5.4 mM Ca^{2+}	3	59.0 ± 10.5	87.0 ± 12.1 [5]	51.0 ± 1.7 [6]	3
9 mM Ca^{2+}	2	70.7 ± 3.1 [7]	85.0 ± 6.1 [8]	12.2 ± 6.2 [9]	0
1 mM Mn^{2+}	2	54.5 ± 0.5 [10]	65.7 ± 5.3 [11]	37.5 ± 5.6 [12]	0

[1] Measurement made at half maximum amplitude.
Control values:
[2] 55 ± 20.0
[3] 23.7 ± 10.3
[4] 69 ± 13.5
[5] 70.3 ± 12.5
[6] 14.6 ± 8.6
[7] 70.7 ± 3.1
[8] 87.3 ± 1.2
[9] 19.4 ± 8.2
[10] 54.5 ± 0.5
[11] 68.8 ± 7.3
[12] 12.9 ± 5.6
[13] Seven myotubes in this group were spontaneously active before TTX was applied. Following TTX two remained spontaneously active, three were blocked and two we were unable to determine.

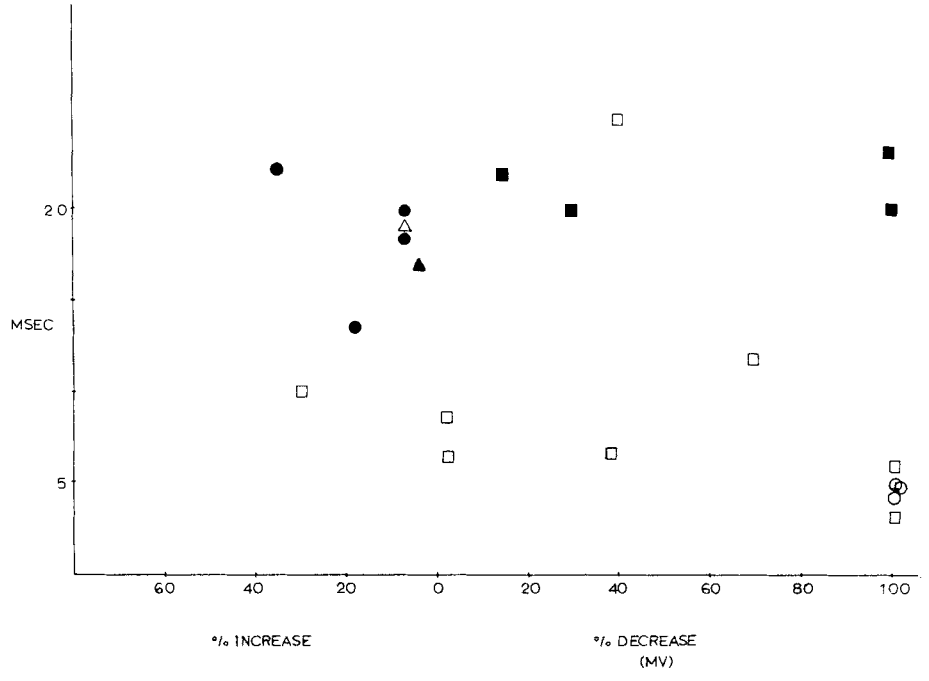

Fig. 5 The relationship between the duration of the action potential, as measured at full width half maximum amplitude, and the effect of TTX, as measured by the percentage decrease in action potential amplitude. The □ represent simple action potentials, ■ are spontaneous action potentials, ○ are the first components of the compound action potentials, ● second peak of compound action potentials, △ average duration of action potentials in 10^{-6} M TTX, and ▲ average duration of action potentials in 10^{-5} M TTX.

TABLE 2

Control			14 mM Na^+		TTX	
Resting [1] potential	Action potential (1)	Action potential (2)	Action potential (1)	Action potential (2)	Action potential (1)	Action potential (2)
31	66	35	31	11 [2]		
33	53	37	28	28 [3]		
46	58	34			0	40
53	80	61			<40	65
45	65	68			0	73
35	13	25			0 [4]	33
43	45	48			0 [5]	34

[1] Resting potentials in 14 mM Na^+ and TTX did not change.
[2] After three minutes in low Na^+.
[3] After about five minutes in low Na^+.
[4] In 10^{-5} M TTX.
[5] In 10^{-6} M TTX.
The frequency of compound action potentials is between about 20–40 per 100 recordings.

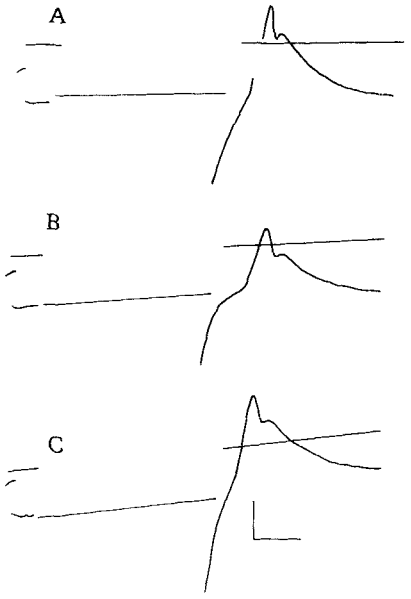

Fig. 6 The effect of sodium replacement with tetraethylammonium on the compound action potential. Calibration signals are 20 mV in amplitude and 20 msec in duration. Current calibration is the same as figure 2. "A" is the control response. "B" is 14 mM sodium 123 mM tetraethylammonium bromide. "C" is the recovery in normal Tyrode solution.

a quantitative model. If sodium is completely replaced by Tris, the action potentials are completely abolished, and in several instances we were able to show that when an action potential was blocked by TTX, replacement of sodium with Tris also blocked the action potential. In some instances, however, we have observed action potentials that are blocked by the replacement of sodium with Tris, but they may only be reduced in amplitude in 10^{-5} M TTX. This observation agrees with observations made by others on the L_6 line (Kidokoro, '73) as well as on denervated rat muscle (Redfern and Thesleff, '71). Again, we were unable to find a relationship between the magnitude of the effect of sodium and the duration of the action potential.

An important test for determining the relationship between the effect of low sodium and TTX is to determine whether the two peaks of the compound action potentials are equally sensitive to reductions in sodium. Table 2 summarizes our observations on complex action potentials in low sodium or in TTX. The reduction in sodium concentration reduced both components, but there is a greater reduction in the first peak compared to the second one (fig. 6). The first peak is reduced by about 50% whereas the second one is reduced by only about 30%. This observation suggests that sodium may be the major ionic component involved with the generation of the first peak, but it is likely that another ion is involved with the generation of the second peak. This observation supports the observations made with TTX, in that the second component of the compound action potentials has a smaller sodium dependence, and is not blocked by TTX. If all the sodium is replaced by Tris, both components of the compound action potentials are blocked.

In an effort to determine what other

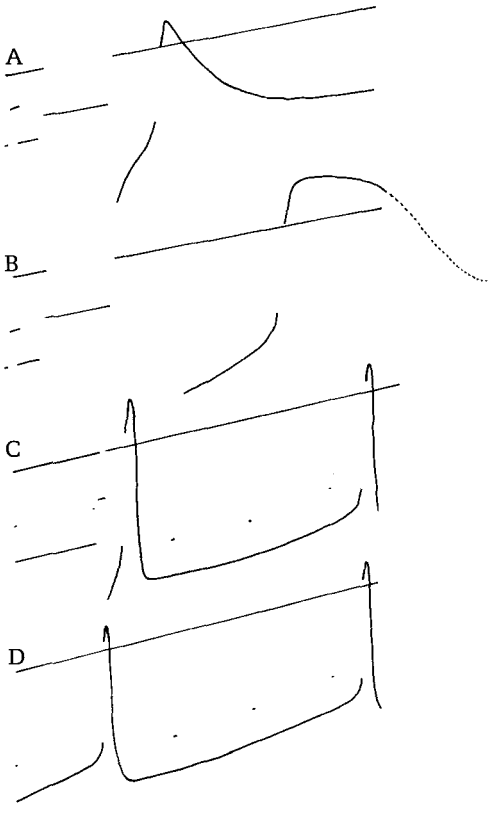

Figure 7a

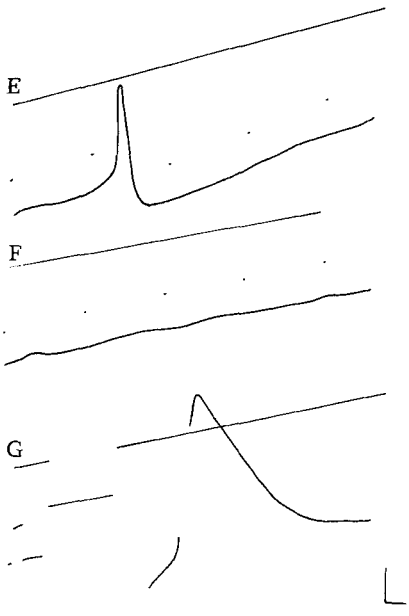

Figure 7b

Fig. 7 The effect of increasing the calcium concentration from 1.8 to 5.4 mM/L. "A" is the control response taken before the calcium concentration was increased. "B" shows the first response caused by stimulation in high calcium. Following the first action potential, which was evoked by hyperpolarizing current, spontaneous activity may occur as shown in "C" and "D". The spontaneous activity disappears in time as shown in "E" and "F". "G" shows the response after the solution has been changed back to normal Tyrode solution. Calibration is the same as shown in figure 2. On slow time scale calibration pulses are separated by 500 msec.

ions may be involved, we increased the external calcium concentration. In some myotubes the change in action potential in high calcium, i.e., 5.4 mM calcium or three times normal, is very striking (fig. 7). There is a rapid change in the amplitude, overshoot, and duration of the action potential in high calcium. In addition, the cells often become spontaneously active as shown in figure 7. The increase in overshoot of the action potential is about 14 mV and represents the size of the change predicted by the Nernst equation if the calcium concentration inside the cell is about 0.1 mM, and the action potential peak approaches the calcium equilibrium potential. This concentration represents a much higher concentration of calcium inside the myotubes than would be expected based upon calcium determinations made on adult cells (Ashley, '70; Baker et al., '71). Until we have measured the internal concentration of calcium in these cells we are reluctant to suggest that calcium is the only ion that is responsible for this action potential, but these experiments certainly implicate a conductance change for calcium. These cells may not have a fully developed regulatory system for calcium and they have a low level of calcium activated ATPase activity in membranes (Podleski, unpub-

lished observations). It is possible, therefore, that the concentration of calcium inside the cells is higher than in the adult muscle. Although we did not measure the sensitivity of the cell shown in figure 7 to TTX, we would expect that TTX would not block this response since the primary permeability change in this cell seems to be to calcium, or possibly calcium plus sodium. In addition to the type of cell that becomes spontaneously active in elevated calcium solutions, we found cells whose response is reduced in high calcium. All the cells, that we tested for sensitivity to TTX, were blocked by TTX.

We have investigated the effects of manganese and lanthanum on these action potentials, since it has been reported that these ions block calcium conductance (Hagiwara and Nakajima, '66; Reuter, '73). The effects of 1 mM manganese is illustrated in figure 8. Manganese always increased the duration of the action potential, and the lengthening is due primarily to a prolongation of the falling phase of the action potential, as opposed to the rising phase. This action of manganese was observed on action potentials that were completely blocked by either TTX or by replacement of sodium. Similar effects of manganese have been reported on frog heart ventricle muscle (Hagiwara and Nakajima, '66). We conclude that the action of manganese is not due to blocking calcium or that there is a small calcium component in the action potentials blocked by TTX and reduced sodium concentration. These effects of manganese could also be explained by a slowing of either the inactivation process of sodium or the activation of potassium. Any of these possibilities could account for the effect of manganese on the falling phase of the action potential. The action of lanthanum was not as consistent as the action of manganese. Lanthanum (0.1–0.2 mM) often lengthened the action potential, in a manner similar to manganese. All these effects were reversible.

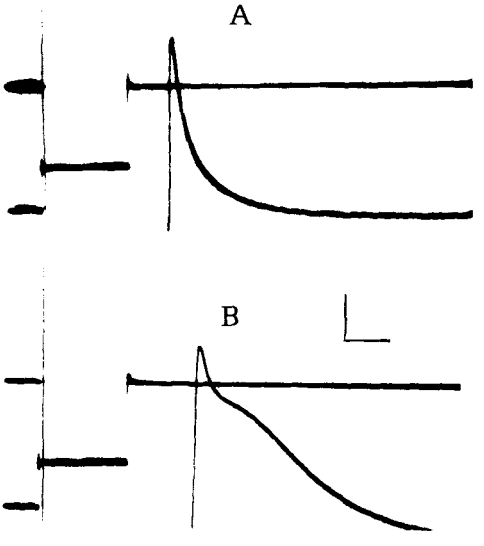

Fig. 8 The effect of 1 mM manganese. "A" is the control action potential in normal Tyrode solution, and "B" is in 1 mM manganese in normal Tyrode solution. Voltage calibration is 20 mV in amplitude and 20 msec in duration. A 20 mV deflection on the current trace represents 5 nA.

Our data on the ion replacements indicate that neither sodium nor chloride play a major role in the maintenance of the resting potential. It seems likely that the predominant ion is potassium, and potassium has been shown to depolarize myotubes (Lawrence, unpublished observations). Calcium may play a regulatory role, in the development of the membrane potential, since high calcium results in a hyperpolarization of the membrane. We have not yet investigated the possible role of active transport in the generation of the resting potential.

Spontaneous electrical activity occurred in high calcium, but was often seen in normal Tyrode solution (figs. 2C, 4A). We have not studied this activity in detail, but we did find that spontaneous action potentials were significantly longer in duration, i.e., 30.7 ± 15.4 msec ($n = 20$), as measured at half maximum amplitude, than evoked action potentials. Also the amplitude of the spontaneous action potentials were significantly greater, 75.5 mV ± (10.6). The resting potential, since it is not steady, is difficult to measure accurately and the value that was used for this calculation was the membrane potential at the inflection point at the beginning of the action potential. Had we used the maximum negative potential, the value used for the resting potential would have been more negative. Spontaneous action potentials can be compound ones as shown in figure 2C, or simple as shown

in figure 4A. If the myotube twitched spontaneously, electrical parameters were found to be the same as those given for the spontaneous action potentials. With the L_6 line we have never observed a twitch to accompany a fast action potential, and 10^{-6} M TTX did not block twitching in the few examples we were able to test. The presence of spontaneous action potentials could not be correlated with the age of the culture. With primary mice cultures, twitching could be observed to accompany fast action potentials, and they were blocked by TTX. We have also found TTX insensitive action potentials in primary cultures.

DISCUSSION

Our results show that excitable responses of the myotubes are extremely diverse. There are responses that resemble adult innervated muscle in that they are completely blocked by TTX and by removal of sodium (Redfern and Thesleff, '71). The concentration of TTX that is required to block the responses in the myotubes is about the same as that required to block innervated muscle, i.e., 10^{-6} M. On the other hand, the myotubes can generate action potentials that are sodium-dependent but not blocked by 10^{-5} M TTX, although these potentials may be reduced or increased slightly in TTX. In this respect the myotubes resemble denervated skeletal muscle (Redfern and Thesleff, '71). We have also recorded action potentials that seem to be due to changes in calcium permeability; this type action potential is not commonly observed in skeletal muscle but exceptions do exist, e.g. barnacle muscle (Hagiwara and Nakajima, '66). The compound action potentials have not been observed in adult skeletal muscle, although these potentials resemble observations made on amphibian and mammalian cardiac fibers (Hagiwara and Nakajima, '66; Mascher and Peper, '69).

In cardiac cells the action potential can be divided into two phases. The fast phase is due to an increase in sodium permeability and it is blocked by TTX. The current during the second slower phase is due to either calcium or to both calcium and sodium, and it is not blocked by TTX. In low sodium these two phases may become separated into two distinct peaks whereas normally they overlap in time. The first component of the compound action potential recorded from myotubes resembles the rapid phase of the action potential recorded from cardiac muscle, and the second (slow) component of the compound action potentials resembles the slow component recorded from cardiac fibers. The simple action potentials recorded from the myotubes that are little affected by TTX would then be expected to be identical to the second component of the compound action potentials. We suggest, therefore, that the recordings we have made from the myotubes represent a spectrum of responses with sodium and TTX sensitivity on one end and calcium-sodium sensitivity and TTX insensitivity at the other end. In between these two classes of response would be responses with varying degrees of each of these two properties. The compound action potentials would represent an intermediate state during which the kinetics of the two systems are sufficiently different to result in a temporal separation of the two peaks. More often, however, the action potentials would be simple ones with varying degrees of sodium and calcium-sodium conductance systems.

The results of others with TTX insensitive, sodium sensitive action potentials have shown that TTX sensitive action potentials have a faster rise time than insensitive ones (Redfern and Thesleff, '71). We were unable to distinguish a difference between the rise rates of TTX sensitive and insensitive action potentials. Our measurements are subject to considerable error, however, since we did not electronically differentiate the action potentials. It is possible, therefore, that TTX sensitive action potentials may rise slightly more rapidly than insensitive ones. Also it is conceivable that action potentials with small calcium components may be recognized by small changes in the fall rate.

There are several possible explanations for the diversity of the responses that have been recorded from the myotubes. The two possibilities we believe to be most likely are that the cells differ genetically or that the different responses represent different developmental states. The ge-

netic complement has been shown to influence electrical excitability of neuroblastoma cells (Minna et al., '71), but we favor the second interpretation because of the resemblance of some of the responses to denervated muscle, and because we have recorded many of the same type of action potentials in primary mouse cultures. In addition, we have found that acetylcholine response in the myotubes is blocked by both d-tubocurarine and atropine (unpublished observations). Both innervated and denervated muscle are blocked by these two compounds, but innervated muscle is much more sensitive to d-tubocurarine than is denervated muscle, whereas the response of the two muscles to atropine is the same (Beranek and Vyskocil, '67). The myotubes have an identical sensitivity to atropine as innervated and denervated rat muscle. The response to d-tubocurarine in the myotubes, however, resembles denervated muscles in that both are less sensitive to d-tubocurarine than the innervated muscle. The acetylcholine response of the myotubes is also about the same as denervated muscle, i.e., 50–100 mV/ncoul. These observations are consistent with the possibility that the action potentials we have recorded represent a sequence of changes that normally occur during myogenesis, up to the time innervation occurs.

It is unfortunate that we cannot determine the precise developmental sequence of these excitable events from our results. The reason for this is that neither the cell cycle nor the fusion process was synchronized in our studies. The variety of responses we recorded was not related simply to the length of time following the plating of the myoblasts, nor to the size of the myotube. We are in the process of studying the development of the excitable components in more detail in an effort to determine the precise time at which these different excitable properties develop. For example, it would be very useful to determine the time a calcium dependent response appears and disappears, and what factors are responsible for these changes.

Recently there have been several reports on the electrical properties of myotubes in culture (Kidokoro, '73; Harris et al., '73; Kano et al., '72; Kano and Shimada, '72). In one instance, the cell line studied was the L_6 line used in our studies, and the results are similar in many respects, but different in others. For example, we both have observed calcium dependent action potentials. There are, however, large differences in the duration of the action potentials between the two studies. Our action potentials were generally between 2 and 30 msec in duration, measured at one half the maximum amplitude, whereas Kidokoro found much longer action potentials, e.g., 100 msec. The variation in the duration of the action potentials is not due to variation in the rise times. The rise times of all the action potentials we have recorded is about 20 V/sec, which agrees well with Kidokoro's results. This means that the main reason for the lengthening in duration is due to a slowing of the falling phase.

There are several possible explanations for the differences between Kidokoro's observations and our own: one, different stimulus parameters; two, differences in the ionic composition of the solutions; three, genetic drift in the cells. We have stimulated the cells with pulses of up to 100 msec in duration, the same used by Kidokoro, and our results were either unchanged or the action potentials disappeared with the longer stimulation pulses. The most notable difference between the solutions used is the lack of magnesium in Kidokoro's. We are currently testing this possibility. We cannot rule out at present the possibility of genetic drift.

As we have indicated before, the measurements we have made on the L_6 line are very similar to the ones we have made on primary mouse cultures. In addition, there are many similarities between our observations and those made by others on primary cultures. Among the various reports, however, there is considerable variation in the wave shape and duration of the action potentials as well as their sensitivity to TTX. For example, Kano and Shimada ('72) found two types of action potentials in primary chick muscle in culture. One was blocked by TTX and the other was not. In this respect their observations are similar to our own. On the other hand the TTX insensitive action potentials were longer in duration than

the ones we observed. Harris et al. ('73) studying myotubes obtained from chick explants and Fischbach et al. ('71) studying chick myotubes did not observe TTX insensitive action potentials nor did they observe action potentials with two components. It seems likely that the differences between these observations are due to variation in states of differentiation. There are, however, no consistent differences between primary cultures and the L_6 line.

Our results, as well as those of others (Kidokoro, '73; Patrick et al., '72; Sytkowski et al., '73) show that fusion is the event that triggers the development of excitability in the L_6 line of myoblasts. The process of fusion is complex and our observations provide some insight as to the time during fusion that excitability must develop. Since two cells must make contact with one another before fusion can take place, we studied single isolated myoblasts as well as confluent ones. We found that these cells were not electrically or chemically excitable. Furthermore, our results show that cells may be electrically coupled to one another and still not show excitable properties. We must conclude, therefore, that excitability develops at a time following the initial contact between the two cells, and the formation of low conducting pathways between the cells. The results differ from those reported by Rash and Fambrough ('73) who used primary cultures and studied the development of chemical excitability. They found that some mononucleated cells were chemically excitable while others were not. When two cells, one excitable and the other not, became electrically coupled, acetylcholine sensitivity spread rapidly to the membrane of a cell that was not excitable. The reasons for the differences between the observations on the L_6 line and chick primary cultures are not clear. The differences may be due to species difference or due to differences between primary and long term culture. We have not yet studied in detail the chemical excitability of primary mouse cultures.

Whether the changes in membrane properties we have observed arise from the synthesis of the new membrane components or a reorganization of the membrane requires further study. The study of the L_6 cells, however, would seem to be an excellent choice for determining the regulatory processes responsible for altering these membrane properties. We are attempting to correlate biochemical changes with these physiological events by studying biochemically the membrane components of the cells.

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Note added in proof: J. B. Harris and M. W. Marshall (Nature New Biology, *243*: 191–192 (1973)) have recently reported in vivo studies on the development of TTX sensitive action potentials in rats. They have found that at birth action potentials recorded in diaphragms are not sensitive to 10^{-6} M TTX. At 20 days after birth the action potentials are blocked by 10^{-6} M TTX. These observations support the hypothesis that the sensitivity to TTX that we have observed is due to a developmental process.