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Role of Substrate and Calcium in Neurite Retraction of Leech Neurons following Depolarization

M. Diana Neely

Department of Pharmacology, Biocenter, University of Basel, CH-4056 Basel, Switzerland

The aim of these experiments was to analyze how depolarization influences neurite outgrowth in leech neurons and what role the substrate and Ca^{2+} play in this response. Neurons in culture were exposed to 60 mM extracellular K^+ for 30 min, which induced retraction of a subset of neurites growing on extracellular matrix substrate (ECM), a response comparable to that observed after electrical stimulation (Grumbacher-Reinert and Nicholls, 1992). After normal medium had been restored, the neurites continued to retract for about 1 hr to approximately 80% of the total starting neurite length. Retraction was reversible and regrowth began after the cells had been in normal medium for about 3 hr. Similar depolarization-induced neurite retraction was observed in both Retzius and anterior pagoda cells. Retraction was inhibited by raised extracellular Mg^{2+}, suggesting a mechanism dependent on calcium. The effect of high K^+ on neurite outgrowth was also influenced by the substrate on which the cells were plated. Cells plated on concanavalin A (ConA) did not retract but continued to extend processes during exposure to high K^+. To understand the different behavior of cells grown on ECM and ConA, the morphology of growth cones was analyzed by scanning electron microscopy. The growth cones of cells grown on ECM and exposed to high K^+ revealed retraction of lamellipodial and filopodial structures. On ConA, however, no differences were observed between growth cones of cells exposed to high K^+ and those of control cells. These results demonstrate the importance of substrate molecules in the responses of growth cones to depolarization and therefore in the differentiation of neurons.

[Key words: leech, neurons, depolarization, substrate, calcium, neurite retraction, growth cone]

A key question in neural development concerns the mechanisms that mediate specificity of axonal outgrowth. In many instances, initial axonal outgrowth is excessive and imprecise, giving rise to an immature network, which must then be remodeled through elimination of superfluous axons to acquire the adult form (Purves and Lichtman, 1980).

The first evidence for local elimination of neuronal processes came from studies of the developing neuromuscular junction (reviewed in Van Essen, 1982). It is now clear, however, that this phenomenon occurs widely in PNS as well as in CNS of vertebrates (Innocenti, 1981; Mariani and Changeux, 1981; Heathcote and Sargent, 1985). A growing body of evidence indicates that process elimination is also important for neural development in invertebrates (Truman and Reiss, 1976; Pipa, 1978; Goodman et al., 1981; Levine and Truman, 1982; Levine et al., 1986; Lnenicka and Murphey, 1989). Thus, several types of neurons of the developing leech grow in many directions and then retract processes (Kuwada and Kramer, 1983; Wallace, 1984; Glover and Mason, 1986; Gao and Macagno, 1987a,b; Jellies et al., 1987; Loer et al., 1987; Baptista and Macagno, 1988; Wolszon and Macagno, 1992).

In vivo studies in vertebrates and invertebrates have indicated that one of the signals influencing axonal outgrowth is neural activity (Wiesel and Hubel, 1963, 1965; Hubel and Wiesel, 1970; Lnenicka and Murphey, 1989; Budnik et al., 1990; Shatz, 1990). Similar observations have been made *in vitro* with cells in defined culture conditions (Anglister et al., 1982; Cohan et al., 1985; Cohan and Kater, 1986; Fields et al., 1990; Grumbacher-Reinert and Nicholls, 1992) where the effects of electrical activity can be mimicked by depolarization with raised extracellular K^+ (Anglister et al., 1982; Campenot, 1984; Sussdorf and Campenot, 1986; Mattson et al., 1988; Robson and Burgoyne, 1989). In some systems influx of Ca^{2+} into the growth cone is involved in depolarization-induced changes in neurite outgrowth (Anglister et al., 1982; Cohan et al., 1987; Mattson and Kater, 1987; Kater et al., 1988; Mattson et al., 1988). In other systems, however, a voltage-dependent influx of Ca^{2+} does not affect neurite extension (Campenot and Draker, 1989; Usowicz et al., 1990; Garyantes and Regehr, 1992).

Extrinsic cues such as molecules in the extracellular matrix (ECM) are also potent regulators of neuronal morphology *in vivo* (for reviews, see Sanes, 1989; Reichardt and Tomaselli, 1991). Neurites of cultured cells are influenced by the substrate with respect to their growth rate, branching pattern, and morphology (Letourneau, 1979; Carbonetto et al., 1983; Chiquet and Acklin, 1986; Chiquet and Nicholls, 1987; Hammarback et al., 1988; Wehrle and Chiquet, 1990; Rivas et al., 1992). Neurites of leech cells plated on ECM are slender, long, and straight, rarely branch, and their growth cones are small. On concanavalin A (ConA), the processes are curved, highly branched, and fasciculated, and their growth cones are broad and flat (Chiquet and Acklin, 1986; Chiquet and Nicholls, 1987; Grumbacher-Reinert, 1989). Leech Retzius cells grown on ECM respond with neurite retraction following electrical stimulation, whereas this response is absent in cells plated on ConA (Grumbacher-Reinert and Nicholls, 1992). In addition to differences in neurite out-

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Correspondence should be addressed to M. Diana Neely, Biocenter, University of Basel, Department of Pharmacology, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

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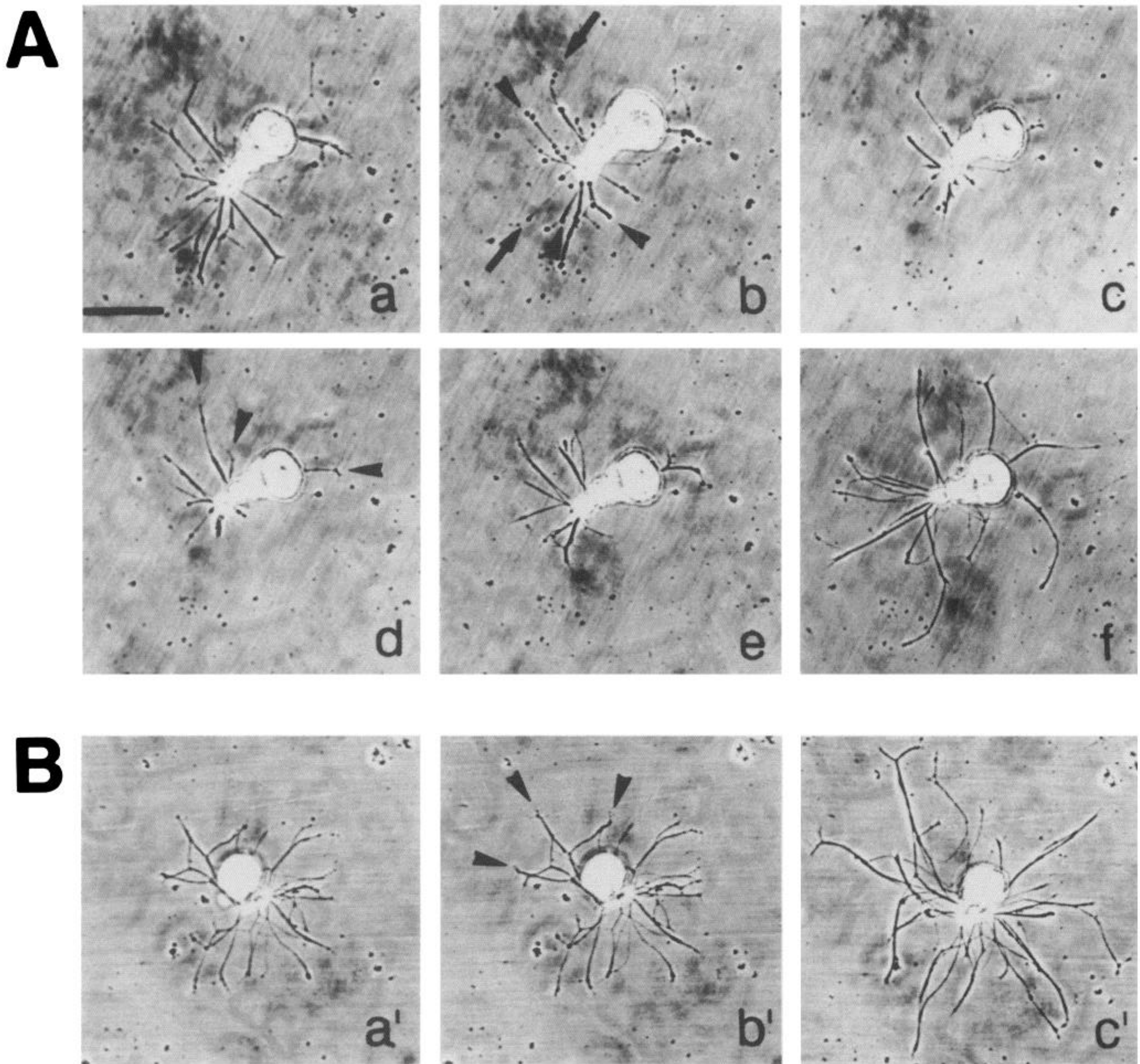


Figure 1. Depolarization induces neurite retraction in leech neurons. *A*, A leech Retzius cell 1 d after plating on ECM (*a*). After 30 min exposure to high K^+ medium (*b*), some of the neurites had retracted extensively (*b, arrowheads*), while others showed partial retraction and rounding up of their growth cones (*b, arrows*). After return to normal medium, retraction continued during the first 1.5 hr (*c*). After 3.5 hr, however, the cell started growing again (*d, arrowheads*) and showed recognizable regrowth after 5.5 hr (*e*). The cell had extended neurites to more than the total starting neurite length after 22.5 hr of recovery (*f*). *B*, A Retzius cell 1 d after plating on ECM (*a'*). No neurite retraction or rounding up of growth cones was observed during the 30 min exposure to Na^+ control medium, but rather the cell continued to extend its neurites (*b', arrowheads*). Extensive growth was observed after 22.5 hr in normal medium (*c'*). Scale bar, 100 μm.

growth, the expression of Ca^{2+} channels on the surface of leech neurons depends on the substrate. Retzius cells plated on ECM show strong voltage-dependent Ca^{2+} currents in their neurites, while those plated on ConA have few Ca^{2+} channels in the processes (Ross et al., 1987, 1988).

The present experiments were designed to analyze the role the substrate plays in depolarization-induced neurite retraction in cultured leech cells. The results show that membrane depolarization by high extracellular K^+ induces changes in growth cone morphology and neurite retraction in cells plated on ECM. These responses were dependent on extracellular Ca^{2+}. In contrast, the morphology of the growth cones and growth of cells on ConA were not altered by the same treatment.

Materials and Methods

Cell culture. The techniques for the identification, isolation, and culture of neurons from the leech CNS have been described in detail elsewhere (Fuchs et al., 1981; Dietzel et al., 1986). In brief, leech (*Hirudo medicinalis*) ganglion chains were dissected out and pinned in a Sylgard-coated dish (Pluess Staufer AG, Oftringen, Switzerland). The capsules enveloping the ganglia were torn open with forceps. The ganglia were incubated with collagenase/dispase (2 mg/ml; Essex Chemie AG, Lucerne, Switzerland) in L-15 medium (GIBCO) supplemented with gen-

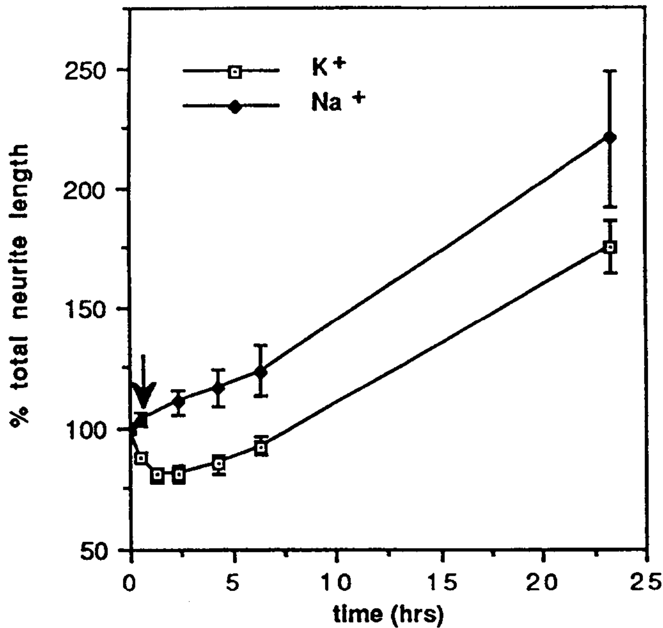


Figure 2. Time course of depolarization-induced neurite retraction. Retzius and AP cells were exposed to elevated K^+ (◻) for 30 min and then returned to normal medium (*arrow*; *n* = 65). Control cells were exposed to Na^+ control medium (◆) (*n* = 15). Total neurite lengths were measured at the times indicated and normalized to the total starting neurite length at time 0. Points represent mean ± SEM.

tamicin sulfate (0.1 mg/ml Garamycin; Essex Chemie AG, Lucerne, Switzerland), glutamine (2 mM), glucose (6 mg/ml), and 2% fetal calf serum (GIBCO) for 1 hr at room temperature. Individual Retzius and anterior pagoda (AP) cells were then removed by suction and plated in microwell culture dishes (Nunc) previously coated either with ConA (2 mg/ml) for 2 hr or with a laminin-enriched EDTA extract of leech extracellular matrix (ECM) (prepared as described in Chiquet et al., 1988) for 4–16 hr. The cells were cultured for 1–6 d at room temperature in L-15 medium with the supplements described above.

The K^+, Ca^{2+}, Mg^{2+}, and Na^+ concentrations in normal L-15 medium are 5.8 mM, 1.3 mM, 1.8 mM, and 145.1 mM, respectively. *High K^+ medium* was prepared by adding isosmolar KCl and $CaCl_2$ solutions to the medium to final concentrations of 60 mM for K^+ and 6 mM for Ca^{2+}. To control for the resulting dilution of the medium, *Na^+ control medium* was prepared by adding isosmolar NaCl (containing 5.8 mM KCl) and $CaCl_2$ solutions to normal medium; final concentrations were 152.4 mM for Na^+ and 6 mM for Ca^{2+}. *High K^+, high Mg^{2+} medium* was prepared by adding isosmolar KCl and $MgCl_2$ to final concentrations of 60 mM and 20 mM for K^+ and Mg^{2+}, respectively.

Determination of neurite length and statistical methods. Cells were observed by phase-contrast microscopy using a Leitz-Diavert microscope with a photo camera as well as by video analysis using a Leitz-Labovert FS microscope equipped with an MTI CCD72 video camera (Dage-MTI, Inc., Michigan City, IN). The software used to measure neurite length was IMAGE-1 (Universal Imaging).

Total neurite length was determined for each cell at different times during the experiment and normalized to the total starting neurite length, which was defined as 100%. Multiple exchanges of medium sometimes caused the neurites to detach from the substrate. In these cells, retraction was usually 100% and the cells did not recover. Therefore, only cells that grew at least 10% during the recovery phase were used for analysis. Seventy-three percent of the cells treated with *high-K^+ medium* and 75% of the cells exposed to *Na^+ control medium* showed more than 10% regrowth during the recovery phase. An unpaired two-tailed Student's *t* test was used for statistical analysis of these experiments. In some experiments cells were scored as retracting or nonretracting. A cell was considered a retracting cell when its neurite length was reduced by at least 10% of the total starting neurite length. For these experiments the 2×2 contingency table test was used for statistical analysis.

Scanning electron microscopy. To prepare the cells for observation with the scanning electron microscope (SEM), they were first fixed for 18 min in 0.4% paraformaldehyde, 0.6% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature. After washing the cells 4 × 5 min with PBS followed by 3 × 3 min with distilled water, they were dehydrated in increasing concentrations of ethanol. Critical point drying was performed in a critical point dryer (Balzers Union) with liquid CO_2. The cells were vacuum coated with a 20–35 nm layer of gold in a sputtering device designed by Balzers Union and observed with a Hitachi S-800 scanning electron microscope at 20 kV.

Results

Time course of depolarization-induced neurite retraction

Leech Retzius and AP neurons that had been in culture for 1–4 d on ECM and were extensively growing were depolarized by raising the extracellular K^+ concentration from 5.8 mM to 60 mM. Intracellular recordings showed that high K^+ depolarized the membrane from −45 mV to −15 mV (data not shown). An exposure of 30 min to raised extracellular K^+ stopped neurite outgrowth and induced neurite retraction. Figure 1*A* shows a Retzius cell 1 d after plating on ECM that had shown extensive neurite outgrowth. Thirty minutes of exposure to 60 mM K^+ resulted in marked changes. Neurite extension ceased completely. Growth cones were observed to round up and neurite retraction began after 15–20 min of exposure to high K^+. After return to normal medium, neurites continued to retract for a further 1.5 hr. The first signs of regrowth appeared after 3.5 hr. After 22.5 hr of recovery the cell had regrown extensively to a total neurite length that exceeded the total starting process length. Cells treated with medium diluted with the same amount of an isosmotic Na^+ solution (Na^+ control medium) did not retract, but continued to extend neurites (Fig. 1*B*).

Figure 2 shows the results for 65 Retzius and AP cells exposed to 60 mM K^+ for 30 min. Maximal retraction was observed in the time between 30 min and 1.5 hr of recovery. At these times the mean total neurite lengths were 80.8 ± 2.6% and 80.1 ± 3.1% of the total starting neurite length, respectively. After 22.5 hr the neurites had grown back to a mean total neurite length of 175.1 ± 11% (*n* = 65). Cells exposed to Na^+ control medium did not retract, but continued to grow (*n* = 15). The difference between total relative neurite length of cells treated with high K^+ medium and of cells in Na^+ control medium was statistically highly significant at all times ($p < 0.01$) except at 23.5 hr ($p < 0.09$).

Exposures to high K^+ longer than 30 min were not more effective. Neurons in culture that were exposed to high K^+ for 4 hr showed mean relative neurite lengths of 85.4 ± 2.7% after 2 hr and 85.3 ± 2.8% after 4 hr exposure. After 22.5 hr of recovery the cells had regrown to a mean neurite length of 116.8 ± 4.9% (*n* = 26) with a growth rate that was comparable to the growth rate of control cells (*n* = 11) (data not shown).

Comparison of depolarization-induced neurite retraction in Retzius and AP neurons

A comparison was made of the responses of Retzius and AP cells exposed to high K^+ medium for 30 min. No significant differences were seen between the two cell types. Maximal retractions were observed to 78.1 ± 3.3% of the total starting neurite length (*n* = 42) for Retzius cells and 79.8 ± 5.7% (*n* = 23) for AP cells. Longer exposures of Retzius cells (*n* = 21) and AP cells (*n* = 22) to high K^+ also showed no differences (data not shown).

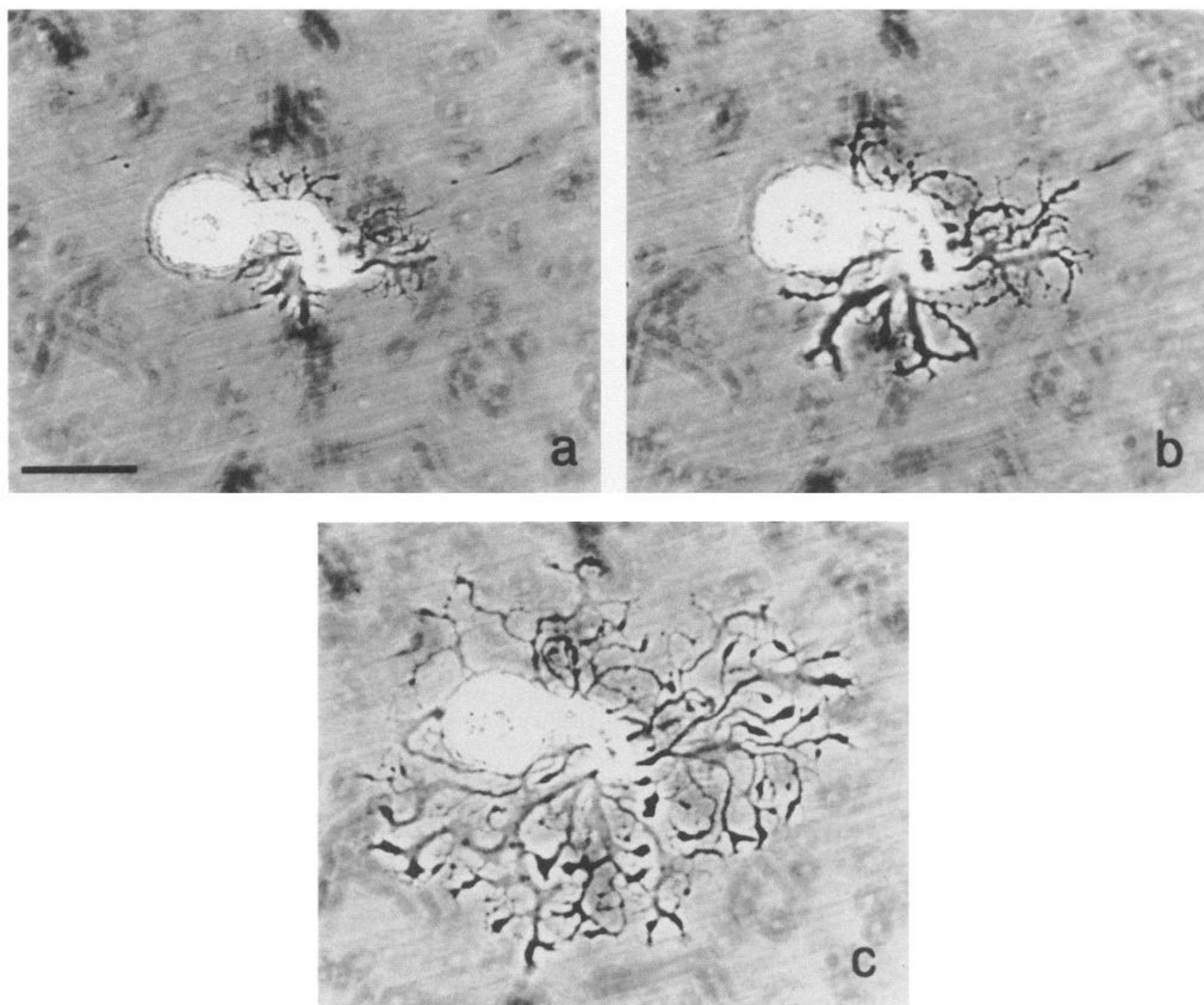


Figure 3. Depolarization does not induce neurite retraction in cells on ConA. An AP cell that had been in culture for 6 hr in normal medium (*a*) was photographed after an additional 4 hr in high K^+ medium (*b*). During this time the cell continued to grow. The medium was then changed back to normal medium and neurite elongation continued during a 14 hr recovery phase (*c*). Scale bar, 100 μm.

Depolarization-induced neurite retraction is substrate dependent

To assess the role played by the substrate in the response of neurons to depolarization, cells plated on ConA were exposed to high K^+. Figure 3 shows an AP cell that was exposed to high K^+ medium for 4 hr. During the high K^+ exposure, extensive outgrowth was observed, which continued during the following 14 hr in normal medium. The same behavior was observed for Retzius and AP cells. Only 7.7% of cells on ConA reacted with neurite retraction after a 2 hr depolarization with high K^+ medium ($n = 26$) (Fig. 4). The response of these cells was significantly different from the observations on cells plated on ECM, where 60.5% showed retraction after a 2 hr depolarization ($n = 43$, $p < 0.01$), but not significantly different from cells on ECM treated with Na^+ control medium ($n = 25$).

To analyze the substrate-dependent behavior at the level of the growth cones, cells plated on ECM or ConA were photographed at the light microscopic level before (Figs. 5*A*, 6*A*) and after (Figs. 5*B*, 6*B*) exposure to high K^+ medium for 30 min and then processed immediately for SEM. Growth cones of control cells plated on ECM were small, 5–10 μm in diameter, and had numerous filopodia (Fig. 5*C*). On neurites that had retracted after high K^+ exposure, a retraction bulb and retraction fiber were observed (Fig. 5*D*). Even on neurites that had not retracted, many of the growth cones were rounded up and the number of filopodia was reduced (Fig. 5*E*). Growth cones of cells plated on ConA in control medium were more spread out than growth cones on ECM (Fig. 6*C*). No differences could be observed between growth cones on ConA exposed to high K^+ medium (Fig. 6*D,E*) or to Na^+ control medium (Fig. 6*C*). No loss of filopodia was apparent, nor was there any rounding up of the lamellipodia in depolarized cells (Fig. 6*D,E*).

Neurite retraction depends on Ca^{2+} entry

Calcium channel distribution on the growth cones of leech neurons depends on the substrate. Neurites of cells plated on ECM show Ca^{2+} currents after electrical stimulation, whereas those growing on ConA have no measurable Ca^{2+} currents (Ross et al., 1987, 1988). Several studies have suggested that experi-

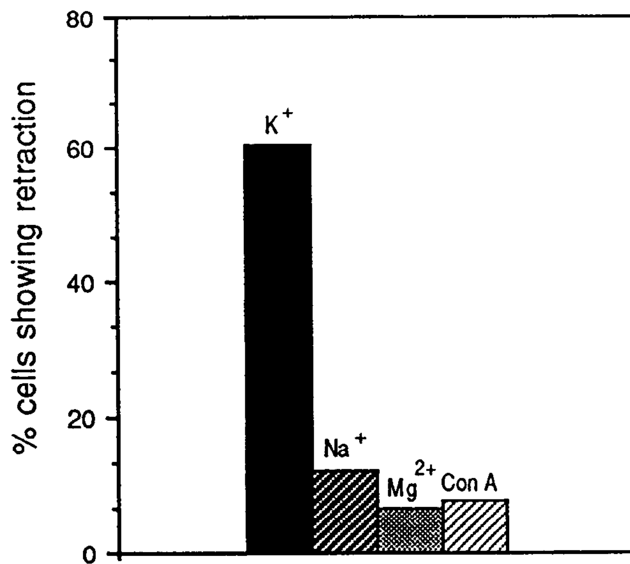


Figure 4. Retraction of leech neurons after 2 hr of depolarization. Retzius and AP cells cultured on ECM were exposed to either high K^+ medium ($n = 43$), Na^+ control medium ($n = 25$), or high K^+, high Mg^{2+} medium ($n = 15$). Of the cells exposed to high K^+ medium, 60.5 % showed at least 10% retraction, whereas only 12.0% of the cells exposed to Na^+ control medium and 6.7% of cells exposed to high K^+, high Mg^{2+} medium responded with retraction. Of cells growing on ConA, 7.7% showed at least 10% retraction after exposure to high K^+ medium ($n = 26$). See Results for statistic analysis.

mentally induced neurite retraction in cultured cells might be mediated by raised intracellular Ca^{2+} (Cohan et al., 1987; Mattson and Kater, 1987; Kater et al., 1988; Mattson et al., 1988). Blocking Ca^{2+} channels reduced, but did not abolish, retraction in leech Retzius cells that had been electrically stimulated (Grumbacher-Reinert and Nicholls, 1992).

To address the question of the role that Ca^{2+} plays in the depolarization-induced neurite retraction of leech neurons, I analyzed the effect of high extracellular Mg^{2+} on their response to high extracellular K^+. Elevated Mg^{2+} was chosen as a Ca^{2+} channel blocker since dihydropyridines do not affect leech channels and inorganic ions such as Cd^{2+} and Mn^{2+} damage leech neurons if applied for longer than a few minutes. Complete elimination of Ca^{2+} from the medium was not possible, because this damages leech cells (J. G. Nicholls, unpublished observation). Mg^{2+} (20 mM) in the medium blocks Ca^{2+} influx into leech neurons reversibly and without damaging the cells or producing other effects (Muller, 1981; Henderson, 1983). Figure 7 shows that 20 mM Mg^{2+} inhibited the K^+-induced neurite retraction and the cells continued to grow ($n = 13$), whereas the mean maximal neurite retraction after 30 min exposure to normal high K^+ medium was 21.5 ± 6.0% of the total starting neurite length ($n = 18$). The difference of total relative neurite length of cells treated with high K^+ medium and of cells exposed to high K^+, high Mg^{2+} medium was statistically significant at 0.5 hr ($p < 0.016$), and highly significant at 1.5 hr, 2.5 hr, and 4.5 hr ($p < 0.01$).

Cells exposed for longer times to high K^+, high Mg^{2+} medium also behave differently from cells exposed to high K^+ medium for the same time. Only 6.6% of the cells treated with high K^+, high Mg^{2+} medium for 2 hr responded with retraction ($n = 15$), compared to 60.5% of the cells exposed to *high K^+ medium* for the same length of time ($n = 43$) (Fig. 4, $p < 0.01$).

The effect of high K^+, high Mg^{2+} medium on the growth cone morphology of cells on ECM was analyzed. Growth cones of an AP cell on ECM were photographed before (Fig. 8*A*) and after (Fig. 8*B*) a 30 min exposure that caused as expected no retraction; rather the neurites continued to elongate (Fig. 8*B*, arrowheads). No loss of filopodia or rounding up of growth cones was observed (Fig. 8*C*). Many of the growth cones showed a flattened morphology (Fig. 8*C*), when compared to control cells on ECM (Fig. 5*C*).

Discussion

This study extends work on the substrate control of neurite outgrowth in cultured leech neurons (Chiquet and Acklin, 1986; Chiquet and Nicholls, 1987). In earlier studies the overall effects of electrical stimulation and the influence of the substrate were analyzed on whole cells (Grumbacher-Reinert and Nicholls, 1992). To follow with more precision the mechanisms by which substrate influences neurite outgrowth, it is important to know which cell organelle is affected and how it responds to stimulation on different substrates. The principal emphasis here is on responses of neuronal growth cones to depolarization including detailed morphological analysis on two different substrates.

For cells grown on ECM, depolarization with high K^+ led first to a rounding up of growth cones, loss of filopodia, and then to a pronounced retraction of a subset of neurites. This effect was reversible and the cells started to regrow about 3 hr after removal of the K^+. Although some variability in the strength of the response was observed in different cell cultures, the variability was not correlated with time in culture or amount of neurite outgrowth (data not shown). This was different in cells that were electrically stimulated, where only cells that had already grown extensively were sensitive to the effects of electrical activity (Grumbacher-Reinert and Nicholls, 1992). On ConA, however, depolarization had no effect on the morphology of the growth cones and indeed the neurons continued to grow indistinguishably from control cells.

These experiments were performed with two different types of leech neurons, the Retzius and AP cells. Both cell types showed the same degree and timing of retraction and recovery on ECM and continued to grow on ConA after depolarization. Therefore, the response to changes in the membrane potential seems not to be cell type specific.

What could be the mechanism for this substrate-dependent response? The differences are unlikely to be due to differential adhesiveness of the two substrates. Leech neurons, when given a choice of ECM and ConA, extend processes on both substrates with equal efficiency and with substrate-specific morphology (Chiquet and Acklin, 1986; Grumbacher-Reinert, 1989). If in fact the neurons adhere better to ConA than to ECM, then the increased adhesion does not affect the efficiency of neurite extension. Other studies have shown that strength of adhesion does not necessarily correlate with the extent of growth (Tomaselli et al., 1986; Gundersen, 1987). For example, many neurons stick strongly to polylysine, but show very reduced neurite outgrowth on this substrate (Chiquet and Acklin, 1986; Chiquet and Nicholls, 1987; Wehrle and Chiquet, 1990). The observation that cells on ConA continue to extend neurites in the presence of high extracellular K^+ suggests that whatever mechanism leads to a cessation of neurite outgrowth of cells on ECM is not functional in cells on ConA.

The ability to detect a signal depends on whether appropriate receptors and channels are expressed and functional within a

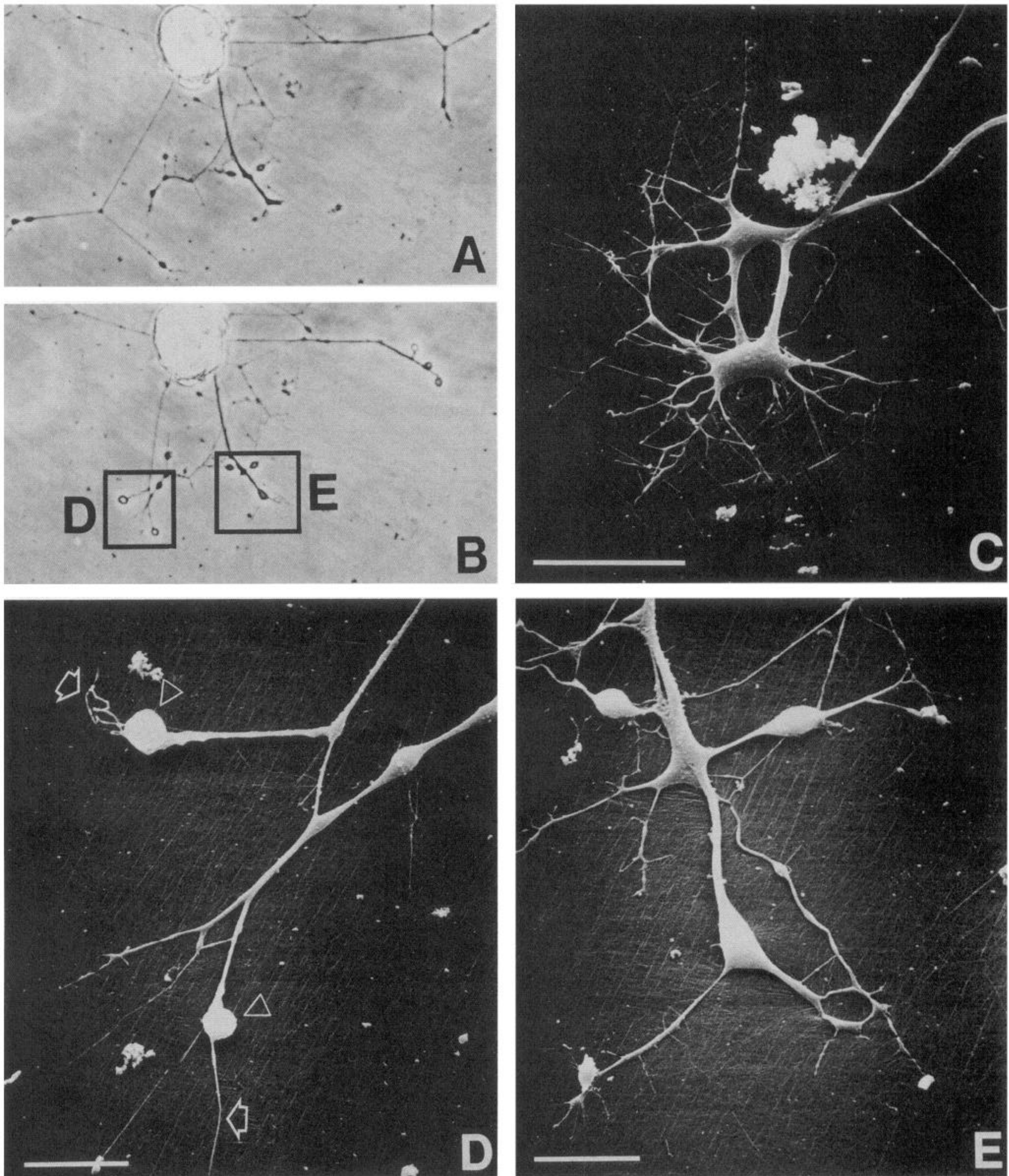


Figure 5. Morphology of growth cones of leech neurons plated on ECM after exposure to high K^+ medium. An AP cell that had been in culture for 1 d was photographed before (*A*) and after (*B*) a 30 min exposure to high K^+ medium. On neurites that had retracted, a retraction bulb (*D, arrowheads*) and retraction fiber (*D, arrows*) were usually observed. Characteristic growth cone changes of neurites that had not retracted included rounding up of lamellipodia and loss of filopodia (compare *E* and *C; C* shows a growth cone in Na^+ control medium). *D* and *E* are SEM views of the *boxed regions* in *B*. Scale bars, 10 μm.

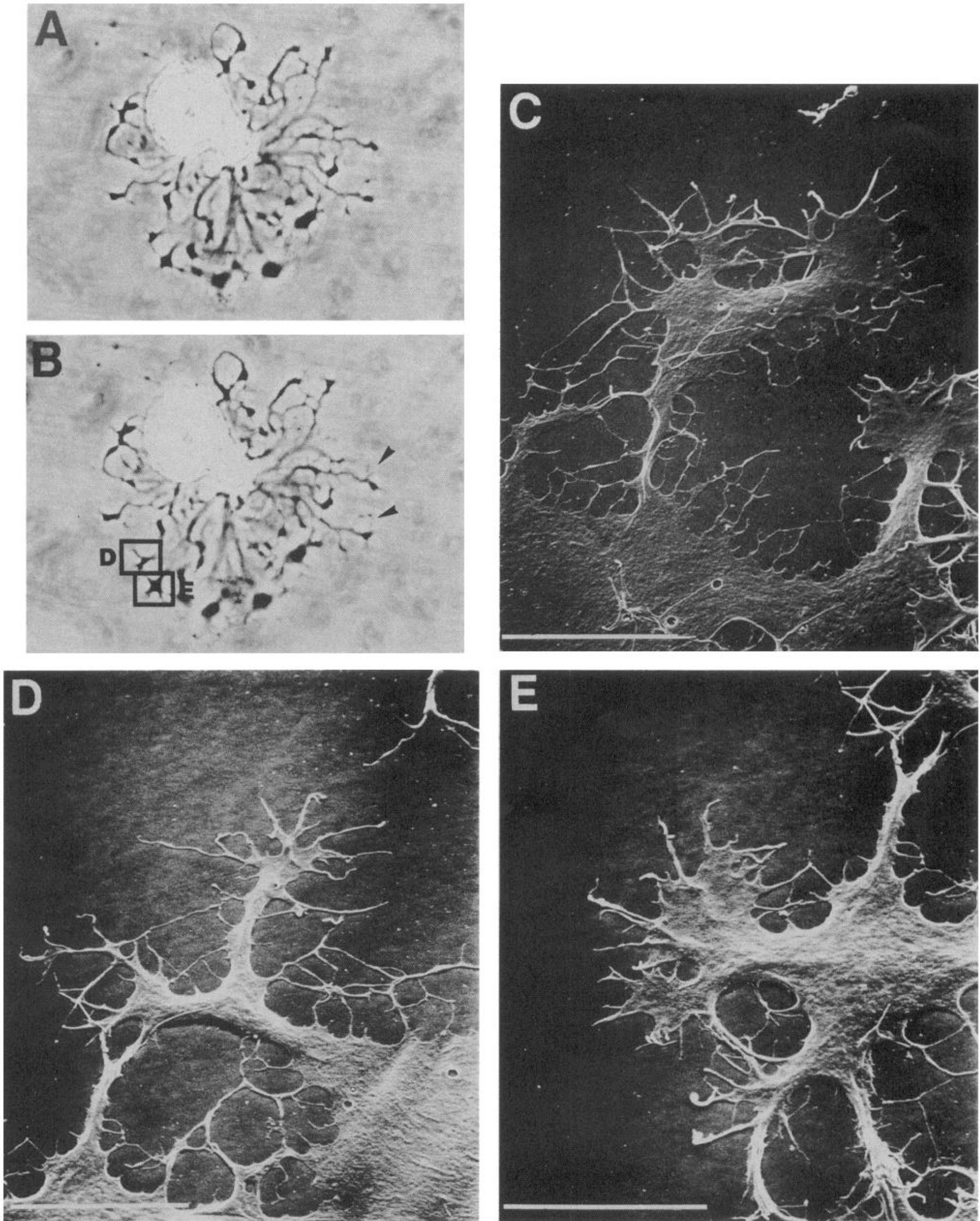
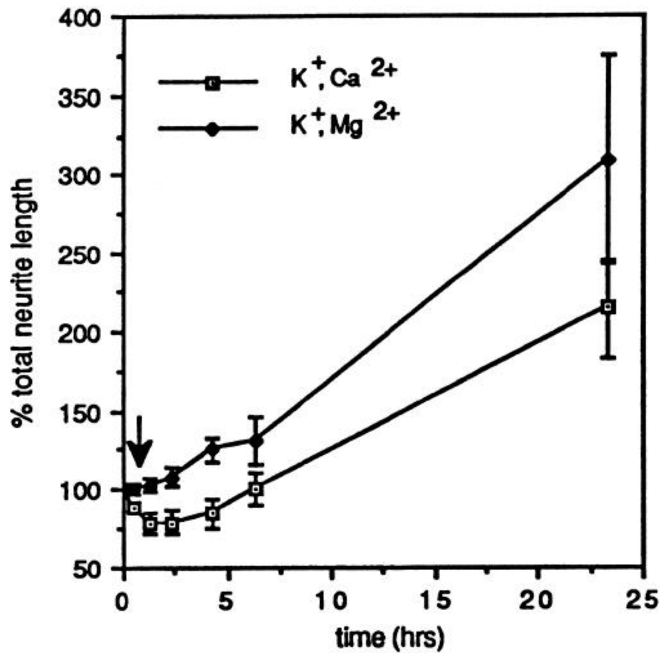


Figure 6. Morphology of growth cones of leech neurons plated on ConA and exposed to high K^+. An AP neuron that had been in culture for 1 d was photographed before (*A*) and after (*B*) a 30 min exposure to high K^+ medium. Some of the neurites grew during this treatment (*B, arrowheads*). No differences were observed between growth cones exposed to high K^+ (*D, E*) or Na^+ control medium (*C*). Broad flat growth cones, extensive lamellipodia, and many filopodia were routinely observed. *D* and *E* are SEM views of the *boxed regions* in *B*. Scale bars, 10 μm.



particular growth cone. I have shown here that depolarization-induced neurite retraction depends on the influx of extracellular Ca^{2+}. If this influx was blocked by high extracellular Mg^{2+}, the typical changes in growth cone morphology and neurite retraction were not observed. To the contrary, some of the neurites extended farther and their growth cones had a slightly broader appearance. Retzius and AP cells growing on ECM show pronounced Ca^{2+} influx in their neurites following action potentials. By contrast, little or no Ca^{2+} entry is detectable in processes of Retzius or AP cells growing on ConA (Ross et al., 1987, 1988). These results suggest that depolarization by high extracellular K^+ of cells on ECM induces a Ca^{2+} influx through voltage-

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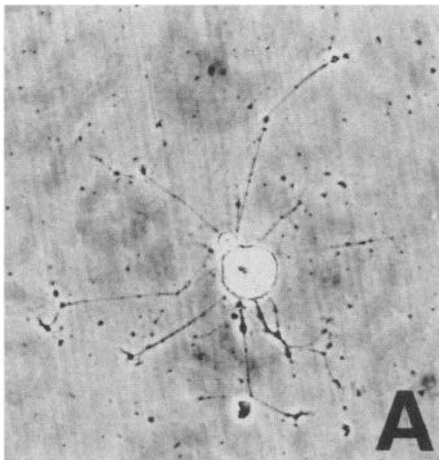
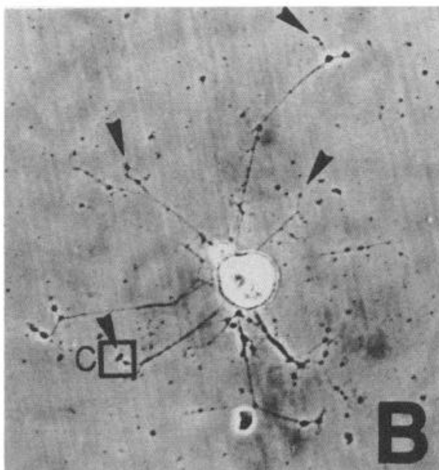
Figure 7. Neurite retraction induced by high K^+ is dependent on Ca^{2+} influx. Leech neurons (Retzius and AP; $n = 13$) exposed to high K^+, high Mg^{2+} medium (◆) for 30 min and then returned to normal medium (*arrow*) did not respond with a change of growth rate and neurite retraction. Cells exposed to high K^+ medium (▣) showed maximal retraction to 78.5% of the total starting neurite length ($n = 18$). Each point represents mean ± SEM.





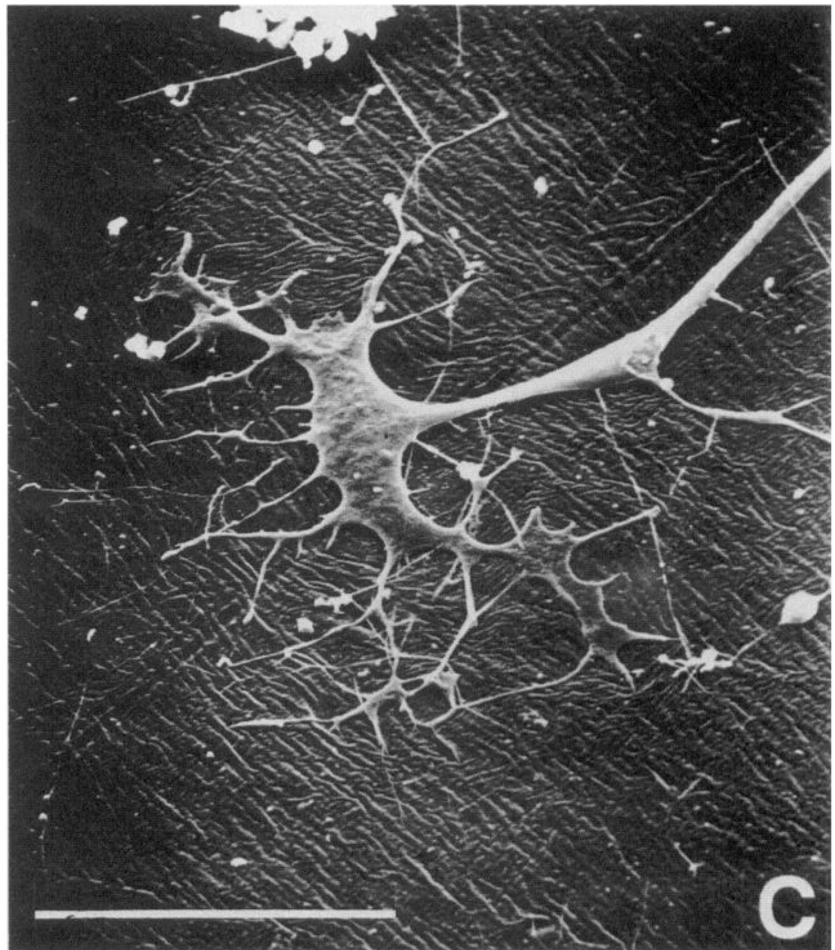


Figure 8. Morphology of growth cones on ECM exposed to high K^+, high Mg^{2+} medium. An AP cell that had been in culture for 1 d was photographed before (*A*) and 30 min after (*B*) exposure to high K^+, high Mg^{2+} medium. Some of the neurites extended farther during this treatment (*B, arrowheads*). No retraction bulbs and fibers, and no loss of filopodia or rounding up of growth cones were apparent (*C*). The growth cones of these cells often had a more flattened broad appearance (*C*), when compared to control cells on ECM (Fig. 6*C*). *C* is an SEM view of the *boxed region* in *B*. Scale bar, 10 μm.

dependent Ca^{2+} channels. This Ca^{2+} influx may then be associated with neurite retraction. In cells on ConA, with no or only few Ca^{2+} channels and far less Ca^{2+} entry, no retraction is observed.

Ca^{2+} has been implicated in the regulation of neurite outgrowth in several other systems, although the results are contradictory. In some systems elevation of intracellular Ca^{2+} causes cessation of neurite outgrowth and retraction (Mattson and Kater, 1987; Mattson et al., 1988); in other systems it enhances neurite outgrowth (Anglister et al., 1982; Suarez-Isla et al., 1984) or has no effect (Campenot and Draker, 1989; Usowicz et al., 1990; Garyantes and Regehr, 1992). The range and optimal levels of calcium concentrations seem very different for different systems (Mattson and Kater, 1987).

Exactly how Ca^{2+} acts to change neurite outgrowth behavior is unclear for leech as well as other types of neurons. Ca^{2+} affects the assembly states of both microtubules (Schliwa et al., 1981) and microfilaments (Adelstein and Eisenberg, 1980; Lankford and Letourneau, 1989) and could therefore act directly upon the cytoskeleton. The earliest changes observed in this study were rounding up of growth cones and loss of filopodia. Since microfilaments are the main cytoskeletal component of the filopodia and the peripheral areas of the growth cone, they might well be a target for entering Ca^{2+}. Experiments to test if microfilaments and other cytoskeletal components are affected by depolarization are in progress.

It is, however, also possible that Ca^{2+} acts more indirectly by influencing other second messenger systems. Recent studies have suggested an involvement of other second messengers in the Ca^{2+}-dependent change of neurite outgrowth (Reboulleau, 1986; Polak et al., 1991). High K^+ has been shown to induce a change in the expression of cytoskeletal proteins (Riederer et al., 1992). The influence of the substrate on K^+-induced change in gene expression is not known, but the substrate under normal conditions has been shown to affect gene expression (Acheson et al., 1986; Werb et al., 1989; Ben-Ze'ev, 1991). The delay of 20 min between the start of depolarization and the beginning of the first changes in growth cone morphology suggests that events subsequent to Ca^{2+} entry are complex and involve several steps.

There remains the question of whether the observed change in neurite outgrowth after depolarization of adult leech neurons in culture is physiologically significant. The response of leech neurons to depolarization with high extracellular K^+ observed in these studies was in many aspects similar to the response of Retzius cells stimulated with extracellular electrodes (Grumbacher-Reinert and Nicholls, 1992). The use of high extracellular K^+ to depolarize cells has several advantages over electrical stimulation. Stimulation of cells with extracellular tungsten electrodes is difficult to perform, and only a limited number of cells can be stimulated at a time. Neurite retraction is a phenomenon also observed during the normal development of the leech nervous system (Wallace, 1984; Gao and Macagno, 1987a,b). In the adult animal, leech neurons start sprouting after injury and target removal and stop growing when they reach their target (Scott and Muller, 1980; Mason and Muller, 1982). Leech neurons that have been removed from the ganglion and put into culture also start to sprout extensively. In addition, leech neurons *in vitro* retain many of their *in vivo* properties (Fuchs et al., 1981; Arechiga et al., 1986; Vyklicky and Nicholls, 1988; Stewart et al., 1989; Acklin and Nicholls, 1990). This substrate-dependent response of leech neurons in culture to depolarization therefore provides us with a valuable system to study one mechanism by which the substrate influences neuronal differentiation.

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