Imaging of $\text{Ca}^{2+}$ Dynamics Within the Presynaptic Terminals of Salamander Rod Photoreceptors

Ernest C. Steele, Jr.,1 Xiaoming Chen, P. Michael Iuvone,2 and Peter R. MacLeish1

1Department of Anatomy and Neurobiology, Neuroscience Institute, Morehouse School of Medicine and 2Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia

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Steele, Ernest C., Jr., Xiaoming Chen, P. Michael Iuvone, and Peter R. MacLeish. Imaging of $\text{Ca}^{2+}$ dynamics within the presynaptic terminals of salamander rod photoreceptors. J Neurophysiol 94: 4544–4553, 2005. First published August 17, 2005; doi:10.1152/jn.01193.2004. Although the overall importance of $\text{Ca}^{2+}$ as a mediator of cell signaling and neurotransmitter release has long been appreciated, the details of $\text{Ca}^{2+}$ dynamics within the inner segments of vertebrate rod photoreceptors are just beginning to be elucidated. Even less is known regarding $\text{Ca}^{2+}$ dynamics within the rod presynaptic terminal compartment. Using fura-2 to report changes in intracellular $\text{Ca}^{2+}$ concentration, we imaged the responses of enzymatically dissociated salamander rod photoreceptors retaining intact axons and presynaptic terminals stimulated with a brief depolarizing puff of KCl (30 mM pipette concentration). In the vast majority of cells, the response was a large increase in $\text{Ca}^{2+}$ levels in the terminal compartment, but not in the soma. In contrast, rods exhibited a substantial elevation in somatic $\text{Ca}^{2+}$ levels when depolarized with a brief puff of 100 mM KCl (pipette concentration). These data are consistent with previously reported differences in $\text{Ca}^{2+}$ buffering mechanisms within the somatic and terminal compartments. Additionally, they may reflect the presence of $\text{Ca}^{2+}$ channels having distinct properties within the membranes of the two compartments. Consistent with this hypothesis, fluorescent immunocytochemistry using an antibody against the L-type $\text{Ca}^{2+}$ channel $\alpha_{1C}$ subunit and semiquantitative confocal microscopy revealed a high concentration of immunoreactivity in the membranes of terminals of intact rods compared with the soma. Further investigations using enzymatically dissociated preparations of intact rod photoreceptors retaining their presynaptic terminals will allow further testing of these and other hypotheses regarding the compartmentalized regulation of $\text{Ca}^{2+}$ dynamics within rod photoreceptors.

INTRODUCTION

Synchronized transmission from photoreceptor cells to second-order neurons in the vertebrate retina is mediated by the neurotransmitter glutamate (Copenhagen and Jahr 1989). Glutamate release from photoreceptors is largely $\text{Ca}^{2+}$ dependent (Dacheux and Miller 1976; Dowling and Ripps 1973) and regulated by $\text{Ca}^{2+}$ influx through dihydropyridine-sensitive L-type channels (Schmitz and Witkovsky 1997), although a calcium-insensitive component of release has been reported (Schwartz 1986). Several studies of salamander rod $\text{Ca}^{2+}$ currents and intracellular free $\text{Ca}^{2+}$ dynamics have been reported previously (Bader et al. 1982; Corey et al. 1984; Krizaj and Copenhagen 1998,2003; Krizaj et al. 1999; Nachman-Clewner et al. 1999; Rabl and Thoreson 2002; Stella et al. 2002; Thoreson and Stella 2000; Thoreson et al. 2002, 2003). All of these, however, describe data obtained from rod inner segments for the most part and cite the difficulty in isolating fully intact rods retaining axons and presynaptic terminals. There remains a paucity of information regarding the details of free $\text{Ca}^{2+}$ dynamics within the presynaptic terminals of these cells.

Using the gentle enzymatic dissociation method developed and described by Bader et al. (1978), we have been able to achieve a high degree of success in isolating fully intact rod photoreceptors from the tiger salamander retina. The integrity of our dissociated cells has allowed us to conduct live imaging studies of depolarization-induced $\text{Ca}^{2+}$ dynamics within the presynaptic terminal compartment of tiger salamander rod photoreceptors. To our knowledge, this represents the first extensive study of depolarization-induced $\text{Ca}^{2+}$ dynamics within intact presynaptic terminals of isolated salamander rods to be reported. Our results confirm previously reported findings regarding $\text{Ca}^{2+}$ dynamics of rod photoreceptor somata. In addition, our results present new and extensive data regarding the $\text{Ca}^{2+}$ dynamics of the presynaptic terminals of rods in response to a gentle and brief depolarizing stimulus. These data suggest a great degree of independence in the regulation of $\text{Ca}^{2+}$ levels within the somatic and terminal compartments and underscore the necessity for further studies of $\text{Ca}^{2+}$ currents and dynamics within intact rods retaining their axons and presynaptic terminals. Parts of this study have been previously published in abstract form (Steele et al. 2002).

METHODS

Animals

Neotenic tiger salamanders (Ambystoma tigrinum) were obtained from Kons Scientific Supply (Germantown, WI) and Charles Sullivan (Nashville, TN). All animal procedures conformed to the humane treatment of animals as prescribed by the Society for Neuroscience and were approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee.

Dissociation and culture of retinal cells

Whole retinas were dissected from isolated eyecups in bright light and digested with 10–14 units/mL of papain (Worthington Biochemical, Lakewood, NJ) for 30 min at 25°C in a salt solution (pH 7.2) bubbled for 10 min with a gas mixture of 95% O2:5% CO2 and composed of the following (in mM): 85 NaCl, 25 NaHCO3, 1 Na pyruvate, 3 KCl, 0.5 NaH2PO4, 0.5 CaCl2, 1 cysteine, 0.02 phenol red. The tissue was then rinsed twice with 10 mL of a salt solution (pH 7.2)
containing (in mM): 108 NaCl, 2 HEPES, 1 Na pyruvate, 0.5 MgCl₂, 0.5 MgSO₄, 0.5 NaH₂PO₄, 1.8 CaCl₂, 3 KCl, 1 NaHCO₃, 0.1 choline chloride, 0.02 phenol red, 16 glucose. The retinae were resuspended in another 10 mL of medium supplemented with 16 mM additional glucose to increase density. The retinae were then triturated in approximately 2 mL of this solution to yield single cells. Dispersed cells were plated onto modified tissue culture dishes in which the bottom plastic was replaced with glass coverslips pretreated with goat anti-mouse IgG secondary antibody and Sal-1 primary antibody (Mac-Leish et al. 1983). Bovine serum albumin (BSA) was added to a final concentration of 50 µg/mL. All of these procedures were carried out in bright light. It is therefore a safe assumption that all photopigment of the outer segments was bleached.

**Chemicals**

Ultrapure salts for cell culture solutions were obtained from J. T. Baker (Phillipsburg, NJ). Unless otherwise noted, all other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Calcium imaging**

Plated cells were preloaded with 1 µg/mL fura-2 AM (Molecular Probes, Eugene, OR), a membrane permeable form of the ratiometric free calcium indicator, fura-2, for 30 min to 1 h at 10°C. Cells were then rinsed three times with the balanced salt solution described above before adding BSA to the medium at a final concentration of 50 µg/mL. Fura-2 was excited at 340 and 380 nm using a Lambda DG-4 ultrahigh-speed wavelength switcher (Sutter Instrument, Novato, CA). Two different imaging systems were used to acquire different portions of the data. Using a 50- to 100-ms exposure time for each wavelength, emission (510 nm) images were captured by a thermoelectrically cooled CCD digital camera (Princeton Scientific Instruments, Monmouth Junction, NJ) controlled by Imaging Workbench 2.2 software (Axon Instruments, Union City, CA) or a Cascade 650 CCD camera (Photometrics, Tucson, AZ) controlled by Metamorph 6.1 software (Universal Imaging) run on a personal computer. Images were acquired at a rate of approximately one 340/380 nm pair/s with the Princeton Scientific camera and one 340/380 nm pair/300 ms with the Photometrics camera. Signals were averaged over regions of interest corresponding to the soma (nucleus and perikaryon), the myoid body, the presynaptic terminal, and the outer segment. The 340/380 nm emission fluorescence ratio, which is known to be proportional to intracellular Ca²⁺ concentration (Grynkiewicz et al. 1985), is shown for most data. Buffered solutions containing 0, 100, 225, 351, 602, 1,350, and 3,900 nM free Ca²⁺ from the Molecular Probes fura-2 calcium imaging calibration kit were imaged with our system. The concentrations were then plotted against the resulting 340/380 nm fluorescence ratios generated by our imaging system, and the data fit with a nonlinear regression, which was used to translate portions of the 340/380 nm fluorescence ratio data to Ca²⁺ concentrations. Depolarizing stimuli were provided by brief puffs (≥1 s) of saline solution composed as above, but containing 30 mM KCl (and a corresponding decrease in NaCl concentration) or 100 mM KCl (and a corresponding decrease in NaCl concentration), ejected by positive pressure using a picospritzer from a micropipette with a large diameter (about 50 µm) opening to ensure whole cells were bathed on ejection. All cells were maintained at 10°C using a cooling stage platform during recordings. Cells were exposed briefly to bright light using the halogen lamp of the microscope before imaging with UV light to suppress the photocurrent.

**Immunocytochemistry**

Retinal cells, dissociated and plated as described above, were fixed for 5 min at room temperature with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. Cells were then rinsed three times with 0.1 M PB (pH 7.4) and blocked for 1 h with a blocking solution containing the following: 0.1 M PB (pH 7.4), 2% BSA, 2% normal goat serum, 0.1% Triton X-100. Cells were incubated at 4°C overnight with primary antibodies diluted in blocking solution as follows: 1/50 anti-Ca₁,1.2 (αIC) antibody (Alomone Labs), 1/10 SV-2 (Developmental Studies Hybridoma Bank, University of Iowa). As a negative control, anti-Ca₁,1.2 antibody was preadsorbed for 1 h at room temperature with the CNI1 antigenic peptide (1 µg peptide/1 µg antibody) before incubation with tissue. Cells were rinsed three times with 0.1 M PB at room temperature and then incubated for 1 h at room temperature with a goat anti-rabbit IgG FITC conjugate (to detect Ca₁,1.2) and goat anti-mouse IgG Texas Red conjugate (to detect SV2) diluted 1/50 in blocking buffer. Cells were then rinsed three times with 0.1 M PB before mounting with permanent antifade mounting medium (Vector Labs). Fluorescent images were acquired using a scanning laser confocal microscope system with Flöviev software (Olympus).

**RESULTS**

Depolarization with 30 mM KCl increases Ca²⁺ levels in presynaptic terminals

A survey was conducted on 52 cells (from six animals), most of which possessed preserved axons and terminal compartments. We chose to use a brief puff (≥1 s) of 30 mM KCl without electrical monitoring with an electrode as our depolarizing stimulus. This allowed us to preserve the endogenous buffering capacity of the cells ensuring a more physiologically relevant response. Changes in intracellular free Ca²⁺ levels were monitored as changes in fura-2 fluorescence (340/380 nm ratio). Figure 1A shows a representative rod cell possessing an intact axon and terminal and Fig. 1B shows the corresponding depolarization-induced changes of intracellular Ca²⁺ level in the presynaptic terminal, somatic, and outer segment compartments of this cell. When cells were bathed with a brief puff (1–3 s) of medium containing a high concentration of KCl (30 mM pipette concentration), the majority (44/52) exhibited a rapid and large increase in the intracellular Ca²⁺ level of the terminal compartment with no or only a very small increase in the somatic compartment. Figure 1C summarizes the statistical information for all 44 responding cells. The mean difference between the basal prestimulus and maximal response 340/380 nm ratio in the terminal compartment was 0.275 ± 0.018 (mean ± SE), whereas in the soma was only 0.016 ± 0.002.

Despite some variation in the levels of response from individual cells, the difference between the overall responses of the terminal and somaic compartments was highly statistically significant (Student’s t-test, P < 0.0001). This result was consistent in cells with an intact outer segment (n = 34) as well as cells without an intact outer segment (n = 10), suggesting that the outer segment plays no significant role in regulating the response observed within the terminal. This is consistent with the notion that the photopigment of the outer segment is bleached as a result of their isolation under bright light conditions from light-adapted animals. To demonstrate that the elevation of Ca²⁺ did not arise simply from the mechanical stimulation of stretch-activated channels in the membrane by the puffing on the depolarizing medium, we stimulated cells (n = 4) with medium containing 30 mM KCl, then 3 mM KCl (the same concentration as the bath), and again with 30 mM KCl. A large increase in Ca²⁺ levels was observed only in the
terminal compartment of the cells and only when 30 mM KCl was applied; no response was observed in either compartment when 3 mM KCl was applied. It should also be noted that, although we sometimes noted a difference in the basal 340/380 nm ratio of the somatic and terminal compartments as shown in Fig. 1B, this was not always the case.

**Somatic Ca\(^{2+}\) increase originates in the terminal and enters soma by the axon**

Eight cells in this survey showed an increase in somatic Ca\(^{2+}\) levels on depolarization with 30 mM KCl. Somatic Ca\(^{2+}\) responses were associated with one of the following conditions: 1) as a result of retraction, the terminal structure protruded from the soma with no discernible axon separating the two compartments; and 2) the test rod had an axon of shorter length and/or larger caliber. The rate of change in Ca\(^{2+}\) levels in the soma appeared faster in cells lacking a clear separation of the two compartments by an axon when compared with cells retaining some separation by an axon of shorter length and heavier caliber. For comparison, we defined the somatic region of interest to be the perikaryon and myoid, and did not include the protruding bulge. These morphological and kinetic correlations suggest that the increase in somatic Ca\(^{2+}\) of cells with short, large caliber axons was probably a result of Ca\(^{2+}\) entry from the terminal by the axon.

**Rods lacking obvious terminals show mixed responses to depolarization**

In the process of enzymatic dissociation of the salamander retina, the axons and terminals of many rods are broken off. Furthermore, we have observed that terminals can and will retract after plating, even if attached to the dish. We hypothesized that cells with these different etiologies would possess different Ca\(^{2+}\) profiles: that is, cells possessing terminals in the dissociation would lack a somatic Ca\(^{2+}\) elevation in response to depolarization, whereas cells that had retracted their terminals bearing Ca\(^{2+}\) channels would exhibit a rise in somatic Ca\(^{2+}\) levels in response to depolarization. We therefore randomly sampled isolated rods (n = 15) lacking distinguishable terminals to determine whether two such profiles were represented.

The depolarization-induced change in somatic Ca\(^{2+}\) levels, as measured by changes in 340/380 nm fluorescence ratio, was determined for a group of 15 cells exhibiting no detectable terminal as shown in Fig. 3A. A scatterplot of the changes in 340/380 nm fluorescence ratios is shown in Fig. 3B. Moving average analysis of these data suggests two groups of responses with distinct means indicated by the shown trend lines. Approximately half of these cells (7/15) responded with a significant increase in somatic Ca\(^{2+}\) levels on depolarization with a brief puff of 30 mM KCl [mean Δ 340/380 nm ratio = 0.1900 ± 0.0469 (SE)] with the remaining cells (8/15) showing little or no response [Δ 340/380 nm ratio = 0.0125 ± 0.0037 (SE)]. As illustrated in Fig. 3C, the increase in somatic Ca\(^{2+}\) of responders is statistically indistinguishable from that observed in the terminals of the cells described above possessing intact axons and terminals (P = 0.114) and significantly higher than that observed in the somas of those cells (P < 0.01). As shown in Fig. 3D, the somatic Ca\(^{2+}\) changes of the nonresponders without a visible terminal was indistinguishable from that observed in the somas of cells with intact axons and terminals (P = 0.4). These results are consistent with the notion that cells
without visible axons or terminals showing a large elevation in somatic Ca\(^{2+}\) after depolarization have retracted their axons and terminal compartments after dissociation and plating, whereas those cells not showing a response lost their axon and terminal compartments during the dissociation process. These results also illustrate the necessity of knowing the history of a rod without a visible terminal.

**Depolarization with 100 mM KCl increases Ca\(^{2+}\) levels in somatic and outer segment compartments**

Others (Krizaj and Copenhagen 1998, 2003) previously reported a consistent, large elevation of somatic and outer segment Ca\(^{2+}\) in response to puffing or perfusing salamander rods with 90 mM KCl. We therefore tested to see whether we could elicit dose-dependent responses by depolarizing intact salamander rods retaining presynaptic terminals first with a brief puff of 30 mM KCl and then a brief puff of 100 mM KCl (pipette concentration).

All cells tested (three of three) responded to a brief puff of 30 mM with an elevation in only the presynaptic terminal compartment. When these same cells were then challenged with a brief puff of 100 mM KCl (pipette concentration), a robust elevation in somatic and outer segment Ca\(^{2+}\) levels was observed in addition to the elevation of terminal Ca\(^{2+}\). Changes in the compartmental Ca\(^{2+}\) levels during stimulation first with 30 mM KCl (A) and then 100 mM KCl (B) are shown in Fig. 4 for a representative cell possessing an intact axon and terminal. Interestingly, the Ca\(^{2+}\) levels showed a gradient across the cell, with a steeper slope and maximum amplitude observed over the nuclear region of the soma closest to the axon, and a reduction in slope and amplitude of the response over the myoid region of the soma and the outer segment. We also noted that the response in the terminal compartment consistently and rapidly reached a plateau after depolarization with a brief puff of 100 mM KCl, but not 30 mM KCl, which it maintained for some time before decreasing again. The level in the soma, however, continued increasing in a linear fashion to an apparently higher level than that of the terminal after depolarization with 100 mM KCl.

The gradient across the rod cell compartments suggests that the elevation of Ca\(^{2+}\) in these compartments might be attributable simply to diffusion of Ca\(^{2+}\) from the terminal by the axon. To test this, we imaged the responses to a brief depolarization with 100 mM KCl (pipette concentration) in isolated rods lacking obvious axons or terminals and lacking a response to a brief depolarization with 30 mM KCl (pipette concentration). All cells (four of four) exhibited a rapid rise within regions of interest corresponding to the nuclear soma, the myoid body, and outer segment compartments after depolarization with a brief puff of 100 mM KCl, but not 30 mM KCl, which it maintained for some time before decreasing again. The level in the soma, however, continued increasing in a linear fashion to an apparently higher level than that of the terminal after depolarization with 100 mM KCl.

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and the absence of a response to 30 mM KCl, we assume these cells to be rods that have lost their axon and terminal structures during the dissociation process, and therefore the elevation in Ca\(^{2+}\)/H\(_{11001}\) within the somatic and outer segment compartments to occur directly within these compartments.

Concentration changes in Ca\(^{2+}\) in rod terminals and somata in response to depolarization with KCl

To estimate the actual concentration changes in terminal and somatic Ca\(^{2+}\), we imaged standards of known free Ca\(^{2+}\) concentration and used a nonlinear regression to generate a standard curve relating 340/380 nm fluorescence to Ca\(^{2+}\) concentration. The mean basal 340/380 nm fluorescence ratio of isolated rod terminals (n = 11) was 0.84 ± 0.07 (SD). This increased to 1.88 ± 0.25 (SD) on depolarization with a brief puff of 30 mM KCl (pipette concentration). Although we can estimate the basal Ca\(^{2+}\) concentration in the terminal to be approximately 100 nM, the maximal response level extends beyond the 39 μM maximum reference point on our standard curve; we can therefore say with certainty only that the terminal reaches some concentration >39 μM. A brief puff of 100 mM KCl (pipette concentration) onto isolated rods lacking an obvious axon or terminal (n = 9) and showing no response to 30 mM KCl (pipette concentration) caused somatic 340/380 nm fluorescence ratios to increase from a basal level of 1.01 ± 0.11 (SD) to 4.24 ± 0.25 (SD). We can estimate the basal somatic Ca\(^{2+}\) concentration to be approximately 350 nM. Again, however, we cannot accurately estimate the final Ca\(^{2+}\) concentration after a brief puff of 100 mM KCl as it falls outside the 39 μM maximum reference point of our standard curve.

L-type Ca\(^{2+}\) channel Ca\(_{1.2}\) (α1C)-like subunits are highly localized to rod terminal membranes

One parsimonious interpretation of the differences in the responses of the terminal and somatic compartments to depolarization with lower and higher concentrations of KCl is that there are simply vast differences in the Ca\(^{2+}\) buffering capacity of the two compartments. This interpretation would be consistent with data reported by Krizaj and Copenhagen (2003). Another interpretation, however, is that differences in the density of and/or identity of Ca\(^{2+}\) channels in the membranes of the two compartments could contribute to the disparity in responses of the two compartments to depolarization. Consistent with this, Nachman-Clewner et al. (1999) previously reported dense patches of immunoreactivity in isolated salamander rod terminals using a commercially available antibody (Alomone Labs) against the L-type Ca\(^{2+}\) channel pore forming subunit Ca\(_{1.2}\) (α1C). We therefore used this antibody to compare the proportional distribution of immuno-
reactivity in the somatic and terminal compartments of isolated salamander rods.

Fluorescent detection of immunoreactivity was carried out with a scanning laser confocal microscope system. As seen in the composite confocal image in Fig. 5A, Cav1.2-like immunoreactivity was highly localized to the rod presynaptic terminal membrane of intact isolated salamander rods, where it appeared to be at a much greater density. Whereas some weak immunostaining of the somatic membrane is observed, it is much less uniform and intense than that observed in the terminal membrane. Most of the terminal immunoreactivity was colocalized with synaptic vesicle protein, suggesting a role in vesicular neurotransmitter release (Fig. 5, B and C). Also shown in Fig. 5 is a large isolated Müller cell body. Extending from the crown of this cell body fine Cav1.2-immunoreactive processes that normally envelop the rods and define the outer limiting membrane can be seen. These may in fact account for some of the immunoreactivity observed on the surface of the rod somata that is fine and threadlike in appearance.

To quantitate the observed difference in somatic and terminal immunoreactivity, we compared the maximum fluorescence intensity of somatic and terminal compartments across the lines indicated in Fig. 5A from individual z-sections of several isolated rods (n = 15). Figure 5, D and E shows the distribution of fluorescent intensity across lines within individual z-sections of the somatic and terminal compartments, respectively, of a representative rod. Consistent with previous data from Nachman-Clewner et al. (1999), we observed differences in the intensity of Cav1.2 immunoreactivity across the presynaptic terminal, consistent with concentrations of Cav1.2-like channels in synaptic ribbons surrounding synaptic invaginations within the presynaptic terminals. Although the actual intensity values varied from cell to cell, the maximum fluorescent intensity of the terminal of any given rod was consistently 3.1-fold greater than the maximum fluorescent intensity of the somatic membrane of the same cell. A Student’s t-test analysis of these data revealed this difference to be highly statistically significant (P = 2.5 × 10⁻¹⁸). Preincubation of the Cav1.2 antibody with the antigenic peptide (CNC1) eliminated immunoreactivity (data not shown). Furthermore, we observed immunopositive bulges on the somata of some rods (Fig. 5F) and large, discrete patches of Cav1.2-like immunoreactivity in the somatic membranes of some (Fig. 5G), but not all (Fig. 5H), rods lacking visible terminals. The lower degree of immunoreactivity observed in cells presumed to have lost their axons and terminals during dissociation, may be a reflection of damage to the cells resulting from this loss before fixation. The immunostaining patterns we report are consistent with the
retraction of the terminal resulting in the Ca\(^{2+}\)/H11001 channels of the terminal membrane becoming an integral component of the somatic membrane. Rods lacking visible terminals or significant somatic Cav1.2 immunoreactivity likely represent cells that lost their terminals during dissociation. These immunocytochemical results identify the L-type Ca\(^{2+}\)/H11001 channel subunit Cav1.2, or a closely related isoform, as a plausible mediator of at least some portion of the observed Ca\(^{2+}\)/H11001 entry into the terminal compartment after depolarization with 30 mM KCl (pipette concentration).

Interestingly, we consistently observed strong immunoreactivity in the outer segment region that appears to correspond to the cilium connecting the somatic and outer segment compartments or to the calycal processes.

**DISCUSSION**

Previous studies of changes in salamander rod free Ca\(^{2+}\) levels used brief exposure or perfusion with 90 mM KCl, a very strong depolarizing stimulus. Furthermore, these studies were focused on somatic Ca\(^{2+}\) changes because of the difficulty of isolation of intact rods retaining axons and presynaptic terminals. Here, we provide the first report of Ca\(^{2+}\) changes in the somatic, outer segment, and, most significantly, the termi-
nal compartments of intact salamander rods in response to a much weaker depolarizing stimulus, a brief puff of 30 mM KCl (pipette concentration). The major finding is that in intact rods this brief depolarizing stimulus elicits large increases in intracellular free Ca$^{2+}$ only in the terminal compartment.

We further demonstrate that rods lacking obvious axons or terminals can have one of two distinct responses to depolarization with 30 mM KCl (pipette concentration): 1) little or no elevation in free Ca$^{2+}$ levels or 2) a significant increase in somatic Ca$^{2+}$ levels. Careful morphological and kinetic comparisons make a compelling case that rods exhibiting the different responses represent cells with distinct histories. Specifically, we suggest that nonresponding rods represent cells whose axons and presynaptic terminals were broken off during the dissociation process, whereas responding rods represent cells that have retracted and reabsorbed their axons and presynaptic terminals.

One apparent paradox in our data from rods lacking obvious axons and terminals is the large mean amplitude of Ca$^{2+}$ elevation in responding rods lacking visible axons or terminals. On absorption of the terminal structure into the soma, the terminal membrane becomes contiguous with that of the soma, thus allowing entry into the soma by the calcium channels of the terminal. If all the terminal calcium channels of the absorbed terminal are redistributed to the somatic membrane, then one might expect that the concentration change in free calcium in the soma after stimulation should be much less than that observed in the terminal compartment in response to the same stimulus as a consequence of the much larger volume of the soma. However, we report here that the level of somatic Ca$^{2+}$ elevation induced by depolarization of cells with a brief puff of 30 mM KCl after retraction of the terminal is similar to that observed in the terminals of cells with an intact axon separating it from the soma.

Because our current knowledge of the identities of the players involved in calcium homeostasis and our understanding of the complex interactions between them is very limited within rod photoreceptors, we cannot accurately predict how reabsorption of the terminal will impact somatic calcium homeostasis. Immunochemical staining of salamander rod and cone photoreceptors with an antibody against the ryanodine receptor (RyR) by Krizaj et al. (2003) reveals a concentration of RyR-mediated stores in the presynaptic compartments of rods and cones. In this same report, Krizaj et al. (2003) also demonstrated a significant level of calcium buffering by the mitochondria within the soma of salamander rods and cones. Krizaj and Copenhagen (1998) and Morgans et al. (1998) also demonstrated that the plasma membrane Ca$^{2+}$ ATPase (PMCA) is highly localized to the terminals and somatic base of rod photoreceptors and plays an important role in Ca$^{2+}$ extrusion. The strategic positioning of the PMCA highlights its importance as a barrier between the terminals and somata, maintaining a separation of calcium dynamics in the two compartments. After reabsorption of the terminal and integration of terminal structures into the soma, the stimulus-induced calcium changes will occur within the context and under the limitations of the new environment, which is a hybrid of what were originally compartmentally separated components. Beyond the obvious redistribution of intracellular stores, other consequences might include a change in the conductance of the calcium channels by phosphorylation or interactions with other calcium-binding proteins of the soma or in the efficacy of amplification of the calcium signal after initial influx of calcium.

One parsimonious interpretation of our study of rods lacking axons and terminals is that no Ca$^{2+}$ channels are present within the somatic membrane. However, in the present study, we were also able to corroborate previous reports of somatic and outer segment Ca$^{2+}$ elevations in rods depolarized with a stronger stimulus, 100 mM KCl (pipette concentration), while confirming that these same cells exhibit no detectable Ca$^{2+}$ elevation in these compartments when depolarized with 30 mM KCl (pipette concentration). These results demonstrate that the response of salamander rods to depolarization is dose dependent, with terminals responding to smaller depolarizing stimuli and somata requiring larger depolarizing stimuli to induce a large increase in somatic Ca$^{2+}$ levels. When this larger stimulus was administered to cells retaining terminals, however, Ca$^{2+}$ levels in the terminal compartment appeared to plateau at approximately the same maximal level observed in response to depolarization with 30 mM KCl (pipette concentration). This plateau may reflect the activation of the Ca$^{2+}$-activated anion channel after initial Ca$^{2+}$ entry into the terminal compartment. This channel was functionally localized to the presynaptic terminals of salamander rods by MacLeish and Nurse (2000). Subsequently, it has been shown by Thoreson et al. (2000, 2003) that Ca$^{2+}$- and Ca$^{2+}$-activated Cl$^{-}$ channels may contribute to the regulation of presynaptic Ca$^{2+}$ currents involved in synaptic transmission from rod photoreceptors.

An alternative interpretation of this plateau in the terminal response is saturation of fura-2 within the terminal compartment arising from a larger surface area/volume in the terminal. Differences in compartmentalization or hydrolysis of fura-2 AM to fura-2 free acid could further compromise accurate reporting of free Ca$^{2+}$ levels within the terminal compartment. Further evidence of fura-2 saturation in our experiments is our inability to accurately estimate the Ca$^{2+}$ concentration within the terminal and soma after depolarization with 100 mM KCl, as a result of obtaining 340/380 nm fluorescence ratios that were far beyond the highest reference point of our calibration curve (about 39 μM). Additional studies using weaker depolarizing stimuli (lower KCl concentrations) and/or shorter duration of depolarizing stimuli might prove beneficial for improved accuracy in estimation of terminal and somatic Ca$^{2+}$ concentrations after depolarization with different KCl concentrations. Alternatively, the use of available Ca$^{2+}$ reporters with higher Kd values than fura-2 may prove useful for studying Ca$^{2+}$ dynamics in response to stronger stimuli.

A significantly lower density of the same channel as that of the terminal membrane and/or a different channel, requiring a stronger depolarizing stimulus, may be present within the somatic membrane and mediate the somatic Ca$^{2+}$ elevations we and others have observed after depolarization with 100 or 90 mM KCl, respectively. The immunocytochemical staining profile using an antibody against the L-type Ca$^{2+}$ channel Ca$^{1.2}$ (α1C) subunit provided in this study argues against uniform distribution of Ca$^{2+}$ channels and supports the notion that this subunit, or a highly related one, is present at a much higher density in the terminal than in the soma and may therefore mediate some portion of the terminal Ca$^{2+}$ changes we observed after depolarization with 30 mM KCl. Consistent with the hypothesis of a higher density of Ca$^{2+}$ channels within...
the terminal membrane, Thoreson et al. (2000) reported the inability to consistently observe single Ca\(^{2+}\) channel activity from somatic membrane patches of isolated salamander rods, while consistently observing Ca\(^{2+}\) channel recordings from patches of terminal membranes or the base of the somatic membranes using the cell attached mode of the patch-clamp technique. These authors also suggested a decreased somatic density of Ca\(^{2+}\) channels in situ.

The observation of prominent staining in the region of the connecting cilium or calycal processes of the isolated rods in the present study is noteworthy. The cilium acts as the main conduit through which vesicles containing newly synthesized proteins, including rhodopsin, are transported from the trans-Golgi complex to the membranes of the outer segment discs (Wolfrum and Schmitt 2000). This transport along the cilium is known to be mediated, at least in part, by the Ca\(^{2+}\)-dependent motors myosin VIIa and kinesin II (Williams 2002). Also, recent data suggest that the light-induced exchanges of the visual G protein transducin between the outer and inner segment of rod photoreceptors occurs through the narrow connecting cilium and that this process is regulated by the centrin Ca\(^{2+}\) concentration of rod photoreceptors occurs through the narrow connecting cilium and that this process is regulated by the centrin Ca\(^{2+}\) binding protein(s) (Pulvermuller et al. 2002). It is therefore an interesting possibility that these Ca\(^{2+}\)-dependent processes may be regulated in part by an L-type Ca\(^{2+}\) channel in the membrane of the connecting cilium.

The understanding that the Ca\(_{1.2}\) immunocytochemical data are merely correlated and were obtained by using a polyclonal antibody against rat brain Ca\(_{1.2}\) on amphibian tissues warrants caution in interpretation. Henderson et al. (2001) previously reported intense labeling of structures that could be rod terminals within the outer plexiform layer of salamander retinal sections using an antibody against the highly related L-type Ca\(^{2+}\) channel Ca\(_{1.3}\) (α1D) subunit. Pharmacological and physiological data also suggest that the L-type Ca\(^{2+}\) channel Ca\(_{1.3}\) (α1D) subunit may mediate most of the Ca\(^{2+}\) current during light-flash responses observed in salamander cones (Wilkinson and Barnes 1996) and tree shrew cones (Taylor and Morgans 1998).

An alternative explanation for the difference in terminal and somatic Ca\(^{2+}\) channel response properties is that the Ca\(^{2+}\) channel pore-forming subunits themselves may represent distinct Ca\(^{2+}\) channel subtypes, isoforms, or splice variants of the same isoform within the two different compartments, each with unique physiological ranges of operation and only one of which is well recognized by the anti-Ca\(_{1.2}\) antibody. Use of different Ca\(^{2+}\) channels (different subtypes, isoforms, or splice variants of the same isoform) with distinct properties within the terminal and somatic membranes could confer a degree of separation between voltage control of transmitter release from the terminal and other functions such as signal forwarding through the soma and regulation of gene expression in the nucleus. Compartmentalization of L-type and T-type channels was previously reported for both isolated and intact retinal mouse bipolar cells (de la Villa et al. 1998; Satoh et al. 1998). L-type currents were previously reported to shape the voltage response of depolarizing bipolar cells of goldfish (Burrow and Lagnado 1997). More recently, Berntson and Morgans (2003) reported evidence for the presence of Ca\(_{1.4}\) (α1F) at the active zones of mouse bipolar cells and suggest a role in regulating glutamate release from bipolar cell terminals. Salamander rods may therefore possess a similar compartmentalization of distinct Ca\(^{2+}\) channel subtypes or isoforms.

Careful consideration of the depolarizing stimuli used in our study suggests that our inability to detect a significant change in somatic intracellular Ca\(^{2+}\) levels in response to the application of 30 mM KCl is likely explained by an insufficient number of somatic Ca\(^{2+}\) channels being opened to produce a detectable signal with our system. The Nernst equation predicts the membrane potential of a cell resting in 3 mM KCl to be depolarized by about 60 mV after a 30 mM KCl stimulus and about 91 mV after a 100 mM KCl stimulus, assuming that the membrane is permeable only to potassium ions. Although these estimations of membrane potential differences do not take into account the depolarizing contribution of the Ca\(^{2+}\) channels or any other channels themselves, they suggest a very large difference in the membrane potential achievable in cells depolarized by 30 mM KCl as compared with 90 or 100 mM KCl, and suggest that the membrane potential of cells depolarized with 30 mM KCl is likely to be about 30 mV more hyperpolarized than that of cells depolarized with 90 or 100 mM KCl. Capovilla et al. (1980) reported a membrane potential of close to −20 mV for amphibian rods exposed to light in the presence of 26 mM potassium. We think that this value is at the upper limit for the membrane potential of light-adapted rods under this condition because the recordings were made with micropipettes, which tend to underestimate the actual value of the membrane potential. In support of this claim is the observation that Bader et al. (1978) reported a resting potential of dark-adapted (HVA) L-type Ca\(^{2+}\) channels on amphibian rods exposed to light in the presence of 26 mM potassium. We think that this value is at the upper limit for the membrane potential of light-adapted rods under this condition because the recordings were made with micropipettes, which tend to underestimate the actual value of the membrane potential. In support of this claim is the observation that Bader et al. (1978) reported a resting potential of dark-adapted rods of −45 mV compared with that of −30 mV reported by Capovilla et al. (1980). Thus we can estimate the membrane potential under our experimental conditions with 30 mM KCl to be closer to −35 mV, just on the edge of the activation threshold typically reported for high-voltage-activated (HVA) L-type Ca\(^{2+}\) channels, and therefore not as likely to activate these channels on the cell body. Similarly, we can estimate the membrane potential under our experimental conditions with 100 mM KCl to be closer to 0 mV, a potential typically associated with maximal Ca\(^{2+}\) currents by HVA L-type Ca\(^{2+}\) channels, thus explaining the observation of maximal elevations in somatic Ca\(^{2+}\) after exposure to this depolarizing stimulus.

Although our results do not allow us to completely and precisely determine which of the above scenarios best explains the compartmentalization of depolarization-induced Ca\(^{2+}\) elevation, they clearly indicate a significant difference in voltage-controlled Ca\(^{2+}\) dynamics of the somatic and terminal compartments and underscore the importance of carefully studying Ca\(^{2+}\) currents and dynamics within the two compartments of intact rods retaining preserved presynaptic terminals, to better understand the cellular basis for these differences.

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REFERENCES


