The Journal of Neuroscience, August 1987, 7(8): 2412–2422

The Influence of Skeletal Muscle on the Electrical Excitability of Dorsal Root Ganglion Neurons in Culture

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Dorsal root ganglion (DRG) neurons from embryonic mice grown in coculture with dissociated skeletal muscle or in skeletal muscle conditioned medium (CM) showed an increased incidence of repetitive firing of action potentials when injected with sustained (60–100 msec) depolarizing current. This is in contrast to DRG neurons grown in monoculture and normal medium, which exhibit such behavior far less frequently. The first action potential showed less sensitivity to block with TTX and more sensitivity to Ca^{2+} channel blockers than the subsequent action potentials. The increased incidence of repetitive firing occurred when CM was added after as few as 2 or as many as 22 d in culture and with as little as 1–7 hr exposure to CM. This effect of CM cannot be mimicked by NGF or by coculture with cells from embryonic spinal cord (Peacock et al., 1973), can be eliminated by heating the CM at 56°C for 30 min, and partially reversed following short exposure to CM. These results indicate that skeletal muscle releases some heat-labile factor(s) that can cause repetitive firing and, in addition, significant decrease in input resistance in the CM-treated neurons and a depression of the anomalous rectification, neither of which could account for the increase in repetitive firing.

Sensory input transduced at specialized receptors and free nerve endings in peripheral tissues is propagated to central targets via all-or-none action potential activity in dorsal root ganglion (DRG) neurons. Variations in action potential properties of DRG neurons can be correlated with conduction velocity (Harper and Lawson, 1985), which may in turn be associated with particular types of sensory modality. The temporal pattern of action potentials varies with functionally identifiable DRG neurons as well. Details of the temporal patterns vary widely among the many sensory modalities, with spike frequency, adaptation rates, sensory thresholds, and conduction velocities ranging over orders of magnitude. The influence of the sensory receptor on the intrinsic properties of the sensory neuron and the relative contributions of the receptor and neuron to the characteristic firing pattern are unclear.

Embryonic and early postnatal (DRG) neurons receive both survival and neurite-promoting support from target and nontarget tissues through a variety of distinguishable factors (Thoenon and Edgar, 1986). The roles such factors play in promoting and maintaining appropriate innervation by these neurons or selective survival associated with specific sensory modalities have been widely studied. Tissue culture can be used as a way to observe the action of differentiating factors on neuronal development. Under such conditions, NGF has been found to promote selective (Yip and Johnson, 1984) or nonselective (Lindsay, 1979) survival of DRG neurons. In addition, extracts from skeletal muscle (Hsu et al., 1984), heart, liver, and brain (Lindsay and Tarbit, 1979), and ocular tissues (Skaper et al., 1982) have been reported to contain survival-promoting factors for DRG neurons in culture. Likewise, conditioned media (CM) from several sources have similar effects in culture. These include CM from cultures of glioma cells (Barde et al., 1978, 1980) and Schwann cells (Varon et al., 1981). However, the effects of either extracts or CM from both target and nontarget tissues on the development of DRG cell membrane excitability have received little attention.

One strategy to elucidate the possible role(s) played by various factors in differentiating specific sensory transduction processes involves culturing embryonic DRG cells with and without their peripheral and central targets. In monolayer culture the DRG neurons are readily accessible to detailed electrophysiological study of the effects of cellular contact and diffusible factors on the development of electrically excitable membrane properties. Thus, this preparation is suitable for investigation of the roles played by extrinsic factors on physiological properties of DRG neurons.

In this study we have used intracellular recording techniques to evaluate exposure of embryonic DRG neurons to skeletal muscle, one of the peripheral target tissues. The CM from cultured skeletal muscle induced dramatic changes in the excitability of some cells, generating the capacity to fire repetitive action potentials in response to steady depolarizing currents.

Materials and Methods

Dissociated cell culture

DRG neurons. DRG cells were dissected from 13- to 14-d-old fetal mice, treated with 0.1% trypsin (type VIII, Sigma), mechanically triturated, and plated on collagen-coated 35 mm plastic dishes (Falcon) at a density of approximately 3.5×10^5 neurons/dish. The cells were

Received Sept. 4, 1986; revised Jan. 15, 1987; accepted Feb. 20, 1987.

We wish to thank Drs. Donald E. R. Meyers and Thomas M. Jessell for reading an earlier version of this manuscript and making helpful suggestions. We also gratefully acknowledge Veronica Smallwood for the preparation of the cell culture.

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0270-6474/87/082412-11$02.00/0

incubated at 37°C in humidified air containing 5% CO_2. The plating medium contained 80% Eagle's medium (DMEM; Gibco), 10% heat-inactivated horse serum (HS; Gibco), 10% heat-inactivated fetal calf serum (FCS; Hazelton), glucose (4.5 g/liter), and 1% glutamine (Gibco). NGF (gift from Drs. G. Guroff, Scholfield, and Rabe) was added at plating at a concentration of 10–30 ng/ml. Four days after plating, the cultures were exposed to 10 mg/ml 5'-fluorodeoxyuridine (FuDr; gift of Hoffmann LaRoche) for 3–4 d to suppress proliferation of non-neuronal cells. The DRG neurons from each dissection were separated into control and experimental groups. The growth medium for both groups contained the same components as plating medium, except that FCS was deleted and HS reduced to 5%. Culture media of all groups were changed twice a week.

Skeletal muscle cells. Cells were obtained from thigh muscles of 19–21 d mouse embryos. The muscle was minced and incubated in a dissecting medium (99% MEM, 1% HEPES, Gibco) with 0.1% trypsin and 0.1% DNAse (type III, Sigma) at 37°C for 30 min. Soybean trypsin inhibitor (0.01%) was added to stop the trypsin activity. The solution was centrifuged, decanted, and resuspended in plating medium (80% DMEM, 10% HS, 10% FCS). The resulting cell suspension was preplated in a 100 mm dish for 10–20 min. Then the muscle cells were plated on collagen-coated 35 mm plastic dishes at a density of 3–5 × 10^5 cells per dish. Four days after plating, the cultures were exposed to FuDr (10 mg/ml). At the first medium change, the cultures were incubated in growth medium (as defined above), which was subsequently changed twice a week. Medium conditioned by the muscle cells was collected, filtered through 0.45 μm filters (Millex), and then diluted 1:1 with fresh growth medium. Collections were made from cultures from days 5–21. In some experiments CM was heat-inactivated at 56°C for 30 min. Feeding schedules were identical for maintenance of all experimental and control plates. Lightly plated muscle cell cultures (2–3 × 10^5 cells/dish) were used for coculture with DRG neurons.

Coculture of DRG neurons and skeletal muscle. The embryonic DRG cell suspension was plated directly onto muscle previously cultured for 7–10 d. The resulting cocultures were grown in medium containing 95% DMEM, 5% HS, and NGF (10–30 ng/ml).

Electrophysiology

Solutions. At the time of the experiments, the culture medium was drawn off and replaced by 1.5 ml Hank's balanced salt solution (HBSS; Gibco) containing (in mM): 142 NaCl, 5.3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 glucose, and 10 HEPES adjusted to pH 7.2–7.4. When divalent cations were used to eliminate Ca^{2+} currents, either Cd^{2+} or Co^{2+} was added to HBSS in place of Ca^{2+}. For the Na^+-free solutions, choline-Cl was isosmotically substituted for NaCl. All modifications were made keeping the tonicity constant at 310 mOsm. TTX, 1 μM, was added to the HBSS as needed. Experiments were performed at room temperature (22–24°C).

Electrodes and electrical recording. Intracellular and whole-cell recordings were made from individual DRG neurons under phase-contrast optics on the modified stage of an inverted microscope (×200–250). The sharp intracellular microelectrodes were filled with 3 M KCl and had resistances of 60–100 MΩ. The whole-cell microelectrodes were filled with solutions containing (in mM): 140 K-gluconate, 2 $MgCl_2$, 1.1 EGTA (with 2.4 mM NaOH), 5 HEPES. This solution was adjusted to pH 7.2–7.4 with KOH and to 310 mOsm with sucrose. The filled pipettes had resistances of 5–8 MΩ. Seals between the microelectrodes and the DRG cell membranes greater than 1 GΩ were routinely established for whole-cell recordings. A bridge circuit was used for current-clamp recordings, which allowed simultaneous current injection and voltage measurement. Records of membrane potential and current were either photographed with a Grass oscilloscope camera or digitized and stored on an on-line digital computer (PDP 11/23 Plessy Peripheral System) for off-line analysis.

Calculations of membrane electrical parameters. The membrane time constant (τ) was evaluated by stimulating cells with small (0.1–0.15 nA) hyperpolarizing current steps 60–100 msec in duration. The amplitude of the voltage response was measured, and the time at which the potential reached 63% of the asymptote was taken to be τ. Input resistance (R_{in}) was calculated from the current–voltage (I–V) relationship obtained near resting potential; the slope of the linear part of the curve was taken as R_{in}. Cell diameters were estimated using an ocular micrometer, and an approximate surface area of the soma was calculated assuming spherical symmetry (i.e., $A = 4\pi r^2$). Specific membrane resistance was then $R_m = AR_{in}$. The estimated specific membrane capacitance, C_m, was derived from $C_m = \tau/R_m$.

Population statistics

Confidence limits. Since we were studying the effects of various growth conditions in culture on physiological parameters, it was not possible to use a cell before treatment as control for that same cell after treatment. It was necessary, therefore, to use population statistics in order to make claims of significant effects. Control and experimental plates (populations) were always sister cultures derived from the same dissection. The criterion for statistically significant differences is 95% confidence limits (i.e., $p < 0.05$).

Statistical tests. We were interested in 2 types of data: continuous and enumerative. For the former (membrane potential, spike duration, etc.), we used Student's t. For the latter (counts of cells with and without repetitive firing, etc.), we used a c^2 test of contingency.

Results

DRG neurons grown in culture will produce one or more action potentials in response to suprathreshold current pulses (Fig. 1*A*). The latency of the first action potential in response to a step stimulus follows the classical pattern of shortening with increasing current. Likewise, if there is more than one action potential, the rate of firing or the total number of action potentials increases with increasing current. We will take as an operational definition of repetitive firing the generation of more than one action potential during a 60–100 msec suprathreshold depolarizing current stimulus. The probability with which one encounters cells capable of generating repetitive firing varies with the culture conditions.

Figure 1 shows examples of typical voltage responses from DRG neurons under current-clamp in response to the activation protocol used in most of our experiments. For small positive or negative current steps, the transmembrane potential exponentially approached a new level with a slow time constant, reflecting the input resistance and capacitance of the cell membrane (Peacock et al., 1973). With more intense negative currents, the initial hyperpolarization was followed by a sag back towards resting levels due to activation of inward (anomalous) time- and voltage-dependent rectification (Mayer and Westbrook, 1983).

Electrical activity of DRG neurons grown in monoculture and coculture

Using whole-cell recording techniques, the majority (83%; Table 1) of DRG neurons grown in culture under standard growth conditions generated single action potentials in response to stimulation with sustained (60–100 msec), suprathreshold depolarizing current pulses (Fig. 1*A*). A minority (17%; Table 1) generated 2 or more action potentials under the same conditions. In contrast, DRG neurons that had been cocultured with skeletal muscle showed high levels of repetitive action potential activity (97%; Table 1). This is a significant increase over cells recorded in the absence of muscle. An example of one such repetitively firing DRG neuron grown in coculture is shown in Figure 1*B*. In those cells showing repetitive firing, increasing the strength of the depolarizing current stimulus increased the rate of action potential generation. In the hyperpolarizing direction, inward rectification similar to control DRG neurons was routinely detected.

Excitability of DRG neurons grown in muscle CM

The increased excitability of the cocultured neurons could have arisen from direct contact with skeletal muscle fibers or from diffusible factors released by the muscle fibers into the medium. It was also possible that an inhibitory factor was released by

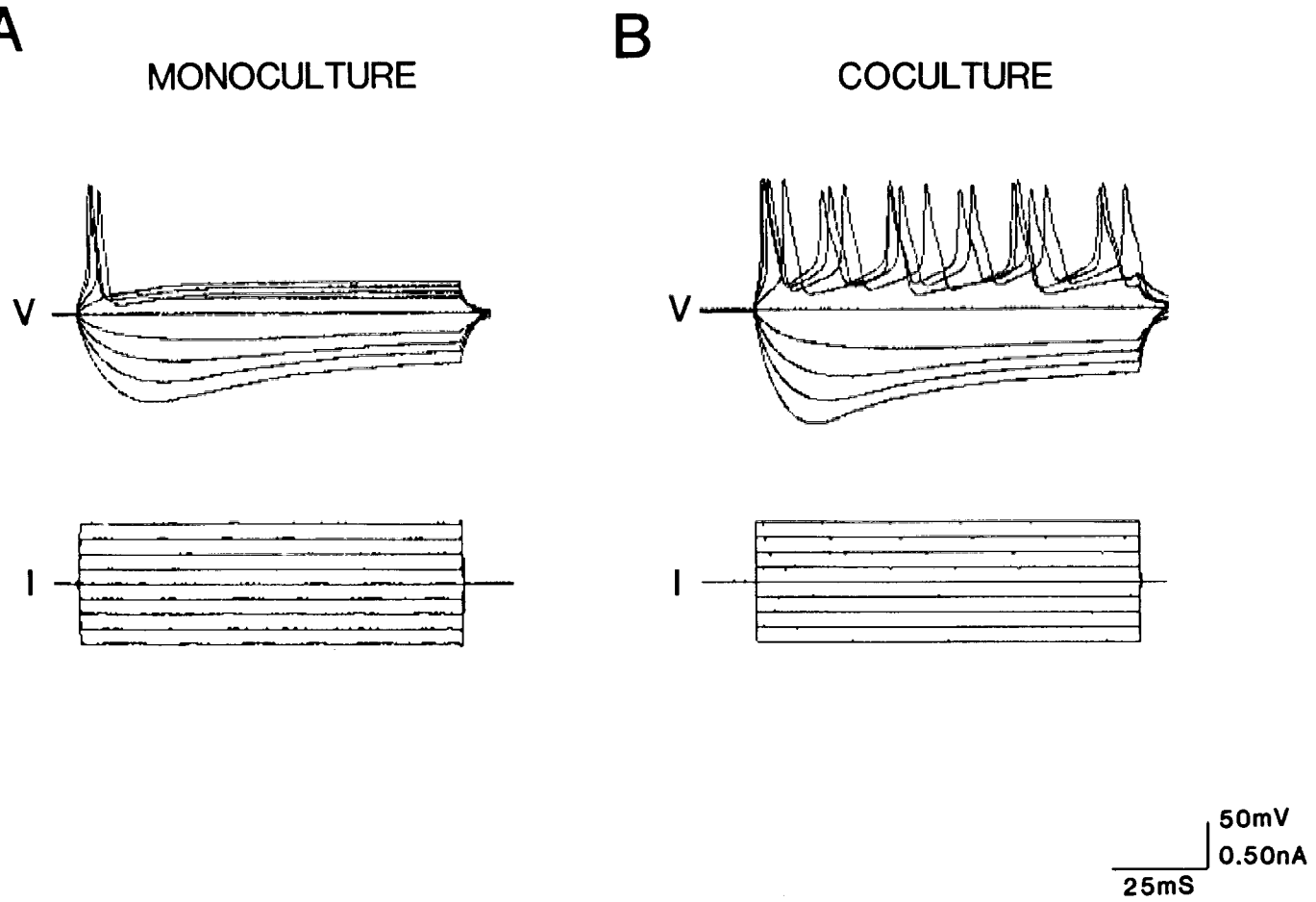

Figure 1. Cocultured DRG neurons are electrically more excitable. Typical voltage responses of DRG neurons to current steps are illustrated. *A*, Example from a recording of a DRG neuron grown in monoculture (control), showing current steps (*below*) and the corresponding voltage responses (*above*). This cell fired a single action potential in response to all suprathreshold depolarizing current steps. *B*, Example of a DRG neuron from the same dissection but grown in coculture with dissociated skeletal muscle, showing repetitive action potential activity in response to all suprathreshold current steps. Membrane potentials were held at −50 mV. The recordings were made with patch electrodes.

some type of ganglion cell and removed from the medium by the muscle cells. To test for the action of soluble factors released by the muscle cultured cells or the removal of an inhibitory factor, we examined the effect of muscle CM on the electrical excitability of DRG neurons. The experimental protocol remained the same with the addition that about one-third of the cells were studied using sharp, high-resistance microelectrode intracellular recording. Of neurons grown in CM, 60% studied with whole-cell recording and 47% of those studied with intracellular recording showed repetitive firing. These results are significantly different from the 28 and 9% in the equivalent control groups (Table 2). It is clear, therefore, that direct contact between the neurons and skeletal muscle is not necessary to induce repetitive firing, while some soluble factor or factors can induce enduring changes in the excitability of DRG neurons.

The large majority of the neurons tested showed similar responses to CM. However, neurons recorded from some dissections showed unusual firing patterns compared with the majority. All of the data from these experiments come from 37 separate dissections. In 6 of these dissections, control or untreated DRG neurons showed an extremely high incidence of repetitive firing (80%), making any action of CM impossible to detect. These were not included in the tables. Our observations suggest that repetitive firing of DRG neurons in monoculture (Table 2) usually occurs in a low percentage of cells. The reason for the unusually high rate of firing in these few dissections was not clear. The neurons from 5 of the 37 dissections showed no repetitive firing under any conditions and these were also not included in the final counts. Of the neurons recorded from the remaining 26 dissections, 24% of control neurons showed repetitive firing, while 56% of the CM-treated neurons showed repetitive firing.

CM effects are heat labile

In order to characterize the factor or factors in the CM contributing to the induction of repetitive firing, we attempted to destroy activity with heat as described in Materials and Methods. Cultures treated with heat-inactivated CM had a significantly lower incidence of repetitive firing than matched cultures treated with normally prepared CM, 14 vs 60%, respectively (Table 2). In dissection-matched controls, 28% of the cells showed repetitive firing. On the other hand, CM frozen for 3–7 d retained its efficacy. These results indicate that medium conditioned by cultured skeletal muscle contains a soluble, heat-labile substance that can affect the excitability recorded in cultured DRG neurons.

Table 1. Comparison of passive and active properties of DRG neurons alone or with skeletal muscle

	Resting membrane potential (mV)	Membrane time constant (msec)	Input resistance (MΩ)	Action potential amplitude (mV)	Action potential duration (msec)	Incidence of repetitive firing (%)
Control	−51.7 ± 5.2	10.42 ± 2.6	287.31 ± 129.5	121.5 ± 8.9	2.55 ± 0.98	17.4
(monoculture)	(46)	(32)	(22)	(45)	(46)	(46)
Experimental	−54.2 ± 4.8	9.41 ± 2.3	256.8 ± 58.3	121.1 ± 8.0	5.0 ± 2.2	97
(cocultured with muscle)	(33)	(24)	(21)	(31)	(23)	(33)

All experiments conducted using the whole-cell recording technique. All values except the percentages are means ± SD. Values in parentheses are number of cells recorded. The differences in action potential duration are statistically significant.

NGF does not alter DRG cell excitability

One substance that might be found in the CM that could contribute to the changes in excitability is NGF. Even though NGF was used in all the DRG cell cultures, it is possible that muscle cultures secreted NGF so that there was a greater concentration of NGF in the CM than in the control media (see Discussion). To test this possibility, control cultures were incubated with varying concentrations of NGF (100 and 200 ng/ml) in the growth medium. Matched CM and control plates (10–30 ng/ml NGF) were simultaneously prepared. Repetitive firing was not recorded in any of 10 NGF-treated or 5 control neurons compared with 2 of 3 CM-treated neurons. Thus, the increased concentrations of NGF did not alter the proportion of cells capable of repetitive action potential activity in response to depolarizing currents, while paired cultures grown in CM showed a high percentage of repetitive firing.

DRG excitability changes with time in culture

In some experimental groups, the time of initial exposure to CM was varied to determine if there was a critical period when the CM could act on DRG neurons in culture. There was an increased incidence of repetitive firing when CM was added after as few as 2 or as many as 22 d in culture. Furthermore, the increased excitability of the DRG neurons was maintained throughout the exposure to CM. Thus, the effect of CM on the excitability of the DRG neurons was found in neurons tested at all periods of maintenance in cultures (6–34 d) following initial exposure to CM on the fourth day in culture.

During the second week in culture and under whole-cell type recording conditions, 11% of control cells and 40% of CM-treated cells showed repetitive firing. The likelihood of repetitive firing increased with time in culture, so that during the fourth week, 77% of control and 91% of CM-treated cells showed repetitive firing. The action of heat-inactivated CM was tested over a smaller range of times. These cultures showed a lower incidence of repetitive firing compared with CM-treated cultures during both the second and third weeks. A higher percentage of repetitive firing was recorded in all populations of neurons maintained in culture for longer periods. The CM-treated cells exhibited a significantly higher incidence of repetitive firing than control DRG neurons over the first 3 weeks in culture but not by the 4th week. This effect of time in culture on excitability is illustrated in Figure 2. The overall tendency of increased repetitive firing with time in culture, in addition to the effect of CM, was also observed using high-resistance microelectrodes for recording.

Effect of acute exposure to CM and its reversibility

DRG neurons were tested for responsiveness to relatively brief applications of CM. In a series of 5 experiments including cells from 5 different dissections, control dishes were exposed to CM for 1–7 hr at 37°C. After this brief exposure, 20 of 27 cells showed repetitive firing, while only 1 of 23 matched control cells exhibited repetitive firing. Thus, in these cells, a short exposure to CM was sufficient to greatly increase the percentage of repetitively firing neurons in a dish. In an overlapping series of experiments, 3 dishes that had been exposed to CM were washed in DMEM at 37°C for 1 to 3.5 hr subsequent to recording. Before wash, 12 of 15 cells showed repetitive firing, while after wash, 5 of 13 showed repetitive firing.

Table 2. Active membrane properties of DRG neurons recorded under various conditions

	Whole-cell recording			Intracellular recording		
	Action potential amplitude (mV)	Action potential duration (msec)	Incidence of repetitive firing (%)	Action potential amplitude (mV)	Action potential duration (msec)	Incidence of repetitive firing (%)
Control	98.08 ± 16.17	2.68 ± 1.16	28	81.56 ± 12.64	1.99 ± 0.92	9
	(89)	(89)	(89)	(121)	(115)	(121)
CM-treated	95.4 ± 14.07	2.55 ± 1.07	60	82.83 ± 10.79	2.35 ± 1.01	47
	(90)	(89)	(90)	(48)	(48)	(48)
Heat-inactivated CM	94.64 ± 14.63	2.31 ± 0.49	14			
	(22)	(22)	(22)			

All values except the percentages are means ± SD. Values in parentheses are number of cells recorded. There are 3 instances of significant difference between whole-cell and intracellular recording. These occur in both measurements of action potential amplitude and in the control action potential duration. The differences in incidence of repetitive firing in control and CM-treated cells are significant using either recording technique. The differences in incidence of repetitive firing in CM and heat-inactivated CM cells are significant.

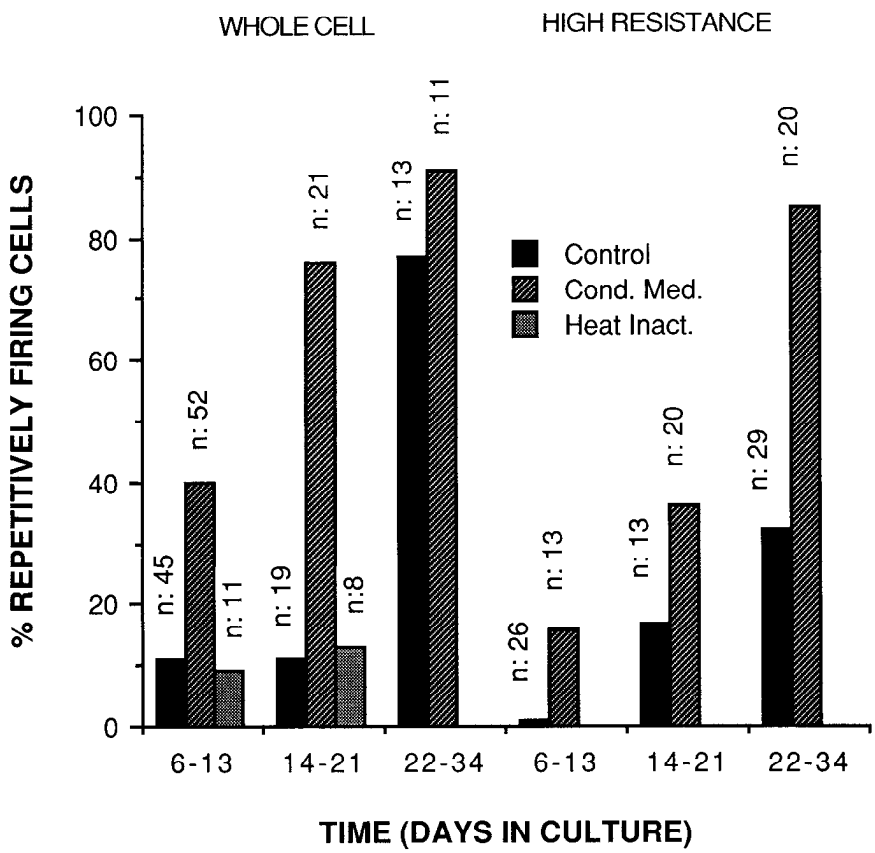

Figure 2. Electrical excitability of DRG neurons varies with time in culture and treatment. Percentage of repetitively firing cells as a function of time in culture. Data obtained using whole-cell recording at *left*; sharp, high-resistance microelectrode intracellular recording, *right*. In both groups, the percentage of repetitively firing cells has been separated into control, CM-treated, and with whole-cell recording, heat-inactivated conditions.

Possible mechanisms underlying repetitive firing

Passive membrane properties

It is possible that passive membrane properties changed during treatment with CM and were partly responsible for the differences between repetitive and nonrepetitive firing cells. Therefore, membrane time constant (τ), input resistance (R_{in}), and resting membrane potential (RMP) were measured in control and CM-treated DRG neurons of various ages. The only significant difference in these parameters was a decrease in input resistance between normal and CM-treated neurons. RMP was near -50 mV for DRG neurons in all the treatment conditions, as measured by either whole-cell or high-resistance microelectrodes (Table 3). There were also no significant differences between the 2 treatment groups for τ.

There were significant differences in the average R_{in} and τ values estimated in whole-cell recordings versus intracellular recordings using high-resistance electrodes. The average R_{in} values for control cells using both methods were 215 and 39 MΩ, respectively, while average τ values were 10.7 and 2.7 msec, respectively. Similar measurements were made under whole-cell recording conditions only (Table 1) for neurons grown in coculture and matched controls.

Specific membrane capacitance (C_m) was derived using the population average of estimated somal surface area and R_{in} to obtain a value of specific membrane resistance that was then divided into the average τ for each treatment condition. Membrane capacitance was normalized in this way to permit comparison between these and other studies (see Discussion). However, independent of the manner in which membrane capacitance was expressed, it did not change with treatment or with method of recording. Thus, essentially all of the difference between values of τ determined from whole-cell recording and high-resistance microelectrode intracellular recording can be accounted for by the difference in R_{in}.

Active membrane properties

Active membrane properties were also examined in control and CM-treated cells of all ages. No significant differences were found in action potential amplitude, spike duration, or threshold between control and CM-treated DRG neurons (see Table 2). A brief plateau on the falling phase of the spike was observed in most DRG neurons (Fig. 1). No differences in action potentials were found between groups of neurons grown for different lengths of time in culture.

The membrane properties of the DRG neurons grown in coculture with skeletal muscle were compared with control cells. No differences were found in the passive and active membrane properties monitored compared to the control cells, except for an increased spike duration in the cocultured DRG neurons. The average spike duration of the DRG neurons in the coculture was 5.0 msec, about twice that of the control cells (Table 1).

In the majority of these experiments, the increased tendency to fire repetitively was monitored as a shift in the behavior of a population of neurons. Thus, it is important to note that although treatment with CM caused an increase in the percentage of neurons showing repetitive firing, some cells in the untreated control group fired repetitively while some neurons in the CM-treated group did not. We compared the range of membrane properties expressed under the various treatment conditions and looked for systematic differences between treatment groups or between single versus repetitively firing neurons

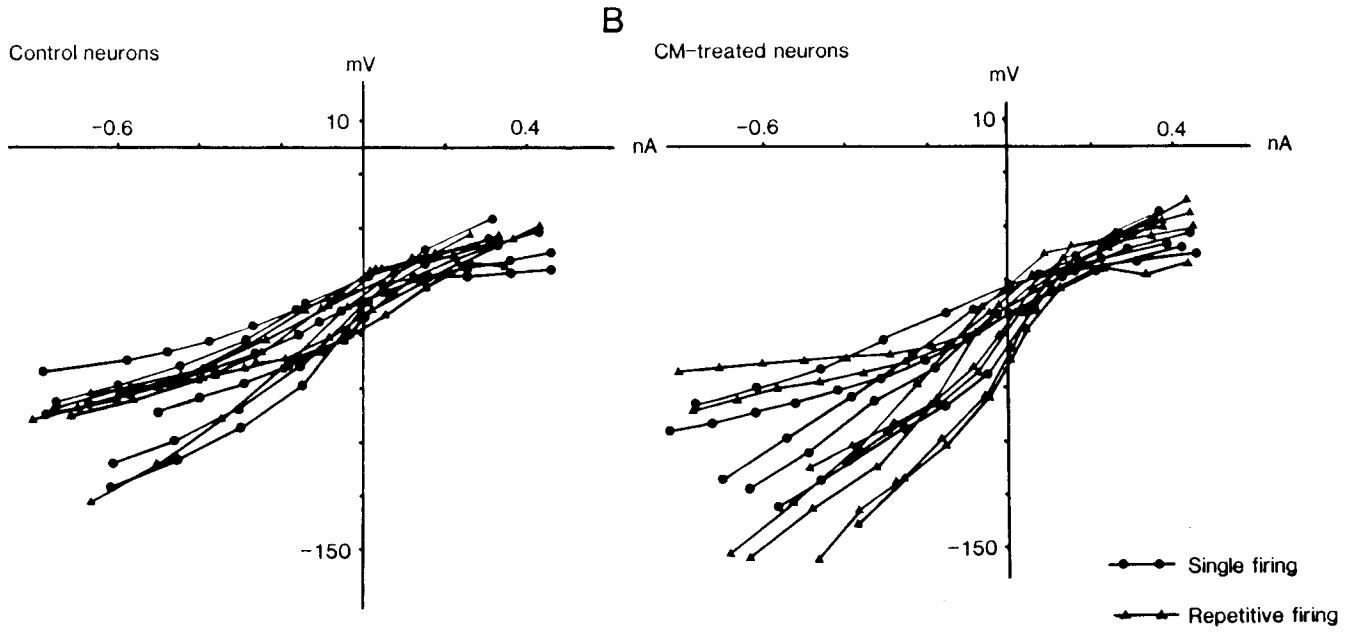

Figure 3. Current–voltage relations of control and CM-treated DRG neurons. Current–voltage relationships of DRG neurons from 4 dissections (15–21 d in culture). *A*, Current–voltage data from 12 control DRG neurons showing a small linear region near the resting potential and membrane rectification on either side. *B*, Current–voltage data from 13 neurons in matched cultures from the same dissections treated with CM. These current–voltage curves also show rectification; however, the slope of the inward rectification is less flattened for many of the CM-treated cells relative to the controls. In both *A* and *B*, *filled circles* represent neurons that fired only single action potentials and *filled triangles* represent neurons that fired repetitive action potentials.

independent of treatment. Data recorded from neurons of the same dissection were compared in order to minimize variability.

Figure 3 shows current–voltage relationships of 25 neurons from 4 dissections recorded over several days. Figure 3*A* has data from 12 control or untreated neurons 15–21 d after plating. There is a small linear region of each curve near the resting potential. Regions of both outward and inward rectification are apparent for both single and repetitively firing neurons. Data from 13 cells treated with CM in matched cultures are given in Figure 3*B*. These current–voltage relationships also show both outward and inward rectification bracketing a linear region similar to the controls. However, the slope of the inward rectification is less flattened for many of the CM-treated cells relative to the control cells in Figure 3*A*. This suggests that less inward rectification is activated under current clamp in CM-treated neurons. Cells that fire both repetitively and singly are included in this group. Cocultured neurons as a group showed less anomalous rectification than controls (not shown), similar to the CM-treated neurons.

One hypothesis for the lack of repetitive firing in control neurons is that inward rectification clamps the membrane potential such that a second spike cannot be generated. This possibility was tested by applying 1 mM CsCl to the soma of the DRG neurons to abolish inward rectification (Mayer and West-

Table 3. Passive membrane properties of DRG neurons recorded under various conditions

	Whole-cell recording				Intracellular recording			
	Resting membrane potential (mV)	Membrane time constant (msec)	Input resistance (MΩ)	Estimated specific capacitance (μF/cm^2)	Resting membrane potential (mV)	Membrane time constant (msec)	Input resistance (MΩ)	Estimated specific capacitance (μF/cm^2)
Control	−52.2 ± 5.8 (89)	10.69 ± 5.3 (89)	215.38 ± 138.6 (65)	2.1†/0.9*	−55.31 ± 6.3 (121)	2.68 ± 1.4 (114)	39.20 ± 21.3 (89)	2.3†/1.0*
Cm-treated	−49.8 ± 8.0 (90)	11.14 ± 5.1 (90)	179.13 ± 117.6 (73)	2.8†/1.2*	−54.02 ± 5.2 (51)	3.23 ± 1.7 (44)	45.69 ± 17.4 (34)	2.7†/1.2*
Heat-inactivated CM	−48.5 ± 8.0 (22)	11.49 ± 5.5 (22)	215.59 ± 108.6 (21)	3.0†/1.3*				

All values except specific capacitance are means ± SD. Values in parentheses are number of cells recorded. Estimated specific capacitance was calculated using the mean area and the mean time constant for each group. Specific capacitance was calculated for rho = 0.0 (†) and rho = 1.3 (*). See Discussion and Brown et al. (1981) for further details. There are 4 instances of significant difference between whole-cell and intracellular recording. These occur in both measurements of time constant and both measurements of input resistance. The change in time constant is probably due to changes in input resistance and is, therefore, not an independent effect. There is also a significant difference between control and CM-treated cells in input resistance using whole-cell recording. Note that although not statistically significant, the effect is opposite using intracellular recording. Note also that the effect seems to be abolished by heat inactivation.

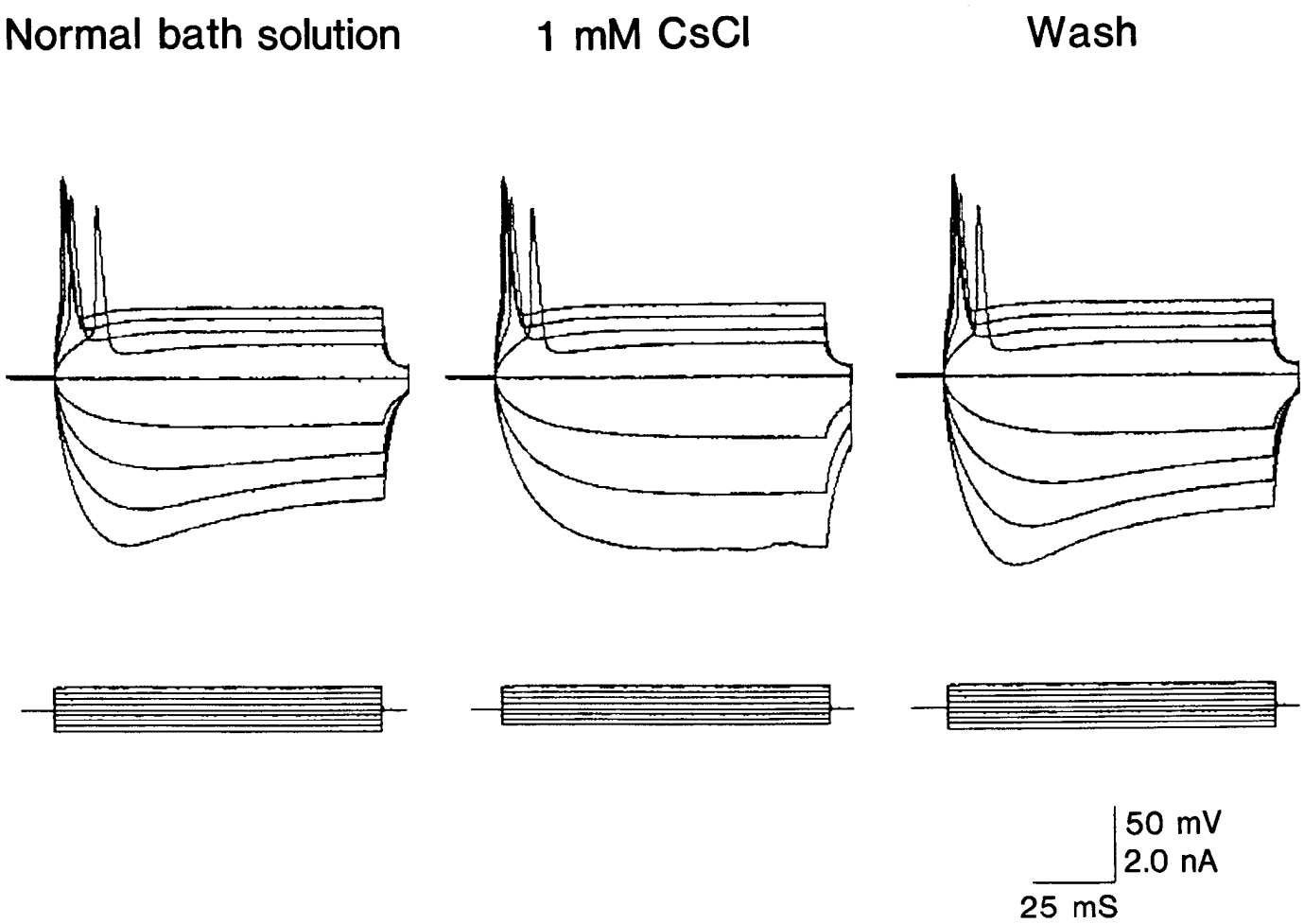

Figure 4. Extracellularly applied 1 mM CsCl blocks inward rectification but does not induce repetitive firing. A series of current steps was applied to a DRG neuron before, during, and after 1 mM CsCl was pressure-applied to the soma. Responses to depolarizing current steps were unchanged by the treatment, while the inward rectification was substantially blocked during CsCl application.

brook, 1983). If this mechanism were important, a nonrepetitively firing cell might be transformed to a repetitively firing cell by applying Cs^+ ions. However, as shown in Figure 4, while the inward rectification was totally abolished by this treatment, there was no change in the tendency of the neurons to fire repetitively ($n = 6$).

Threshold properties of the first and subsequent action potentials

Comparison of parameters of the initial action potential elicited from control and CM-treated neurons during a sustained depolarizing current step revealed no significant differences (Table 2). However, the first action potential in cocultured neurons had significantly increased duration relative to controls. The other significant physiological action of coculture and CM on DRG neurons was to lower the threshold for repetitive firing from an essentially infinite value to a finite value; that is, nonrepetitively firing neurons could not be induced to generate a second action potential even when 3 or 4 times the rheobase (threshold current) for the first action potential was injected. Neurons that did fire repetitively showed increased numbers of action potentials with increased depolarizing current. This observation suggested the possibility of important differences between the first and subsequent action potentials recorded from any given cell. Two approaches were used to evaluate several parameters of the repetitive firing in these cells.

We first determined the distribution of current intensities required to elicit repetitive action potential activity. Neurons in both populations that exhibited repetitive firing were selected. The results are illustrated in Figure 5, which is a cumulative distribution function of the percentage of neurons above threshold for repetitive firing at a given depolarizing current intensity. The distributions for control and CM-treated neurons are not very different.

The second approach was to plot the number of action potentials generated as a function of current intensity, as shown in Figure 6. Control data are plotted for 7 neurons and are indicated by dotted lines. These data are scattered over the entire range of current intensities up to 1 nA. The data from 15 CM-treated neurons are indicated by the solid lines. The latter show heavy clustering below 0.5 nA, even for the highest frequencies achieved, except for 2 cells that fall in a much higher threshold range. In general, the slope of the frequency versus current intensity curves for the CM-treated cells is steeper than for control cells.

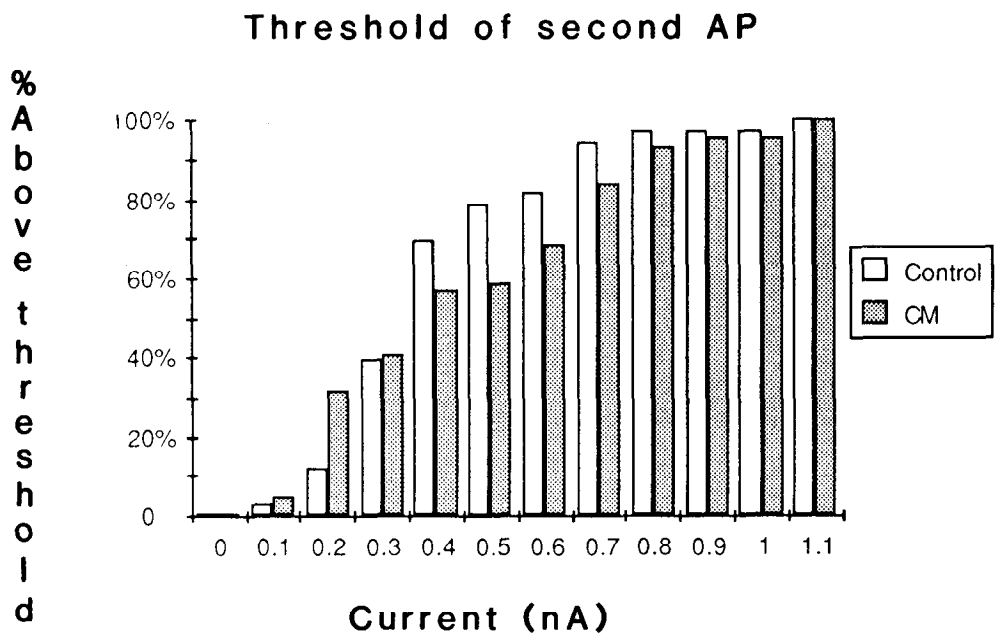

Figure 5. Distribution of current intensities required to elicit secondary action potentials in 33 control neurons and 44 CM-treated neurons. The number of neurons firing more than a single action potential is plotted at a series of different current intensities up to 1.1 nA, which is a maximal value for all the cells that showed repetitive firing.

Ionic properties of the first and subsequent action potentials

The appearance of a finite rheobase for repetitive firing in some cells but not others suggested that the ionic mechanisms of the first and subsequent action potentials might be different. To determine the role of Na^+ and Ca^{2+} conductances in repetitive firing, recordings were made following addition of blockers of specific ion channels or removal of specific ions from the bathing medium. Figure 7*A* shows that sodium-free solution applied by pressure pipette abolished all but the first action potential and increased its duration. This may not be the most critical test of sodium dependence of an action potential since displacing the ambient sodium will be both incomplete and spatially variable. Hence, the susceptibility of the second action potential to sodium-free medium application at the soma and proximal neurites was particularly striking. The first action potential was significantly affected by application of sodium-free medium only when the divalent cation calcium channel blockers Co^{2+} or Cd^{2+} were in the bath, either in the presence or absence of calcium (Fig. 7*B*). In addition, repetitive firing was not blocked when Co^{2+} or Cd^{2+} was added to the bath in the presence of normal sodium (Fig. 7*B*, top panel). Finally, when TTX was pressure-applied to the soma or proximal neurites, only the secondary action potentials were blocked completely (Fig. 7*C*). The effect on the first action potential was variable but usually small and incomplete ($n = 8$). The first action potential generally remained when TTX was applied or divalent cation blockers were in the bath but could then be blocked by the application of sodium-free medium. Table 4 is a summary of results for all of these experiments. These data show that the second and subsequent spikes of a train are not blocked by Co^{2+} or Cd^{2+} but are blocked by TTX or sodium-free conditions. Thus, these repetitively triggered action potentials are clearly different from the first spike, which has an important calcium-dependent component and is less sensitive to TTX and sodium-free medium.

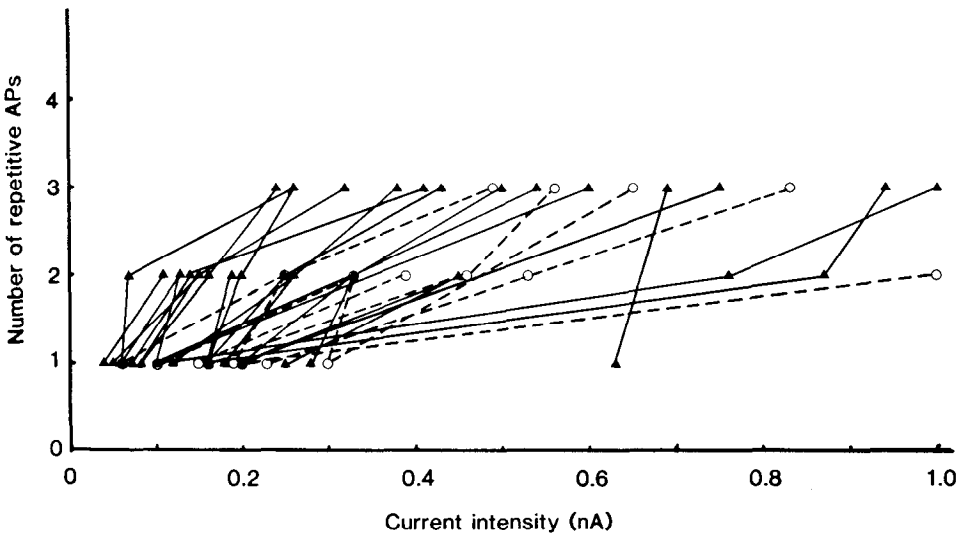

Figure 6. Plot of the number of action potentials fired as a function of current intensity shown for individual neurons, 7 controls and 15 CM-treated. The control data (*dotted line*) are scattered over the entire range of current intensities up to 1 nA. Data from CM-treated neurons (*solid line*) show some clustering below 0.5 nA current stimulus.

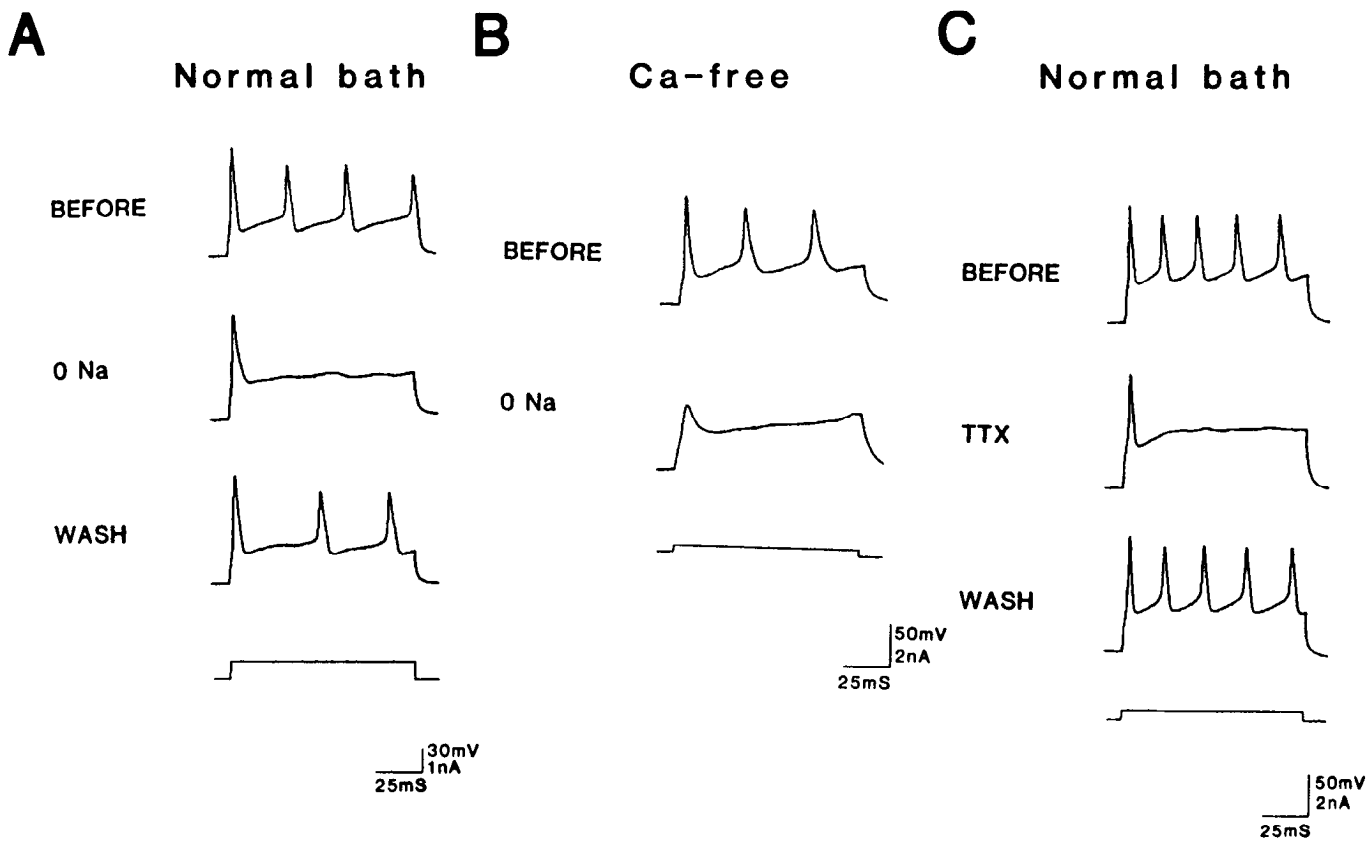

Figure 7. Ionic dependence of repetitive action potential activity induced by coculture and CM. *A*, Effect of Na^+-free medium applied to a DRG neuron grown in coculture with skeletal muscle. A depolarizing current pulse was applied to the neuron before, during, and after perfusion of the soma with Na^+-free solution from a perfusion pipette. *B*, Effect of Na^+-free medium on action potential activity of a cocultured DRG neuron recorded in Ca^{2+}-free medium containing 200 μM $CdCl_2$. The protocol was the same as in *A*. *C*, Effect of 1 μM TTX on repetitive firing in DRG neuron grown in skeletal muscle CM. Protocol same as in *A*. In all cases, the control (BEFORE) solution contained 142 mM NaCl.

Discussion

The results presented here show that the electrical excitability of mouse DRG neurons developing in tissue culture can be altered by association with skeletal muscle via either coculture or a soluble, heat-labile factor(s) present in muscle CM. The effect of CM is seen on DRG neurons after 1–5 weeks in culture and with short or long duration exposure to CM. The action of CM after short exposure was partially reversible. The effect of muscle on excitability of DRG neurons in culture is particularly apparent when the neurons are grown in coculture with muscle and contrasts sharply with the excitability of DRG neurons grown in monoculture (97 vs 17%) or in coculture with spinal cord neurons. Peacock et al. (1973) reported that 47% of the DRG neurons grown with spinal cord showed repetitive firing measured after times in culture ranging from 1 week to several months. This level of firing is consistent with our observation of increased firing at longer times in culture and considerably less than the levels recorded after 3 weeks in culture in CM or at any time in coculture with muscle. Thus, the effect of muscle on DRG neuronal excitability is not mimicked by central neurons or glial cells.

A reasonable conclusion from our results is that a significant proportion of the cells in the dorsal root ganglion maintained *in vitro* respond to soluble factors diffusing from muscle. However, a larger percentage of neurons grown in coculture with skeletal muscle show more repetitive firing than those exposed to CM. Thus, coculture with skeletal muscle appears to have a more dramatic effect on DRG neuron excitability than CM from skeletal muscle. This effect could be due to a larger concentration of the factor(s) in the coculture dishes or nerve–muscle contact could enhance the excitability of neurons in addition to the effect of diffusible substances.

Table 4. Effects of blockers of ion channels on the repetitive firing

	First spike	Subsequent spikes
TTX	– (5/8) ↓ (3/8)	⇊ (8/8)
0 Na^+	– (12/15) ↓ (3/15)	⇊ (15/15)
0 Na^+ + Co^{2+}	↓ (2/4) ⇊ (2/4)	⇊ (4/4)

Symbols: ⇊, blocked; ↓, depressed; –, no effect. Numbers in parentheses are number of cells responding of total cells tested.

Action of the CM

NGF has long been thought to play a role as a retrograde trophic messenger from the target tissue to the neuron (Thoenen and Edgar, 1986). Recent evidence shows the presence of messenger RNA sequences homologous to B NGF-cDNA in embryonic rat muscle (Hulst and Bennett, 1986). In our experiments, rel-

atively low concentrations of NGF were included in all cultures (controls, CM-treated, and skeletal muscle coculture) as a necessary trophic factor. It is possible that the CM from the embryonic muscle cultures was adding significantly to the levels of NGF and that this NGF supplement produced the increased excitability. However, when higher doses of NGF were added and compared with the action of CM, no obvious relationship between DRG excitability and higher doses of NGF was observed. Therefore, it is unlikely that NGF is the heat-labile factor associated with the expression of repetitive firing. Although the identity of the material(s) responsible for increased repetitive firing is still unknown, the heat lability does seem to point to a protein or peptide.

An important issue is whether the increase in repetitive firing with exposure to CM is due to direct or indirect action on DRG neurons. For example, if the CM nonselectively increased neuronal survival, the resulting increase in neuronal density could change the rate of development of excitability, as can occur with spinal cord neurons in culture (Westbrook and Brenneman, 1984). Alternatively, CM could promote selective survival of a population of DRG neurons with a greater likelihood of firing repetitively in response to depolarizing current. Another indirect action could be through the background cells, which might respond to CM and release substances that, in turn, affect excitability of the neurons. The rapid onset of the excitability increase following exposure to CM (1–7 hr) argues against a slow, trophic action of CM, including selection of subpopulations of DRG neurons.

Physiological consequences of skeletal muscle CM

Passive membrane properties

The only significant action of the CM on the passive membrane properties of the DRG neurons was a decrease in input resistance at the soma. This increased leak would tend to prevent firing of action potentials since the threshold for action potential firing is directly related to the leak. Therefore, there was no action of CM on passive membrane properties sufficient to produce the observed changes in firing pattern. However, it is interesting to note that the estimates of the specific membrane capacitance (C_m) of the DRG neurons listed in Table 1 are higher than those reported by other authors (Peacock et al., 1973; Brown et al., 1981). We assumed a spherical, neurite-free anatomy (i.e., ratio of neurite input conductance to soma input conductance, $r = 0$). The electrotonic structure and specific membrane properties of the mouse DRG neurons in tissue culture have been well documented by Brown et al. (1981). According to their calculation, the mean value of r was 1.3; if we use this value, the estimates of C_m of our DRG neurons would be in the range of 0.9–1.3 $\mu F/cm^2$, which agrees with their results.

Action potential duration

Correlation between cell size, conduction velocity, and action potential duration could be a physiological means of classifying subpopulations of mature DRG neurons (Yoshida and Matsuda, 1979; Harper and Lawson, 1985). Furthermore, these parameters could be developmentally regulated (Yoshida and Matsuda, 1979; Fulton, 1986). Variation of action potential duration in DRGs has been attributed to combinations of 2 or 3 empirically separate currents: TTX-sensitive sodium current, TTX-resistant sodium current, and calcium current (Dichter and Fischbach, 1977; Ransom and Holz, 1977; Matsuda et al., 1978; Yoshida et al., 1978; Fukuda and Kameyama, 1980; Fulton, 1986). Thus, the relative contribution of these currents to the depolarized phase of the action potential may be a means of identifying cell type or subpopulation, as well as level of development.

It has been observed that younger neurons (embryonic and early postnatal) have long duration action potentials, with both a TTX-resistant sodium conductance and a calcium conductance contributing to the depolarizing phase. In maturing DRG neurons, TTX sensitivity develops in a subpopulation of the cells with an associated action potential with decreased duration. Such action potentials are associated with A type, fast-conducting neurons (Yoshida and Matsuda, 1979; Harper and Lawson, 1985; Fulton 1986). Transition in culture from minimal TTX sensitivity in all cells to some cells showing shorter-duration action potentials with TTX sensitivity can also be seen after very long times in culture (>100 d) with embryonic DRG neurons (Matsuda et al., 1978). In our experiments, there was no correlation between cell size and action potential duration (unpublished observation), suggesting no tendency to show physiological signs of maturation. This is consistent with the embryonic source of neurons and the relatively short times in culture (<60 d).

The duration of the action potential of DRG neurons in coculture with skeletal muscle was longer than in control and CM-treated cells. This might be caused by the changes of the inward Ca^{2+} current and/or outward K^+ currents. A decrease of the voltage- (or calcium-) dependent outward current could underlie both the change of duration of the action potential as well as the increased tendency to fire repetitively. Blockade of outward current by 4-aminopyridine or tetraethylammonium chloride produces repetitive firing and spike broadening in these neurons (unpublished observations).

Ionic components of the first and subsequent action potentials

TTX alone was never totally effective in blocking the first action potential. The minimal effect of zero sodium applied by perfusion pipette can be partially accounted for by the difficulty in producing a true low-sodium condition with this method of application. Zero sodium in the bath, however, was still not sufficient to block the first action potential, suggesting an important calcium component. When recorded in zero sodium Ringer, the remaining action potential was depressed or blocked by Co^{2+} or Cd^{2+}. This is consistent with other observations of action potential ionic dependence in DRG neurons (Dichter and Fischbach, 1977; Ransom and Holz, 1977; Matsuda et al., 1978; Yoshida et al., 1978).

In contrast, the secondary or subsequent action potentials were insensitive to Cd^{2+} or Co^{2+} and extremely sensitive to either TTX or zero sodium, independent of the mode of application. This is similar to the observations of Yoshida et al. (1978) and Fulton (1986). TTX and zero sodium were also very effective when applied to the neurites at sites distant from the soma. These data may suggest that the site of initiation of the subsequent action potentials is different from that of the first action potentials. It is also interesting to note the differential sensitivity of the first and subsequent action potentials to blockers, even though both have shoulders on the repolarizing phase. Since the shoulder on the falling phase of the action potential is associated with calcium conductance (Dichter and Fischbach, 1977), its appearance on the TTX-sensitive secondary action potentials may be related to the somal location of the recording electrode.

The site of spike initiation may be neuritic, but as it invades the soma, calcium conductance is activated.

The TTX and sodium sensitivity of the subsequent action potentials implies a requirement of a minimum number of sodium channels to allow the membrane potential to surpass threshold for repetitive firing (Hodgkin and Huxley, 1952). It has been shown for spinal cord neurons that over early times in culture there is an increase in the number of sodium channels, which can account for the increased rate of rise of the action potential and is also consistent with the decreased threshold and increase in repetitive firing (MacDermott and Westbrook, 1986). In our experiments, the increased tendency to fire repetitively with time in culture may partially reflect the increase in neurite outgrowth and associated appearance of sodium channels. An increase in repetitive firing in chick DRG neurons with time in culture has also been noted by Handa (1977).

Although a minimum density of sodium channels on the neurites is a prerequisite for repetitive firing and thus for the effect of CM, sodium channels are not necessarily the site of action of CM. Indeed, our data require a more complex explanation than simple lowering of the threshold for action potentials, since the threshold for single spikes is widely distributed regardless of treatment and propensity for repetitive firing. Likewise, the threshold for the production of second and subsequent spikes is not a simple monotonic function of the threshold for the production of the first spike.

Summary

The physiological properties of these embryonic mouse DRG neurons grown in tissue culture, including the absence of correlation between cell diameter and action potential duration, are consistent with the immature state of these neurons. We have shown that at this potentially formative time, these cells show sustained physiological alteration in the presence of embryonic skeletal muscle or the associated CM. The specificity of the action of skeletal muscle compared to the action of other peripheral target tissues remains to be tested. However, the neuritic site of the direct or indirect enhancement of TTX-sensitive sodium channel activation is particularly interesting since changes in neuritic properties might be more relevant to the activity of the maturing neurons *in vivo*. The CM-induced presence of neuritic action potentials in response to somal stimulation suggests that the response of distal neurites to normal sensory input *in vivo* could also be considerably modified.

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