

# Neuronal Galvanotropism is Independent of External $\text{Ca}^{2+}$ Entry or Internal $\text{Ca}^{2+}$ Gradients

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Received 21 February, 2000; accepted 30 June, 2000

**ABSTRACT:** The mechanism by which growing neurites sense and respond to small applied electrical fields is not known, but there is some evidence that the entry of  $\text{Ca}^{2+}$  from the external medium, with the subsequent formation of intracellular  $\text{Ca}^{2+}$  gradients, is important in this process. We have employed two approaches to test this idea. *Xenopus* spinal neurites were exposed to electrical fields in a culture medium in which  $\text{Ca}^{2+}$  was chelated to very low levels compared to the normal extracellular concentration of 2 mM. In other experiments, loading the neurites with the calcium buffer, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), disrupted the putative internal  $\text{Ca}^{2+}$  gradients, and the effects on the electrical response were determined. Fields of 100 mV/mm were applied for 12 h, and no difference was detected in the cathodal turning response between the treated neurites and the untreated controls. Using the Differential Growth Index

(DGI), an asymmetry index, to quantitate the turning response, we recorded DGIs of  $-0.64$ ,  $-0.65$ , and  $-0.62$  for control cells, cells in  $\text{Ca}^{2+}$ -free medium, and cells preloaded with BAPTA, respectively. Furthermore, we detected an increase in neurite length for those neurons cultured in  $\text{Ca}^{2+}$ -free medium; they were 1.5–1.7 times as long as neurites from neurons cultured in normal  $\text{Ca}^{2+}$  medium. Likewise, we found that BAPTA-loaded neurites were longer than control neurites. Our data indicate that neuronal galvanotropism is independent of the entry of external  $\text{Ca}^{2+}$  or of internal  $\text{Ca}^{2+}$  gradients. Both cell-permeant agonistic and antagonistic analogs of cyclic 3',5'-adenosine monophosphate (cAMP) increased the response to applied electrical fields. © 2000 John Wiley & Sons, Inc. *J Neurobiol* 45: 30–38, 2000

**Keywords:** calcium; galvanotropism; neurite outgrowth; neurite length

## INTRODUCTION

An important unresolved problem in neurobiology is the mechanisms of patterning of the developing nervous system. The intricate pathways of neuronal circuits have been traced and mapped throughout embryological nervous systems as well as in adult nervous systems. However, there is much to be understood about how developing neurites know which pathway to take. Studies on neurite outgrowth suggest that a number of factors, including protein distribution, cell-cell attachment, and electric fields, may play

roles in this process. These guidance cues, particularly electric fields, have been well studied *in vitro* and have been found to have significant effects on neurite outgrowth. Furthermore, applied electric fields influence neurite growth rates as well as orientation (Hinkle et al., 1981; Hotary and Robinson, 1990; Jaffe and Poo, 1979; McCaig and Robinson, 1982; McCaig and Erskine, 1995; Patel and Poo, 1982). Neurons from *Xenopus* embryos have been shown to respond to fields of less than 10 mV/mm by growing toward the cathode (Hinkle et al., 1981). Not only do these neurons respond by orienting their neurites toward the cathode in an applied field, they also extend longer processes in the presence of a field (McCaig, 1989a). The mechanisms responsible for the electric field me-

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diated growth response (neuronal galvanotropism) are not known.

Studies on both amphibian and avian embryos have shown that electric fields exist in the developing embryo. The cells of the outer epithelium of the developing frog embryo are arranged such that sodium is electrogenically transferred from the outside, generating an inwardly positive transepithelial potential (TEP). This potential is the result of  $\text{Na}^+$  diffusion into the epithelial cells through channels localized at the apical surface, and the outward movement of sodium into the basolateral spaces via localized  $\text{Na}^+/\text{K}^+$ -ATPases. There are localized, developmentally programmed leaks through the epithelium that allow current to flow to the outside and subsequently re-enter the general epithelium. In this phenomenon lies the basis for our interest in neuronal development in an electric field. As cells migrate or grow within the embryo, they will be exposed to any fields generated by the mechanism described above.

In the case of *Xenopus*, it has been shown that an amiloride-sensitive TEP of more than +50 mV develops by the early neurula stage (McCaig and Robinson, 1982; Rajnicek et al., 1988). Furthermore, the blastopore acts as a leak, which results in an intense outward current that reaches a density of  $100 \mu\text{A}/\text{cm}^2$  by stage 20, creating fields as large as 40 mV/mm. This is considerably larger than the minimum field required to produce a directional effect on growing neurites and neural crest cells in culture (Hotary and Robinson, 1994; Robinson and Stump, 1984). The disruption of the endogenous fields produced severe developmental abnormalities in *Xenopus* embryos including the failure of the neural tube to close, reduced head and eye development, ectopic cement glands, extrusion of cells from the blastopore, and a failure to form functional cilia (Hotary and Robinson, 1994). A similar situation exists in the chick embryo (Hotary and Robinson, 1992; Hotary and Robinson, 1990).

Calcium has been widely thought to play a controlling role in neurite outgrowth. Imaging of the distribution of  $\text{Ca}^{2+}$  in the growth cone has revealed the existence of  $\text{Ca}^{2+}$  gradients (Cohan et al., 1987) in *Helisoma* neurons. Those experiments gave rise to the idea that there is a window of  $\text{Ca}^{2+}$  concentration in the growth cone that is required for growth, and concentrations above or below that level are inhibitory (Kater and Mills, 1991). Gundersen and Barrett (1980), who found that chick dorsal root ganglion neurons turned toward a region of higher external  $\text{Ca}^{2+}$  in the presence of A23187, a calcium ionophore, presented evidence that gradients of  $\text{Ca}^{2+}$  could be

involved in neurite guidance. McCaig (1989b) showed that  $\text{La}^{3+}$  and  $\text{Co}^{2+}$ , calcium channel blockers, arrested growth and caused retraction of embryonic *Xenopus* spinal neurites, suggesting that  $\text{Ca}^{2+}$  entry from the extracellular medium was necessary for growth and maintenance of neurite integrity.

On the other hand, it has been reported that *Xenopus* neurites do not require external  $\text{Ca}^{2+}$  for growth (Bixby and Spitzer, 1984; Robinson and Muncy, 1986), and indeed, extend longer neurites in the absence of external  $\text{Ca}^{2+}$  (Gu and Spitzer, 1995). Furthermore, if the neurites are loaded with the  $\text{Ca}^{2+}$  chelator, BAPTA, in order to collapse intracellular  $\text{Ca}^{2+}$  gradients, growth is not impaired (Gu and Spitzer, 1995). This has led to the suggestion that  $\text{Ca}^{2+}$  acts as a neuronal stop signal, causing growth to cease when growth cone  $\text{Ca}^{2+}$  reaches a certain level (Gomez and Spitzer, 1999). In the case of neuronal galvanotropism,  $\text{Ca}^{2+}$  has also been thought to have an important involvement. It has been suggested that the redistribution of critical membrane components, such as calcium channels, may be redistributed via "lateral electrophoresis" (Poo and Robinson, 1977; Poo et al., 1979), leading to the formation of lateral cytoplasmic gradients of  $\text{Ca}^{2+}$  (Robinson, 1985). Another means by which applied electrical fields might affect  $\text{Ca}^{2+}$  distribution is by opening voltage-gated channels on the cathode-facing side, which will be depolarized. Evidence for this was presented by Bedlack et al. (1992) and Davenport and Kater (1992), who found that N1E-115 mouse neuroblastoma cells and *Helisoma* neurons formed lateral  $\text{Ca}^{2+}$  gradients in response to large electric fields.

In view of the inconclusive nature of the evidence about calcium's role in neuronal growth and galvanotropism, we have examined the effect of the rigorous absence of extracellular  $\text{Ca}^{2+}$  on the response of embryonic *Xenopus* neurons to applied fields. We have also determined the effects on galvanotropism of loading the neurons with BAPTA prior to field application. We find that galvanotropism is independent of these treatments. Additionally, we confirm the observations of Gu and Spitzer (1995), that the neurons extend longer processes when subjected to these treatments.

Furthermore, we have explored the effects of cyclic nucleotides on nerve galvanotropism. Unlike Song et al. (1998), who found that *Xenopus* neurites would reverse their response to soluble guidance factors by altering cyclic nucleotide levels, we did not measure a reversal in the turning response in reaction to similar alterations of cyclic nucleotide concentrations.

## METHODS

Cells were cultured according to the methods described in Hinkle et al. (1981), with certain modifications. Briefly, neural tubes were dissected from stage 20–22 *Xenopus laevis* embryos in Steinberg's solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.83 mM  $\text{MgSO}_4$ , 1.16 mM  $\text{CaCl}_2$ , Mallinckrodt, Paris, KY, 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES); pH 7.8). These tubes were deaggregated in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Steinberg's solution. Deaggregated cells were pipetted into the well of the culture dish apparatus (described below), with alternating drops of cells, suspended in a minimal amount of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Steinberg's and culture medium [Steinberg's solution supplemented with 2% penicillin streptomycin (Sigma, St. Louis, MO) and 1% fetal bovine serum (Sigma)], until the cells had dispersed throughout the length of the channel. The supplemented Steinberg's solution was adequate to maintain these embryonic neurons in a healthy state for the duration of the experiments, as the cells had considerable reserves of yolk.

### Cells Cultured in $\text{Ca}^{2+}$ -Free Medium

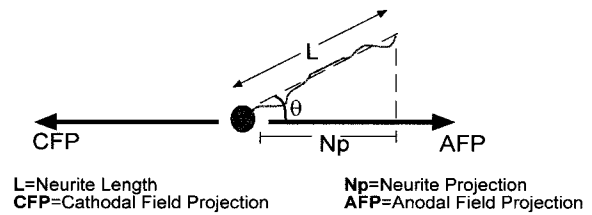
Cells were initially cultured in culture medium for 1 h, to allow cells to adhere to the culture dishes. After 1 h, normal culture medium was replaced with  $\text{Ca}^{2+}$ -free culture medium in which 1.5 mM  $\text{MgCl}_2$  was used in the place of calcium and 2 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was added. Taking into account the amount of calcium present in the serum, we calculated that the final  $\text{Ca}^{2+}$  concentration in the medium was less than 1 nM. To exchange medium, normal culture medium was removed at one end of the channel with absorbent paper while simultaneously the  $\text{Ca}^{2+}$ -free culture medium was added into the opposite end. Coverslips were rinsed before use for about 1 h in a solution of 100 mM EGTA to remove surface-bound calcium. The agar bridges were also made with  $\text{Ca}^{2+}$ -free culture medium.

### Cells Treated with BAPTA-AM

Cells were cultured in 2  $\mu\text{M}$  BAPTA-AM in 0.1% DMSO (dimethylsulfoxide) for 2.5 hrs, immediately prior to the application of the electrical field. Controls were also treated with 0.1% DMSO. After the 2.5 h incubation, BAPTA-containing medium was removed and replaced with normal culture medium using the procedure described above for medium exchange.

### Cells Treated with Cyclic Nucleotide Analogs

Cells were cultured and exposed to electric fields as described above, except that 1 mM 8-Br-cGMP (Sigma), 0.2 mM RP-cAMPS (Alexis, San Diego, CA), or 0.2 mM SP-cAMPS (Alexis) was added to the medium in which the cells were



**Figure 1** Determination of the Differential Growth Index. To assess growth polarity, mean neurite projection (NP) toward the cathode (CFP) or anode (AFP) was measured. NP is calculated by multiplying  $\cos\theta$  by the neurite length ( $L$ ). The neurite projection toward the anode and cathode were used to calculate the DGI. The DGI is total anodal projection minus total cathodal projection divided by the sum of the two. In this scheme, a gray cell body and extending process represents the neuron. The arrows indicate projection to the anode or cathode.

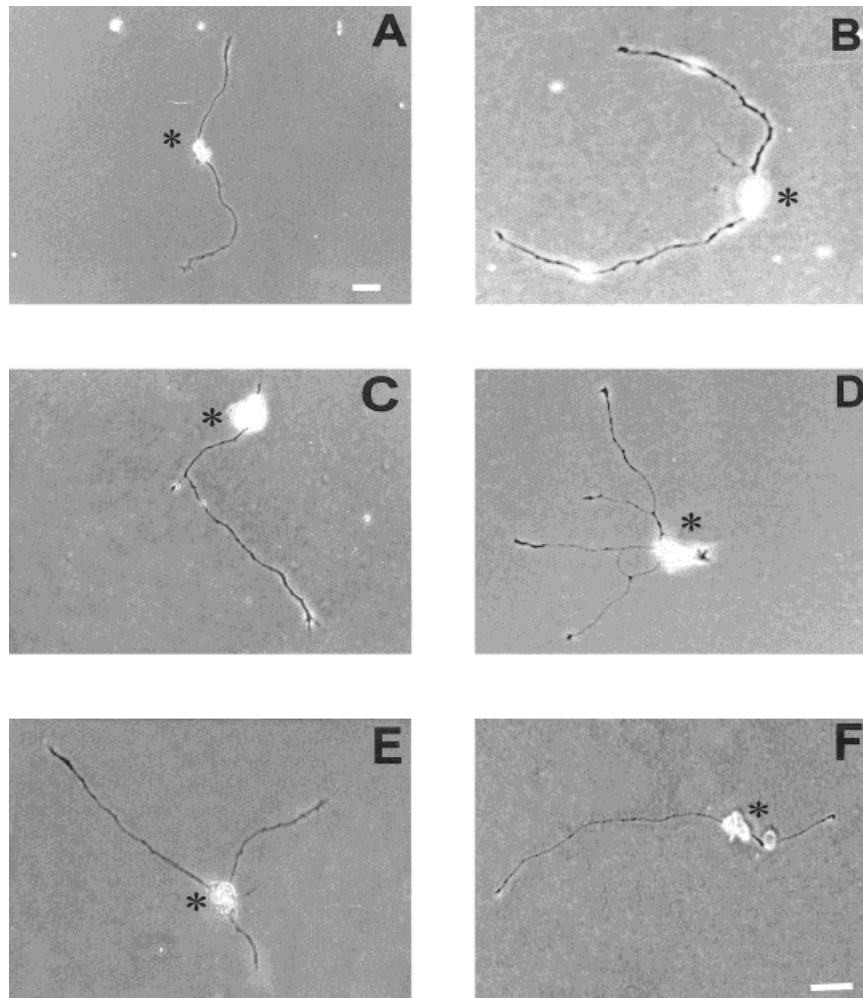
exposed to the fields. Controls in normal medium were conducted simultaneously.

### Field Treatment

Cells were confined to a 60 mm  $\times$  1 cm  $\times$  160  $\mu\text{m}$  space within a 100 mm diameter tissue culture plastic dish. To create the growth channel, two 24  $\times$  60 mm glass coverslips (Thomas Scientific, Swedesboro, NJ) were aligned parallel to each other, separated by 1 cm and stuck to the bottom of the dish with silicone grease. A third coverslip with the same dimensions was laid on top of the first two. This created an open-ended rectangular chamber through which culture medium could easily pass. Two agar-filled bridges were placed on either side of the open-ended channel with the opposite ends of the bridges immersed in a Steinberg's bath containing  $\text{Ag}^+/\text{AgCl}$  electrodes. Approximately 0.18 mA of current was passed through the chamber from a constant current source, producing a field of 100 mV/mm for about 12 h. The voltage difference between the ends of the chamber before, after, and sometimes during each experiment was measured to assure that the electric potential had not fluctuated by more than 0.15 V from the set level of 6.0 V; thus the field in the chambers did not vary by more than 2.5%. If greater fluctuation occurred, the cultures were not included in the analysis.

### Data Collection

Using an inverted phase contrast microscope with a photographic camera attached, 35 mm photographs (Kodak, Rochester, NY, TMAX and Tech Pan film) were taken of the neurites with either a 10 $\times$  or 20 $\times$  objective. The negatives were enlarged such that the neurites could be traced on a digitizing tablet. Neurite tracings were entered directly into the computer and were analyzed using a vector analysis program (Cork and Rajnicek, 1990). In order to assess the asymmetry of the neurite growth, the projection of each



**Figure 2** Photographs of spinal neurites taken approximately 12 h after field application or nonfield treatment. Cells were cultured in the absence of a field (A, C, E) or in the presence of a field of 100 mV/mm with the cathode to the left (B, D, F). Asterisks identify the cell bodies. (A, B) Neurites cultured in normal Steinberg's medium containing 2 mM calcium (C, D) Neurites cultured in  $Ca^{2+}$ -free Steinberg's medium (E, F) Neurites loaded with BAPTA for 2.5 h before culturing in normal Steinberg's medium. Scale bars = 10  $\mu$ m.

neurite on the electric field vector was determined using the length and angle of deviation from the electric field vector (Fig. 1). From these measurements, the total anodal projection (TAP) and the total cathodal projection (TCP) were determined, and then the differential growth index (DGI) was calculated [DGI = (TAP - TCP)/(TAP + TCP)]. If all neurites projected toward the cathode, a DGI of -1 would result, while total neurite projection toward the anode would produce a DGI of +1. Random neurite projection is indicated by a value of zero. Data from cultures without field application were randomly assigned cathode or anode, by the flip of a coin prior to analysis. To obtain the percent neurite projection toward the anode (%AP) or cathode (%CP) we used the total number of anode facing neurites (TNP) and the total number of cathode facing neurites (TNM) in the following equations: %AP =  $100 \times (TNP/n)$ ;

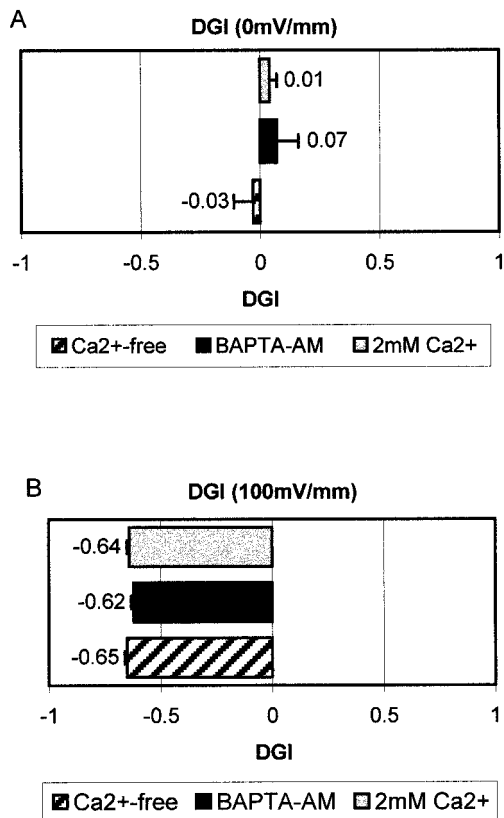
%CP =  $100 \times (TNM/n)$ ; where 'n' equals the total number of neurites.

Statistics for "within group" analysis was done using the z-score. Data are presented as means  $\pm$  the standard error of the means. For correlation between groups, the chi-square and ANOVA tests were used.

## RESULTS

### Response to Electric Fields in 2 mM $Ca^{2+}$

First, the response of cells to an electric field in the normal  $Ca^{2+}$  concentration of 2 mM was determined.



**Figure 3** (A, B) Measurements of the DGI for neurite cultures with and without field application. A DGI of 1 is representative of total neurite projection toward the anode, while a DGI of  $-1$  is representative of total neurite projection toward the cathode. (A) Average DGI of three control experiments in which no field was applied (B) Average DGI for each experimental culture in which a field strength of 100 mV/mm was applied for the duration of 12 h. Z-scores calculated for (B) give a  $p$  value of .0001 for condition, indicating a significant difference between field and non-field treatment. SEM is given for each value. An ANOVA test, used to determine if there was any correlation between culture conditions during field application, yielded a  $p$  value of 0.38.

A field of 100 mV/mm was applied for an average of 12 h, beginning immediately after plating cells. Control cultures, which were run with each experiment, were set up identically except that they were not connected to the power supply. At the end of the treatment, all cells projecting neurites in the chambers were photographed for later analysis (Fig. 2). Neurites projected randomly in the absence of an electric field, but neurite projection was markedly cathodal in field-treated cultures ( $p = .0001$ ) (Fig. 3). This is consistent with the findings of others (Hinkle et al., 1981; Patel and Poo, 1982).

### Ca<sup>2+</sup>-Free, 2 mM EGTA

Neurites were cultured in Steinberg's solution for 1 h to allow them to adhere securely to the culture dishes. We found that culturing neurites in Ca<sup>2+</sup>-free medium from the start resulted in the dramatic loss of cells due to their reduced adhesion. After 1 h in medium containing 2 mM Ca<sup>2+</sup>, the culture medium was replaced with Ca<sup>2+</sup>-free medium containing 2 mM EGTA, and the field was applied. Both field-treated and untreated cultures extended neurites just as those in normal culture medium, except that they were longer on average. Also, field-treated neurites projected toward the cathode just as those neurites in 2 mM Ca<sup>2+</sup> did (Fig. 3). Student's  $t$  test showed significance at  $p = .0001$ . Photographs were taken 12 h after medium exchange (Fig. 2).

### BAPTA-AM

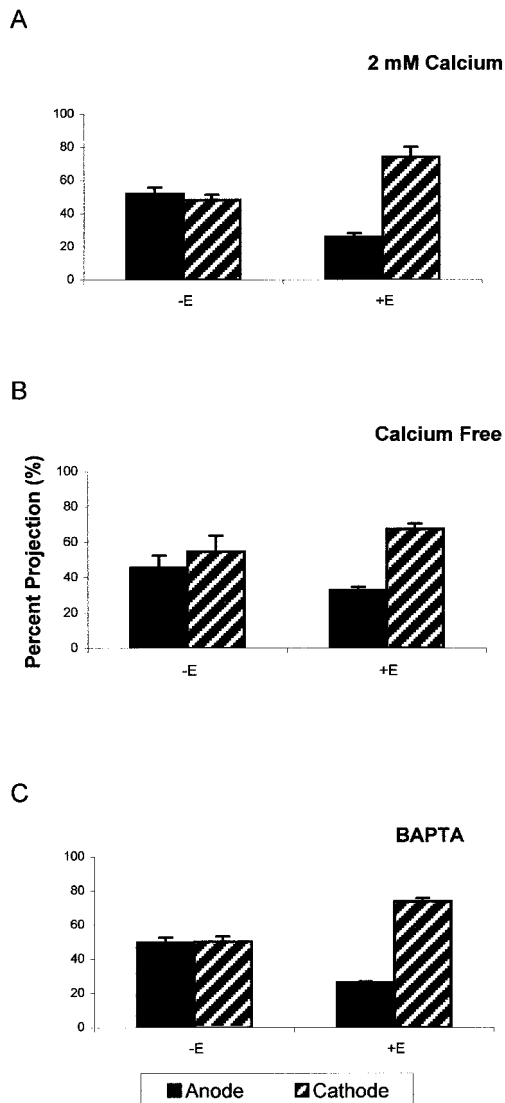
Cells were initially plated in culture medium containing 2  $\mu$ M BAPTA-AM. After 2.5 h, medium was exchanged with normal culture medium. Neurite projection in nonfield treated dishes was random. Neurites cultured in a field of 100 mV/mm grew toward the cathode to the same extent as controls with field application but without BAPTA-AM treatment (Fig. 3). The cathodally directed response was significant at  $p = .0001$ . Photographs were taken 12 h after plating cells (Fig. 2). These neurites were also longer than controls.

### Turning Response

The turning response of these neurites in an electric field under normal conditions (2 mM Ca<sup>2+</sup>) has been characterized by the ability of a neurite initially projecting perpendicular to the field, to turn toward the cathode when an electric field is globally or focally applied (Hinkle et al., 1981; Patel and Poo, 1984). Our experiments with field treatment confirmed that neurites do respond cathodally to field application. Furthermore, the turning response of cells in Ca<sup>2+</sup>-free medium or BAPTA-loaded medium also responded cathodally. To quantitatively assess neurite projection either to the cathode or anode, we employed an asymmetric index as described by Cork and Rajnicek (1990), the DGI (see Methods). The results of these analyses indicate that neurite response to the field application is not different from control experiments ( $p = .38$ ) (Fig. 3). Specifically, the DGI of neurites cultured in 2 mM Ca<sup>2+</sup> at 100 mV/mm field strength for 12 h was  $-0.64$  and similarly, the DGI of Ca<sup>2+</sup>-free cultures was  $-0.65$  and  $-0.62$  for BAPTA-AM treated cultures.

## Neurite Projection

To further characterize the effect of field application on neurite projection, we calculated the percent neurite projection toward the anode (%AP) and cathode (%CP) for each culture condition. For this asymmetry index a line was drawn through the cell body perpendicular to the direction of the field, and the end of the neurite was characterized as lying either on the anodal or cathodal side of that line. An expected frequency of 0.50 or 50% projection toward the cathode and anode field axes would represent true random growth without field application [Fig. 4(a)]. Upon field application, neurite projection favors the cathode and the CP increased to 74% while the AP was only 26% [Fig. 4(a)]. Similarly, when cultures were treated with  $\text{Ca}^{2+}$ -free medium and no field, neurite projection was random (45% AP and 55% CP) [Fig. 4(b)]. How-



ever, after 12 h field exposure in  $\text{Ca}^{2+}$ -free medium, CP was 67% while AP was 32% [Fig. 4(b)]. Similar results were obtained from cultures pretreated with 2  $\mu\text{M}$  BAPTA-AM. After 12 h field application, CP was 74% while AP was 26% [Fig. 4(c)].

## Neurite Length

Analysis of neurite length between and within experimental treatments has shown that neurites not only respond to electric fields in calcium free conditions, but they also extend longer processes (Table 1). For measurements done on neurites cultured in 2 mM  $\text{Ca}^{2+}$ , the neurite lengths were measured as  $77 \pm 5 \mu\text{m}$  without field treatment and  $85 \pm 5 \mu\text{m}$  with field application ( $p = .20$ ). For neurite length measurements done with neurites cultured in calcium free medium, we measured an average neurite length of  $131 \pm 3 \mu\text{m}$ , with and without field application. Similarly, neurites cultured in BAPTA-AM were also extended longer than those in 2 mM  $\text{Ca}^{2+}$  with neurite length measurements of  $95 \pm 2 \mu\text{m}$  without field application and  $92 \pm 1 \mu\text{m}$  with field application.

## Effects of Cyclic Nucleotide Analogs

In view of the remarkable effects of cyclic nucleotide analogs on the responses of *Xenopus* spinal neurons to gradients of neurotropic factors (Song et al., 1998), we exposed neurons to electric fields in the presence of the cGMP agonistic analog, 8-Br-cGMP, the cAMP agonistic analog, Sp-cAMPS, and the cAMP antago-

**Figure 4** Measurement of neurite projection toward the anode or cathode (%AP or %CP). Neurites that did not respond by growing to either pole were scored as having a perpendicular projection (data not shown). (A) Cells cultured in 2 mM  $\text{Ca}^{2+}$ . Random growth without field application (-E) is represented by a 50% projection in both directions. After field application (+E), 74% of the neurites projected toward the cathode. (B) Cells cultured in  $\text{Ca}^{2+}$ -free medium. After field application (+E), 67% of the neurites projected toward the cathode in comparison to neurites with nonfield treatment (-E), where 54% projected cathodally. (C) Cells that were treated with BAPTA prior to media exchange with normal Steinberg's. After field application (+E), 74% of the neurites projected toward the cathode versus the 50% that projected cathodally in the nonfield treatment group (-E). For each condition, neurite projection was random in the absence of field application condition (left, A, B, and C). Chi-square analysis indicated that the percent projection toward the cathode for cultures in B ( $.05 < p > .02$ ) and C ( $p < .0001$ ) with field application were not different from cultures in A based on control data.

**Table 1** Neurite Lengths for Each Culture Condition Measured With and Without Field Application

	100 mV/mm	<i>n</i>	0 mV/mm	<i>n</i>
Control	85.3 ± 5 μm	344	77.3 ± 5 μm	118
Ca <sup>2+</sup> -free	131 ± 3 μm	669	131 ± 4 μm	230
BAPTA	91.5 ± 1 μm	1018	95.3 ± 2 μm	200

For neurites cultured in the absence of Ca<sup>2+</sup> or loaded with BAPTA, neurites extended longer than neurites cultured in 2 mM Ca<sup>2+</sup> ( $p < .0001$ ). Photographs used to measure neurite length were taken 12 h after culture set-up. The data are listed as the means ± SEM for each value.

nistic analog, Rp-cAMPS. As shown in Table 2, the cGMP agonist had no detectable effect on the response to fields, while both the cAMP agonist and antagonist significantly increased the turning response.

## DISCUSSION

Reports indicating that neurite growth is stunted when Ca<sup>2+</sup> channels are blocked using La<sup>3+</sup> or Co<sup>2+</sup> suggest that Ca<sup>2+</sup> entry is necessary for neurite growth (Mattson and Kater, 1987; McCaig, 1989). We confirm here that Ca<sup>2+</sup> is not necessary for neurite growth and furthermore, that neurites respond to an electric field in a manner similar to neurites cultured in 2 mM Ca<sup>2+</sup>. It has recently been shown that growing *Xenopus* neurites in Ca<sup>2+</sup>-free medium decreases basal Ca<sup>2+</sup> levels in the growth cone from 130 nM to 59 nM (Zheng, 2000), so we are assured that our treatment does indeed strongly affect intracellular Ca<sup>2+</sup> levels. We further found that neurites grew considerably longer in the Ca<sup>2+</sup>-free medium, in agreement with the observations of others.

To investigate the role of any intracellular calcium contributions to galvanotropism, we loaded our cell cultures with a calcium chelator, BAPTA-AM. As described elsewhere, BAPTA-AM crosses the cell membrane and disrupts internal calcium gradients. We found that not only do these BAPTA-loaded cells grow under these conditions, but they also respond to an applied electric field. Again, the neurites that were BAPTA-loaded responded cathodally to the same extent as those neurites cultured in 2 mM Ca<sup>2+</sup>. We conclude that the response of *Xenopus* spinal neurites to physiological electric fields is independent of external Ca<sup>2+</sup> and does not require the formation of intracellular Ca<sup>2+</sup> gradients.

The lack of an effect on galvanotropism is unexpected in view of earlier findings that relatively large applied electric fields cause the formation of lateral

gradients of Ca<sup>2+</sup> in the growth cones of other neuronal types (Bedlack et al., 1992; Davenport and Kater, 1992). Additionally, there is evidence derived from inhibitor studies that *Xenopus* neuronal galvanotropism is dependent on entry of Ca<sup>2+</sup> through plasma membrane channels and the release of Ca<sup>2+</sup> from internal stores (Stewart et al., 1995). Our findings are also unexpected in view of more recent work on *Xenopus* spinal neurites, which indicates that cytosolic Ca<sup>2+</sup> gradients, created by localized photorelease of Ca<sup>2+</sup> within the growth cone, can cause neurite turning (Zheng, 2000).

We suggest that the mechanism of neurites' response to external electrical fields is fundamentally different from the mechanism by which these same cells respond to directional signals that involve a spatially-graded activation of membrane receptors. The fact that the neurites have an unimpaired response to fields during the rigorous reduction of extracellular Ca<sup>2+</sup> to less than 1 nM strongly argues against a requirement for Ca<sup>2+</sup> entry in the electrically mediated turning response. The unimpaired response following BAPTA loading argues against a requirement for intracellular Ca<sup>2+</sup> gradients. Providing a substantial concentration of a mobile buffer acts to collapse Ca<sup>2+</sup> gradients (Speksnijder et al., 1989).

In experiments using gradients of soluble guidance factors to determine the mechanistic properties of neurite turning, reports have indicated that Ca<sup>2+</sup> and cyclic nucleotides are involved. We detected no effect on galvanotropism by applying a cell-permeant agonist of cGMP, but surprisingly, we found that both the agonistic and antagonistic cAMP analogs enhanced the galvanotropic response significantly. It is important to point out that the agonist and antagonist that we used were identical to the ones used in the studies cited above, as were the cells that we used. It appears that endogenous levels of cAMP are near the least optimal concentration, and effectively increasing or

**Table 2** DGIs for Cyclic Nucleotide Analogs Treated With and Without Field Application

Analog	100 mV/mm	0 mV/mm
None	-0.64 (405)	0.01 (221)
8-Br-cGMP	-0.69 (215)	0.18 (42)
SP-cAMPS	-0.81* (37)	0.15 (48)
RP-cAMPS	-0.83* (735)	-0.06 (278)

Each analog was added directly to the culture media at the time the cells were plated. Numbers in parentheses following the DGI are the number of neurites used for data analysis. \*Significantly different from controls (absent of cyclic nucleotide analog) at 90% ANOVA (F 5, 20) = 3.457  $p = .0206$ .

decreasing cAMP concentration increases the sensitivity of the neurons to electrical fields.

The conclusion that  $\text{Ca}^{2+}$  entry is not involved in neuronal galvanotropism is counter to that of Stewart et al. (1995). However, in their study, they used a variety of pharmacological blockers of voltage-gated calcium channels and blockers of calcium release from intracellular stores, without evidence of the effects of those reagents on the calcium physiology of *Xenopus* neurites. In addition to effects on cathodal turning, Stewart et al. (1995) reported that the calcium channel blockers reduced the rate of growth of neurites and the order of effectiveness in that regard was identical to the order of effectiveness in inhibitory turning.

Our conclusion that neuronal galvanotropism is independent of calcium is fully consistent with the findings of Brown and Loew (1994). They examined the mechanism of cathodal migration in two fibroblastic cell lines, NIH 3T3 and SV101, in fields of 400 mV/mm. They established that cells do not elevate cytosolic  $\text{Ca}^{2+}$  in response to depolarization, and do not form gradients of  $\text{Ca}^{2+}$  in response to applied electric fields. Cathodal motility was also unaffected by the absence of extracellular calcium.

Clearly, gradients of  $\text{Ca}^{2+}$  within the growth cone are capable of inducing changes in growth direction (Zheng, 2000) and some extracellular vectorial signals act via  $\text{Ca}^{2+}$  gradients (Hong et al., 2000). Our effort to obtain ratiometric images of lateral  $\text{Ca}^{2+}$  distribution in growth cones responding to electrical fields in the absence of external calcium failed. The growth cones in  $\text{Ca}^{2+}$ -free medium or in medium loaded with BAPTA are greatly reduced in lateral extent from those in normal  $\text{Ca}^{2+}$ . In addition, the basal  $\text{Ca}^{2+}$  levels are considerably reduced, into the range where fluorescent indicators are insufficiently sensitive to measure these levels.

Although our findings contribute information concerning what *is not* controlling the electric field-mediated turning response in neurites, we are still left to determine what *is* the mechanism of the turning response. Perhaps protein redistribution, not necessarily ion channels, occurs in a manner that elicits the turning response. It is known that proteins redistribute in the presence of an electric field (Poo and Robinson, 1977). One possible class of proteins that may be involved is the cytoskeleton associated proteins that are associated with the adhesion of cells to their substrates. If these proteins accumulate on the cathodal side, it is possible that growth in the cathodal direction would be favored.

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