Calcium Ion Distribution in Nascent Pioneer Axons and Coupled Preaxonogenesis Neurons *in situ*

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The "calcium hypothesis" of regulation of growth cone motility and neurite elongation has derived from analysis of a variety of neurons growing *in vitro*. It proposes that calcium ion concentration within growth cones is an important regulator of motility and growth. We now extend this analysis by investigating calcium concentrations within growth cones and nascent neurites of identified embryonic neurons growing on their normal substrate *in situ*.

The pair of T11 pioneer neurons are the first to extend axons in limb buds of grasshopper embryos. Their growth cones migrate along a stereotyped pathway, where they encounter a series of guidance cues, including preaxonogenesis afferent neurons (guidepost cells). By injecting a pioneer neuron with fura-2 dye, we measured calcium concentration in the injected neuron, and in cells to which it became dye-coupled, at successive stages of pioneer outgrowth through the limb.

Pioneer neurons undergoing axonogenesis had calcium concentrations in the above-100-nM range characteristic of a variety of neuron types studied *in vitro*. In pioneer neurons not yet in contact with guidepost cells, a shallow calcium concentration gradient, highest at the growth cone, was often observed. Guidepost cells that had not begun axonogenesis had lower calcium concentrations, in the 65 nM range. Therefore, an increase in cytosolic calcium concentration may be associated with the onset of axonogenesis. Nascent pioneer neurons were fura-2 dye-coupled to each other and established coupling with contacted guidepost cells. Calcium concentration measurements along pioneer neurites suggest that calcium ions also are transferred from pioneer neurons to these coupled guidepost cells. Thus, calcium concentration within nascent pioneer neurites appears to be altered by contact with specific *in situ* guidance substrates.

In growth cones studied *in vitro*, calcium ion concentration appears to be an important feature that integrates environmental input and, in turn, regulates behavior (Anglister et al., 1982; Bolsover and Spector, 1986; Connor, 1986; Mattson and Kater, 1987; Kater et al., 1988; Lipscombe et al., 1988; Forscher, 1989; Lankford and Letourneau, 1989; Silver et al., 1989, 1990; Streit

and Lux, 1989). In a variety of cell types, growth cone motility and neurite elongation have been correlated with intracellular calcium concentration (Anglister et al., 1982; Connor, 1986; Cohan et al., 1987; Goldberg, 1988; Lankford and Letourneau, 1989; Silver et al., 1989, 1990; Tolkovsky et al., 1990). Interactions with both soluble and substrate molecules influence intracellular calcium levels. Exposure of growth cones to a variety of neurotransmitters can arrest or otherwise regulate growth (Haydon et al., 1984; Connor et al., 1987; Mattson et al., 1988; McCobb and Kater, 1988; McCobb et al., 1988). This effect appears to be mediated, at least in part, by alteration of intracellular calcium levels through voltage-gated calcium channels (Cohan and Kater, 1986; Cohan et al., 1987; Kater et al., 1988). Binding of antibodies to the cell surface adhesion molecules L1 and N-CAM, or cell-cell adhesion, also can elevate intracellular calcium (Schuch et al., 1989). Extracellular matrix molecules and their receptors also appear to be linked to intracellular second-messenger systems (Bixby, 1989). In addition to the direct effect of binding ligands, adhesion may initiate secondary effects through stretch-activated or stretch-inactivated potassium ion channels in growth cones (Morris and Sigurdson, 1989; Sigurdson and Morris, 1989). Voltage changes generated by these ion fluxes are also thought likely to effect intracellular calcium levels.

Within the growth cone, calcium ion concentration changes could regulate motility and elongation through a variety of cellular processes (Forscher, 1989). The ability of agents such as phalloidin and taxol, which affect actin or tubulin polymerization, to block or reverse the effects of calcium ionophores suggests that a major route for calcium control of the growth cone is through regulation of the stability of cytoskeletal elements (Lankford and Letourneau, 1989).

This information, derived from cell lines and from regrowth of dissociated cells in culture, makes it desirable to examine the relationship between calcium concentration and growth cone behavior of embryonic neurons *in situ*. Among the most intensively studied growth cones *in situ* are those of insect embryos and imaginal disks. The accessibility and identifiability of these neurons facilitates lineage, genetic, and pathway analysis, and in addition, many extracellular matrix, matrix receptor, and cell adhesion molecules are now known to have both insect and vertebrate homologues (Anderson, 1988; Fessler and Fessler, 1989). Embryonic insect neurons and muscle cells also appear to have several conventional types of calcium channels (Wei and Salkoff, 1986; Byerly and Leung, 1988; Leung et al., 1989). We have here begun examination of one set of insect neurons, the afferent pioneer neurons in grasshopper embryonic limb buds.

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The pair of Ti1 pioneer neurons are the first afferent neurons to initiate axonogenesis in the limb buds of grasshopper embryos (Bate, 1976; Keshishian, 1980) and establish a pathway (Fig. 1) essential for normal development of the PNS (Klose and Bentley, 1989). Their growth cones migrate along a highly stereotyped route to the CNS (Ho and Goodman, 1982; Keshishian and Bentley, 1983; Caudy and Bentley, 1986; Lefcort and Bentley, 1987). While their initial outgrowth may be oriented by internal cell polarity (Lefcort and Bentley, 1989), they are subsequently guided by a hierarchical series of extrinsic cues that includes a set of identified preaxonogenesis afferent neurons (guidepost neurons) and limb segment boundary cells, and possibly by an epithelial polarity cue. In some limb regions, growth cones normally interact extensively with the basal lamina (Anderson and Tucker, 1988; Condic and Bentley, 1989a), but the presence of basal lamina is not a requirement for migration along the normal route (Condic and Bentley, 1989c). The growth cones migrate under tension and rapidly establish adhesive contacts directly with the surfaces of boundary cells and guidepost cells (Condic and Bentley, 1989b). Guidance cues appear to be located by filopodial exploration (Bentley and Toroian-Raymond, 1986). Time-lapse video photography of growth cones migrating on the *in situ* substrate shows that, in some limb locations, steering is mediated by asymmetrical veil extension or by selective branch retraction (O'Connor et al., 1990). However, at other sites, a single filopodial contact with a high-affinity substrate is sufficient to reorient the growth cone (O'Connor et al., 1990). This phenomenon suggests that intracellular signaling mechanisms may be involved in growth cone steering. To begin an analysis of possible signaling mechanisms, we here investigate calcium ion distribution in pioneer neurons prior to and during axonogenesis.

Preliminary results have appeared previously in abstract form (Guthrie et al., 1989).

Materials and Methods

Schistocerca americana eggs at the 30–35% stages of development (Caudy and Bentley, 1986) were obtained from a colony maintained at Berkeley. Embryos were removed from the eggs in saline (Lefcort and Bentley, 1987); the segments anterior to the third thoracic segment (T3; metathoracic) were removed, and the anterior surfaces of the T3 limb buds were lowered onto a strongly adhesive poly-L-lysine coated (5 mg/ml) glass coverslip. Adherent limbs were opened along the posterior aspect with a glass needle, the epithelium was unrolled onto the coverslip surface, and the interior mesodermal cells were removed with a suction pipette. With Nomarski optics on a Zeiss IM405 inverted microscope, the Ti1 pioneer neurons could then be directly viewed on the interior (basal) surface of the epithelium. Preparations were maintained on the stage at $23 \pm 2^\circ\text{C}$.

Neuron cell bodies were penetrated with thin-walled microelectrodes containing fura-2 (pentapotassium salt; 10 mM in H_2O ; Molecular Probes, Eugene, OR). Fura-2 was injected with a 1-nA hyperpolarizing current (50% duty cycle) for 2–5 min. Average intracellular concentrations estimated by comparison with calibrated glass microcapillary tubes filled with known concentrations of fura-2 were as follows: Ti1 cell bodies, 600 μM ($n = 10$); Ti1 axons, 360 μM ($n = 10$); Ti1 growth cones, 270 μM ($n = 4$); Fe1 cell bodies, 120 μM ($n = 5$); Tr1 cell bodies, 42 μM ($n = 4$). These concentrations were sufficient for visualization of the cell bodies, axons, branches, and growth cones. While filopodia often could be observed, calcium ion concentrations in filopodia were not measured because of the high (cell-damaging) levels of illumination required. The fura-2 was allowed to diffuse through the cells for a minimum of 15 min before observations were taken. For most cells, two to six images were taken over the course of the next hour. However, because these growth cones migrate slowly (about 5 hr for each 1% of development at 30°C), we did not follow the migration of individual growth cones

Figure 1. A schematic diagram of outgrowth of the Ti1 pioneer axons through a 31–34%-stage limb bud. The sibling Ti1 cell bodies lie in the tibia, and their growth cones migrate proximally across several prospective limb segment boundaries to reach the CNS. Typical locations of the growth cones at successive developmental stages are indicated by the corresponding numbers (31.5–34.0). Along their route, the growth cones orient toward, contact, and become dye-coupled to a series of preaxonogenesis (guidepost) neurons (Fe1, Tr1, Cx1 pair). Cytosolic calcium ion concentration (indicated by shading density) is higher in the pioneer neurons than in the preaxonogenesis neurons. Ti-Fe, tibia-femur boundary; Fe-Tr, femur-trochanter boundary; Tr-Cx, trochanter-coxa boundary. Dorsal, up; proximal, to the right.

over a significant portion of their pathway. Rather, these readings should be regarded as “snapshots” taken at specific developmental stages.

Dye coupling to other cells and calcium ion concentrations were determined from the fluorescence of the fura-2. Efficient passage of the UV excitation light was obtained using a quartz collector on an HBO 50-W mercury lamp and a $40 \times \text{NA} = 1.3$ oil Nikon Fluorite objective. The excitation light was directed through a computer-controlled shutter/filter wheel system for the two fura-2 excitation wavelengths (350 ± 10 nm and 380 ± 10 nm). Neutral-density filters were inserted in the excitation path as necessary to reduce bleaching and saturation of fluorescence. The image was directed to an intensified CCD camera (Quantex Corp., Sunnyvale, CA), and the output of the camera was analyzed by a Quantex QX7 Image/Acquisition/Analysis System. Calcium concentrations were estimated from the ratio of emission intensities (495-nm long-pass filter) at two excitation wavelengths, according to the equation $[\text{Ca}^{2+}] = K_D \cdot [(R - R_{\min}) / (R_{\max} - R)] \cdot F_0 / F_s$ (see Grynkiewicz et al., 1985). For this system, $K_D = 224$, $R_{\min} = 0.48$, $R_{\max} = 13.25$, and $F_0 / F_s = 10$. For all images, a mask was created from the raw fluorescent image to restrict analysis to the cell bodies and processes of the pioneers and guidepost cells.

Because we were interested in obtaining calcium ion concentrations from regions of the cells that varied considerably in thickness, a concern was the relationship between optical path length and computed calcium ion concentration in this system. To calibrate this relationship, a tapering microelectrode was filled with a solution of 90 μM fura-2. The microelectrode was then placed on the microscope stage, and using an optical micrometer, readings were taken at positions where the microelectrode ranged from 31 to 2.5 μm in diameter. A 4.6% decrease in the ratios of fluorescence emissions used to calculate calcium concentration was recorded over the 92% decrease in optical path length (from 31 to 2.5 μm). Note that the shorter optical path length resulted in a slightly lower computed calcium concentration. This calibration indicates that the computed calcium concentration was relatively unaffected by optical path length within the range appropriate here, and that the shorter optical path length at the growth cone would not bias the computed calcium concentration to a higher level relative to the cell body.

For photoinactivation experiments, a 20- μm -diameter circular illumination spot was focused on the target, with a 380-nm excitation filter and a neutral-density filter in the pathway. The filter wheel was then rotated to an open slot, and unfiltered UV light was delivered to the target for 60 sec.

At the completion of *in situ* observations, embryos were fixed overnight in 4% formaldehyde in saline and labeled with serum antibodies against horseradish peroxidase with the protocol of Caudy and Bentley

(1986). These antibodies selectively label insect neurons (Jan and Jan, 1982; Snow et al., 1987) and show the positions of pioneer growth cone filopodia, as well as other nascent neurons. Staging of growth cone location along the pathway was based upon these images (cf. Figs. 2, 3; see also Caudy and Bentley, 1986).

The results are based upon examination of 62 T1l neurons (31 pairs), 20 preaxonogenesis Fe1 (guidepost) neurons, and 12 preaxonogenesis Tr1 (guidepost) neurons in 31 embryos at the following stages: 30.5–31.5%, $n = 6$; 32.0–32.5%, $n = 5$; 33.0–33.5%, $n = 9$; 34.0–34.5%, $n = 11$.

Results

Calcium ion concentration in cell bodies of pioneer neurons

To estimate calcium ion concentration in cell bodies of pioneer neurons, we selected a subset of cells that satisfied a set of criteria designed to minimize possible effects of the dissection or impalement of cells. First, at all stages of development examined, the pair of pioneer neurons is strongly dye-coupled (Keshishian, 1980). Consequently, within a few minutes of filling of one pioneer, the sibling cell also became filled with fura-2 (Fig. 2). When the initially filled cell was damaged by the microelectrode penetration, its calcium ion concentration was much higher than that of its sibling. Because calcium might flow from the damaged to the undamaged cell, we eliminated all cell pairs whose calcium ion concentrations differed by more than 15%. Second, we eliminated all cell pairs that had along their axons a "hotspot" of more than 150% of the average calcium concentration in the rest of the cell, because such cells might have been stretched or nicked during the dissection. Third, we eliminated possibly unhealthy cell pairs whose average calcium ion concentration was more than 150% of that of the pairs remaining after the first two criteria had been satisfied. This left a set of 18 pioneer neurons (nine pairs) in nine embryos roughly equally distributed in age from 30.5% to 34.0%. The average calcium ion concentration in cell bodies of this set was 102.3 ± 1.3 nM ($\pm$ SEM). With less stringent elimination criteria allowing double the average calcium concentration, we observed 48 cells (24 pairs) with an average concentration of 113 ± 6.3 nM.

Distribution of calcium ion concentration along nascent pioneer neurons

To evaluate the possibility of a graded distribution of calcium ions along growing pioneer neurons, we compared calcium ion concentrations at the cell body and at the growth cone, using

Figure 4. Time course of the establishment of fura-2 coupling between the T1l pioneer axons and the Fe1 and Tr1 guidepost cells. Pioneer neurons were filled with fura-2 by intracellular injection, and guidepost cells were subsequently observed for coupling (numbers of filled T1l neurons at each stage shown at top of columns).

the set of optimal-condition neurons defined above. From these, cell pairs were eliminated that did not have growth cones at least a cell-body diameter in length or that had become dye-coupled to the Fe1 or Tr1 preaxonogenesis neurons. This left a set of 12 pioneer neurons (six pairs). In this set, the average calcium ion concentration was 101.8 ± 1.3 nM in the cell bodies and 118.1 ± 6.8 nM in the growth cones. Thus, the calcium ion concentration was higher in the growth cones by an average of about 16 nM. It seems possible, therefore, that growing pioneer neurons sometimes have a shallow gradient with a higher calcium ion concentration at the growth cone (Fig. 2B–D).

Calcium ion concentration in preaxonogenesis afferent neurons

Migrating pioneer neuron growth cones encounter and dye-couple to two preaxonogenesis guidepost neurons, Fe1 and Tr1,

Figure 2. Cytosolic calcium ion concentrations in T1l pioneer neurons during early axonogenesis, before coupling is established with guidepost neurons. One cell of each pair has been intracellularly injected with fura-2; as the cell pair is strongly dye-coupled, the sibling has passively filled with fura-2 by diffusion. Fura-2 concentrations are similar in both cells. In A–D, color represents calcium ion concentration, as indicated on the color scale in A. Cells are typically in the 100–115-nM range. Along the length of the cell, a shallow calcium ion concentration is often seen, with the growth cones being at a slightly higher level. A, 30.5% stage (onset of axonogenesis); B, 31.5%; C, 31.5%; D, 32.5%. In D, the two growth cones have separated (arrowhead, ventral growth cone). After the fura-2 measurements, limb were fixed and labeled with neuron-selective (anti-HRP) antibodies to reveal the disposition of filopodia and placement of guidepost neurons. D' shows the antibody labeling of D (arrowhead, ventral growth cone). Dorsal, up; proximal, to the right. Scale bars: D, 25 μ m for A–D; D', 30 μ m.

Figure 3. Cytosolic calcium ion concentrations in the T1l pioneer neurons, and in the Fe1 and Tr1 guidepost neurons after the establishment of dye coupling. A–C, One cell of each pioneer pair was intracellularly injected with fura-2 dye. The sibling T1l neuron and coupled guidepost cells were then passively filled with fura-2 by diffusion. Cytosolic calcium ion concentrations are represented by color, as shown on the color scale in C. A–C', Neuron-selective (anti-HRP) antibody labeling of the cells in A–C, showing the disposition of pioneer growth cone filopodia and the placement of guidepost cells. A and A', A 33%-stage pioneer pair; though the pioneer growth cones are extending dorsally and ventrally along the Tr–Cx segment boundary (see Fig. 1) and are in contact with the Tr1 guidepost cell, fura-2 dye coupling is not yet established. The pioneers are coupled to Fe1 (arrow). Cytosolic calcium levels are higher in the pioneers than in cell Fe1; within the pioneers, the lowest calcium levels are in the vicinity of Fe1. B and B', Pioneer neurons at a slightly later stage than in A are coupled to both cell Fe1 (open arrows, B and B') and cell Tr1 (solid arrows, B and B'). Both guidepost cells have lower cytosolic calcium levels than the pioneers. C and C', A 33.5% pioneer pair is extending growth cones ventrally along the Tr–Cx boundary (stage shown in Fig. 1). Cell Fe1 (open arrow, C') is unusually displaced from the T1l axons and is not coupled to them. Cell Tr1 (solid arrows, C and C') is coupled to the pioneers and has a lower cytosolic calcium level. Dorsal, up; proximal, to the right. Scale bars: C, 25 μ m for A–C; 20 μ m for A'–C'.

along their pathway in the distal limb. In limbs of the appropriate stages, fura-2 passes into these preaxonogenesis neurons within a few minutes of reaching them (Figs. 3, 4, 5*B*, 6*A*). Following this initial filling, the fura-2 concentration appears to remain stable in these cells for relatively long periods (>1 hr). Consequently, this indirect filling makes it possible to assess calcium ion concentration in these cells (but only after dye coupling with the pioneer neurons has been established). Cells were considered for this set that had stable levels of fura-2 at an estimated concentration of 30 nM or above (see Materials and Methods). Most cells in this group had similar calcium concentrations (see below); a few cells were eliminated that had calcium concentrations more than double this level and thus appeared damaged. The seven Fe1 neurons satisfying these criteria had an average calcium ion concentration of 65.9 ± 4.5 nM. Six Tr1 neurons satisfying these criteria had an average calcium ion concentration of 66.8 ± 4.2 nM. The Ti1, Fe1, and Tr1 cells are all afferent neurons. While the Ti1 neurons initiate axonogenesis at the 30.5% stage, Fe1 and Tr1 axonogenesis is delayed until after the 35% stage. At the stages when we assayed them, the Fe1 and Tr1 neurons are several hours preaxonogenesis, whereas the Ti1 neurons are initiating or undergoing axonogenesis. The average calcium ion concentration of both preaxonogenesis (Fe1, Tr1) neurons was about 66 nM, whereas that of the Ti1 neurons, undergoing axonogenesis, was above 100 nM.

The timing of fura-2 coupling between pioneer neurons and preaxonogenesis neurons

We wished to determine how soon after pioneer growth cones encountered guidepost neurons fura-2 coupling became established. As mentioned above, we did not monitor the process of establishment of coupling in the time frame of our observations. Rather, coupling was observed as an all-or-nothing situation: either fura-2 was transferred to the coupled cells within about 10 min, or it did not transfer in the observation period. (There was a single exception where initially uncoupled Fe1 and Tr1 cells both became coupled within 20 min; it seems likely that this was due to the restoration of coupling lost due to some aspect of the dissection or injection.) We tested coupling between the pioneers and cell Fe1 in 31 embryos at various stages of

development (Fig. 4). No coupling was observed in limbs at the 32.0% stage or earlier, but by the 33.0% stage or later, all Fe1 cells were coupled to the pioneers (with the exception of a single limb with an ectopic Fe1). These data suggest that the Fe1 cells usually become fura-2-coupled to the pioneers at about the 32.5% stage.

We also tested coupling between the Tr1 cell and the pioneers in the same set of 31 embryos (Fig. 4). No coupling was observed at the 33.0% stage or at earlier stages, but all cells were coupled by the 34.0% stage. This suggests that fura-2 coupling occurs between these cells at about the 33.5% stage. Thus, for both the Fe1 cell and the Tr1 cell, the lag between arrival of the pioneer growth cones at the cell and the establishment of coupling that would allow passage of fura-2 observable with our system was about 0.5% of development.

As has been reported using other dyes (Keshishian, 1980), epithelial cells were strongly intercoupled, and mesodermal cells were also strongly intercoupled when tested with fura-2. However, we observed no dye coupling at the stages tested between pioneer neurons and epithelial segment boundary cells at the tibia-femur, femur-trochanter, or trochanter-coxa boundaries.

Calcium ion transfer between pioneer neurons and guidepost cells

Because the nascent pioneer axons appear to have a higher internal calcium ion concentration than the guidepost neurons, and because these cells become dye coupled, calcium ions might diffuse down their concentration gradient from the pioneer neurites into the guidepost neurons. Several observations suggest that this is the case. First, pioneer growth cones that are at stages close to, but preceding, coupling to Fe1 or Tr1 (Figs. 2*D*, 3*A*) often had a higher calcium ion concentration than growth cones of later-stage neurons that had established coupling (Fig. 3*B,C*). This pattern is consistent with the possibility that coupling of a nascent axon to a guidepost cell can lower calcium ion concentration within a nearby growth cone.

Calcium ions might also pass locally from pioneer axons to guidepost cells in the region where they are coupled. We examined 12 instances where pioneer axons crossed a guidepost cell that was coupled and that was at a substantially (>30 nM) lower calcium ion concentration than the average concentration

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Figure 5. Guidepost cells appear to act as calcium sinks for coupled pioneer axons. Although pioneer axons often have lower apparent calcium levels where they cross over, or pass close to, guidepost cells, optical estimation of calcium concentration in these cases of close adjacency is subject to possible summation and light-scatter errors. Such errors can be discounted if the pioneer axons pass at a distance from the coupled guidepost cell. *A*, Immunofluorescent labeling shows a preparation where guidepost cell Fe1 (*open arrow*) is displaced more than a cell diameter from the pioneer axons, with fine processes extending between the axons and Fe1. *B*, Intracellular injection of fura-2 into one pioneer neuron cell body reveals that Fe1 (*open arrow*) is nevertheless dye-coupled to the pioneers. Calcium ion concentrations are represented by color, as shown on *color scale* in *B*. *Solid arrows*, cell Tr1. *Dorsal*, up; *proximal*, to the right. Scale bars: *A*, 20 μ m; *B*, 25 μ m. *C*, Although in this preparation the calcium levels in the pioneer cell bodies were elevated by the microelectrode penetration, measurement of cytosolic calcium levels along the pioneer axons nonetheless reveals a reduction in calcium concentration in the vicinity of cell Fe1. Therefore, the measured decreases in calcium values within the pioneer axons near coupled guidepost cells appear to reflect a true reduction in calcium, rather than spatial summing errors. The *open ovals* indicate the positions and calcium concentrations of the Fe1 and Tr1 guidepost cells.

Figure 6. Effect of irradiation uncoupling of a guidepost cell. *A*, An intracellularly injected 33.5%-stage Ti1 pioneer neuron is fura-2 coupled to both cell Fe1 (*open arrow*) and cell Tr1 (*solid arrow*). Both guidepost cells have a lower cytosolic calcium concentration than the pioneer axons. Within the pioneer axons, the calcium level is slightly depressed in the vicinity of cell Tr1. *B*, Six minutes after irradiating the perimeter of cell Tr1 with a 20- μ m-diameter circular UV beam (*broken line*), cytosolic calcium levels in both cell Tr1 and in the pioneer axon have risen above 1000 nM (see *B'*). *C*, Twenty minutes after the irradiation, cell Tr1 has died and become uncoupled from the pioneer axon. In the pioneer axon, cytosolic calcium concentration has returned toward normal levels, but is higher in the vicinity of cell Tr1 than it was when the pioneer was coupled to Tr1. *A'-C'* show calcium ion concentrations along the Ti1 axons in *A*, *B*, and *C*, respectively. Calcium ion concentrations are represented by color, as shown on *color scale* in *A*. *Dorsal*, up; *proximal*, to the right. Scale bar, 25 μ m for *A-C*.

throughout the pioneer neuron. In 11 of these cases, the calcium concentration in the axon was lower where it crossed the guidepost cell than elsewhere in the axon (Fig. 3*A,B*). Because of the possibility of optical cross talk between the pioneer axons and the guidepost cell bodies (the fura-2 fluorescence from axons directly above the guidepost cell algebraically summing with the fluorescence within the guidepost cell itself), the most reliable measurements were taken from axons that did not pass directly over guidepost cells; in these cases, calcium concentration also was lower in the portion of the axons in closest proximity to the guidepost cells. In one preparation (Fig. 5), cell Fe1 was displaced more than a cell diameter away from the pioneer axons (though connected by fine processes), but calcium ion concentration was still lower in the axon region close to Fe1. A similar situation was seen in the vicinity of cell Tr1, where the two pioneer axons separated slightly before reaching Tr1 (Fig. 5). Therefore, calcium ion concentrations do appear to be lower in the regions of pioneer axons that are close to coupled guidepost cells.

If coupling to guidepost cells locally lowers calcium concentration within pioneer neurons, then uncoupling of the cells ought to block this effect. To test this possibility, we killed coupled guidepost cells by focal UV irradiation in five limbs. A UV beam, approximately 20 μ m in diameter, was positioned to overlap the guidepost cell by about one third of its diameter at the cell site farthest from the pioneer axons, and the cell was irradiated for 60 sec (Fig. 6). In each of these cases, the guidepost cell was initially strongly coupled to the pioneer neurons, and calcium ion concentration was substantially lower (>30 nM) in the guidepost cell than in the pioneer neuron. Following irradiation, the outcome in each case was the same: the guidepost cell changed to a high calcium ion level (and usually subsequently died). The pioneer neurons exhibited a transient increase in calcium ion concentration, but then returned toward normal levels, with calcium ion concentration in the vicinity of the guidepost cell no longer at a lower concentration than in the remainder of the axon. This is the expected outcome if coupling to the guidepost cell had been contributing to the locally decreased calcium ion level within the pioneer axons.

Discussion

While cytosolic calcium concentration in growth cones of cell lines and primary cultures of dissociated neurons has been extensively documented, little information is available for neurons *in situ*. We report here the levels and spatial distribution of free calcium ions in pioneer neurons undergoing axonogenesis on their *in situ* substrate. These concentrations are higher than those of other afferent neurons that are in the preaxonogenesis stage of differentiation. We also report that free calcium concentration is reduced in nascent neurites, and probably in growth cones, after the growth cones encounter the high-affinity guidance substrates provided by preaxonogenesis neurons. These results are also among the first data on calcium concentrations in insect growth cones.

Calcium ion concentration in embryonic neurons

In the set of 18 pioneer neurons most stringently selected to exclude injured cells, the calcium concentration in cell bodies averaged about 100 nM. With more relaxed criteria, including a set of 48 neurons, calcium concentration was about 115 nM. By filling cells with intracellular injection rather than fura-2 AM

loading, ambiguities resulting from preferential association of the dye with intracellular organelles are avoided (Almers and Neher, 1985). Moreover, data were used only from cell pairs with similar calcium levels. Consequently, one cell of each pair was filled neither by transmembrane methods nor by injection, but through gap junctions. The data from these cells should be free of both preferential dye association and cell damage errors.

The 100–115 nM free calcium concentration observed in these insect pioneer neurons is comparable to levels recorded from many cell types. Reports include rat diencephalic neuron cell bodies (60–80 nM) and growth cones (up to 200 nM; Connor, 1986), snail neuron growth cones (about 130 nM; Cohan et al., 1987), frog sympathetic neuron cell bodies (80 nM) and growth cones (130 nM; Lipscombe et al., 1988), mouse N1E-115 neuroblastoma cell motile and advancing growth cones (60 nM; Silver et al., 1989), chick dorsal root ganglion cell growth cones (200–800 nM; Lankford and Letourneau, 1989), *Xenopus* spinal neuron cell bodies (60–80 nM) and growth cones (70–200 nM; Sun and Poo, 1987), and rat hippocampal neuron cell bodies (100 nM; Mattson et al., 1988).

We also recorded calcium concentrations in the cell bodies of 2 other identified embryonic afferent neurons, Fe1 and Tr1, that were in earlier stages of differentiation and that would initiate axonogenesis after the pioneers. The values in both these cell types were in close agreement in the 65-nM range and were thus lower than those in the Tr1 afferent neurons, which were undergoing axonogenesis. This dissimilarity could be because of an intrinsic difference in the calcium levels of these neurons, or it could be associated with their preaxonogenesis state. The latter explanation seems more likely because the later behavior of the growth cones of these cells is very similar to that of the pioneer growth cones (Keshishian and Bentley, 1983). While this is the only information of which we are aware on calcium levels in embryonic neurons *in situ* before the initiation of axonogenesis, there are several comparisons of calcium levels in growing and nongrowing neurons and cell lines *in vitro*. In rat embryonic diencephalic neurons (Connor, 1986) and in identified neurons of the snail *Helisoma*, calcium levels were higher in growing than in nongrowing cells. Mouse N1E-115 neuroblastoma cells had growth cones that were motile and advanced at relatively low calcium levels (60 ± 5 nM; Silver et al., 1989). These calcium concentrations were only slightly higher than concentrations in quiescent growth cones (55 ± 7 nM). Because we did not alter calcium concentration in pioneer growth cones or make time-lapse observations of the (calcium-lowering) effect of coupling to guidepost cells, we have no comparable data on the consequences of low calcium concentration for pioneer growth cone motility or elongation rate.

In some pioneer neurons, we observed a shallow gradient of calcium ion concentration, highest at the growth cone and lowest at the cell body. Other neurons did not have a gradient. Whether this feature might distinguish growing and nongrowing neurons was not determined. Using fura-2, gradients have been observed in rat diencephalic neurons (Connor, 1986) and *Helisoma* neurons (Cohan et al., 1987). In N1E-115 cells, extracellular recordings suggested that more voltage-activated calcium channels were present on the growth cone than in neurites (Anglister et al., 1982; see also Bolsover and Spector, 1986). Patch clamping revealed higher channel densities on growth cones than on neurites of PC12 cells (Streit and Lux, 1989), but not on slowly growing frog sympathetic neurons (Lipscombe et al., 1988). What role these gradients might play in outgrowth is not clear, and it

may be that they are an epiphenomenon of mechanisms for controlling calcium level within the growth cone.

Ion manipulation and patch-clamp analyses of processes of embryonic insect (*Drosophila*) neurons and muscle cells *in vitro* have demonstrated a set of voltage-sensitive channels that are, in general, similar to those found on molluscan neurons (Wei and Salkoff, 1986; Byerly and Leung, 1988; Leung et al., 1989). This set includes calcium channels, found in relatively high density, which appear to include L-type (Pauron et al., 1987) and possibly other types of calcium channels. Growth cones of some molluscan neurons have stretch-activated and also stretch-inactivated potassium-selective ion channels, and the current fluxes through these channels may affect voltage-sensitive calcium channels (Morris and Sigurdson, 1989; Sigurdson and Morris, 1989). The T11 pioneer neurites maintain a substantial level of tension that effects rapid shortening of the neurite if either the cell body or the growth cone unadheres from the substrate (Condic and Bentley, 1989a). Because the pioneer growth cones evidently adhere directly to the plasma membrane of the preaxonogenesis neurons (Condic and Bentley, 1989b), tension-mediated calcium influxes, if present, might play a role in their behavior.

Coupling between growing pioneer neurons and preaxonogenesis neurons

Development of cells in the limb bud includes a set of spatially and temporally selective dye-coupling events. Mesodermal cells in the limb are extensively intercoupled, as are epithelial cells (Keshishian, 1980). The pioneer mother cell within the epithelium is initially dye-coupled to other epithelial cells, but it loses that coupling as it undergoes division and emerges from the epithelium. The sibling pioneer neurons remain coupled to each other, at least until their growth cones enter the CNS and also become coupled to all of the preaxonogenesis afferent neurons they encounter en route. Motor neuron growth cones emerging from the CNS do not, in general, become coupled to afferent neurons. However, one identified motor growth cone leaves the motor bundle with which it exits the CNS and fasciculates with the pioneer axons to cross from the ventral to the dorsal side of the limb; during this excursion, it becomes dye-coupled to the pioneer axons (Myers et al., 1990).

Using a monoclonal antibody against Lucifer yellow, Taghert et al. (1982) reported that some grasshopper central growth cones establish selective coupling through filopodia, and this also is possible between T11 pioneer growth cone filopodia and preaxonogenesis afferent neurons. With fura-2, we did not generally observe significant coupling between T11 growth cones and preaxonogenesis neuron Fe1 until the 32.5% stage, and with neuron Tr1, until the 33.5% stage. In both these cases, therefore, the growth cones were past the preaxonogenesis neurons before this coupling was observed. This discrepancy may not be significant because it may well be that, with our less sensitive method, more extensive dye coupling must be established before dye transfer is detectable. Thus, substantial coupling definitely is present soon after the growth cone passes the cell, and it may occur earlier.

This dye coupling is likely to be mediated by gap junctions, which have been extensively characterized in insects (Bennett, 1978; Spray and Bennett, 1985; Rytse, 1989). These arthropod-type channels, which are slightly larger than those of vertebrate gap junctions, are about 14 Å in diameter and transfer negatively charged and neutral particles up to at least 1000 MW. Accep-

tance characteristics for positively charged particles have been less well characterized, but at least some divalent cations pass through the channels. Thus, once coupling is established, transfer of calcium ions from the pioneer neurons, with a higher internal calcium ion concentration, to the preaxonogenesis neurons with the lower calcium ion concentration, should occur, as it does in other cell types (Saez et al., 1989). With fura-2 imaging, we observed that calcium ion concentration appears reduced within pioneer neurites in the vicinity of coupled preaxonogenesis neurons. If a coupled preaxonogenesis neuron is killed with a UV microbeam, then the local reduction in calcium concentration within the pioneer neurite is no longer observed. Although the possibility that some of this effect may be due to irradiation of pioneer neuron filopodia has not been excluded, it seems likely that calcium ions do flow through gap junctions of these coupled cells.

Transfer of calcium ions through gap junctions has been shown to be an important intercellular signaling mechanism between some coupled cells (Dunlap et al., 1987; Brehm et al., 1989). The calcium current considered here would be expected to elevate calcium concentration within the preaxonogenesis neurons and to reduce calcium concentration in the pioneer growth cones and neurites. What role, if any, this interaction might play has not been determined. The Fe1 and Tr1 preaxonogenesis neurons have been shown to remain capable of axonogenesis and pathfinding even if contact with pioneer growth cones is prevented (Keshishian and Bentley, 1983; Klose and Bentley, 1989). Whether the hypothesized reduction in calcium concentration within the pioneer growth cone or nascent neurite contributes to the high affinity for the preaxonogenesis neuron substrate remains to be tested. The results reported here do suggest, however, that the calcium ion concentration within pioneer growth cones and neurites extending on their *in situ* substrate is selectively altered by encounters with specific guidance features, the preaxonogenesis neurons.

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