Ion Channel Compartments in Photoreceptors: Evidence from Salamander Rods with Intact and Ablated Terminals

Peter R. MacLeish and Colin A. Nurse

Neuroscience Institute
Dept. Anatomy and Neurobiology
Morehouse School of Medicine
Atlanta, Georgia 30310-1495

1 Present address:
Department of Biology
McMaster University
Hamilton, Ontario,
Canada, L8S 4K1

Running Head: Ion channel compartments in salamander rods

Contact Information:

Dr. Peter R. MacLeish
Neuroscience Institute
Morehouse School of Medicine
720 Westview Drive, S.W.
Atlanta, Georgia 30310-1495
Phone: (404) 756-5786
Fax : (404) 752-1078
E-mail: pmacleish@msm.edu
Abstract

Vertebrate photoreceptors are highly polarized sensory cells in which several different ionic currents have been characterized. In the present study, we used whole-cell, voltage-clamp and optical imaging techniques, the former combined with micro-surgical manipulations, and simultaneous recording of membrane current and intracellular calcium signals to investigate the spatial distribution of ion channels within isolated salamander rods. In recordings from intact rods with visible terminals, evidence for five previously-identified ionic currents was obtained. These include two Ca\(^{2+}\)-dependent currents, i.e. a Ca\(^{2+}\)-dependent chloride current \(I_{Cl(Ca)}\) and a large-conductance Ca\(^{2+}\)-and voltage-dependent K\(^+\) or BK current \(I_{K(Ca)}\), and three voltage-dependent currents, i.e. a delayed-rectifier type current \(I_{K(V)}\), a hyperpolarization-activated cation current \(I_h\), and a dihydropyridine-sensitive L-type calcium current \(I_{Ca}\). Of these, \(I_{Cl(Ca)}\) was highly correlated with the presence of a terminal; rods with visible terminals expressed \(I_{Cl(Ca)}\) without exception (n=125), whereas ~71% of rods (40/56) without visible terminals lacked \(I_{Cl(Ca)}\). More significantly, \(I_{Cl(Ca)}\) was absent from all rods (n=33) which had their terminals ablated, and recordings from the same cell before and after terminal ablation led, in all cases (n=10), to the loss of \(I_{Cl(Ca)}\). In contrast, \(I_{K(Ca)}\), \(I_{K(V)}\), and \(I_h\) remained largely intact after terminal ablation, suggesting that they arose principally from ion channels located in the soma and/or inner segment. The outward \(I_{K(Ca)}\) in terminal-ablated rods was reversibly suppressed on ‘puffing’ a Ca\(^{2+}\)-free extracellular solution over the soma, and was markedly enhanced by the L-type Ca\(^{2+}\) channel agonist, Bay K 8644 (0.1-2 M). These data indicate that rod photoreceptors possess discrete targeting mechanisms that preferentially sort ion channels mediating \(I_{Cl(Ca)}\) to the terminal.
**Introduction**

Neurons and other cell types express various ion channels and receptors that are distributed within distinct compartments or domains. This differential distribution appears critical for the ability of neurons and receptor cells to receive and transduce internal or external signals, and relay appropriate physiological responses (Trimmer and Rhodes, 2004). There is compelling evidence for a heterogeneity in the distribution of ion channels that underlie membrane conductances (Pyott et al. 2004; Trimmer and Rhodes, 2004). For example, a combination of patch-clamp recording and optical imaging in tissue slices has shown that L-type Ca\(^{2+}\) channels are concentrated in presynaptic terminals of cerebellar basket cells (Llano et al. 1997) and retinal bipolar cells (Protti and Llano, 1998), giving rise to discrete hot spots of Ca\(^{2+}\) entry. This spatial heterogeneity is not restricted to Ca\(^{2+}\) channels, since patch-clamp and immunofluorescence studies have also revealed a preferential sorting of voltage-gated K\(^{+}\), as well as voltage- and Ca\(^{2+}\)-activated BK, channels between the soma and terminals in the central nervous system (Southan and Robertson, 1998; Trimmer and Rhodes, 2004; Misonou et al. 2006). The Ca\(^{2+}\)-activated BK channels, encoded by Slo1 \(\alpha\) subunits, have been well-studied and their expression pattern indicates a high accumulation in axons and nerve terminals of central neurons (Trimmer and Rhodes, 2004; Misonou et al. 2006). On the other hand, Ca\(^{2+}\)-activated BK channels were surprisingly found clustered in the the apex of mouse inner hair cells away from the basal synaptic sites (Pyott et al. 2004), thereby raising questions about their physiological function.

Vertebrate photoreceptors represent a class of highly polarized cells, whose morphology is characterized by two major specializations (see Wässle and Boycott,
At the apical end, the outer segment is specialized for phototransduction, whereas at the basal pole, the synaptic terminal releases neurotransmitter onto second order cells. Several studies suggest that the light-induced voltage response and modulation of transmitter release from photoreceptors are determined, not only by events in the outer segment, but also by a variety of conductance mechanisms in the soma/inner segment and terminals (Attwell et al. 1982; Bader et al. 1982; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Yagi and MacLeish, 1994). In isolated light-adapted salamander rods, five currents have been identified in voltage clamp studies (Attwell et al. 1982; Bader et al. 1982; Corey et al. 1984). These consist of two Ca\(^{2+}\)-dependent currents, i.e. Ca\(^{2+}\)-activated K\(^+\) current (I\(_{\text{K(Ca)}}\)) and Ca\(^{2+}\)-activated Cl\(^-\) current (I\(_{\text{Cl(Ca)}}\)), and three voltage-dependent currents, i.e. a hyperpolarization-activated current I\(_{\text{h}}\), a TEA-sensitive delayed rectifier type current I\(_{\text{K(V)}}\), and a dihydropyridine-sensitive L-type calcium current (I\(_{\text{Ca}}\)). There is strong evidence that the L-type Ca\(^{2+}\) channel density is highest in the synaptic terminal of salamander and mammalian rod photoreceptors, giving rise to non-uniform or compartmentalized rises in intracellular calcium (Morgans et al. 1998; Nachman-Clewnner et al., 1999; Krizaj and Copenhagen, 2002; Steele et al. 2005). However, little is known about the relative distribution of the remaining ion channels, though Ca\(^{2+}\)-activated K\(^+\) or BK channels were reported to be highly localized in salamander rod terminals (Xu and Slaughter, 2005). To address this, we used patch clamp techniques to record whole-cell currents in isolated salamander rods that were either morphologically intact or lacking terminals as a result of the dissociation procedure or micro-ablation. We obtained evidence for the presence of functional L-type Ca\(^{2+}\) channels in rods with ablated terminals. Interestingly, the two Ca\(^{2+}\)-dependent currents,
ICl(Ca) and IK(Ca) showed a different spatial distribution with ICl(Ca) being highly localized to the rod terminal. In contrast, IK(Ca) and the other voltage-dependent currents IK(V) and Ih remained relatively unchanged in terminal-ablated rods, suggesting they arose principally from the soma and inner segment. We also used simultaneous recording of membrane current and of changes in intracellular calcium, using fura-2, to confirm which cellular compartment is contribution to ICl(Ca).

Materials and Methods

Cell dissociation: Dissociation of salamander retina was carried out using enzymatic digestion with papain, followed by trituration, as previously described (Lam 1972; MacLeish et al. 1983). The cell suspension was plated into central wells of modified 35 mm culture dishes that were previously coated with Sal-1 antibody to aid cell attachment (MacLeish et al. 1983). The well was formed by sealing a glass cover slip to the underside of the culture dish in which a hole was drilled. The surface was prepared by first adding 100 µl of affinity-purified goat anti-mouse antibody (0.2 mg/ml) for > 1 hr. The well was rinsed once with salt solution, and then 100 µl of Sal-1 hybridoma supernatant were added for > 1 hr. Unbound antibody was removed by rinsing three times with salt solution before cells were plated. The dispersed cells were grown in a medium containing 108 mM NaCl, 3 mM KCl, 2 mM HEPES, 1 mM NaHCO₃, 0.5 mM NaH₂PO₄, 1 mM sodium pyruvate, 0.5 mM MgCl₂, 0.5 mM MgSO₄, 16 mM glucose, 1.8 mM CaCl₂, and 100 µg/ml bovine serum albumin (BSA). Cells were maintained in a humidified chamber at 10°C in air, for up to 3 days.
**Whole-cell recording:** The methods followed those of Hamill et al. (1981). Briefly, dishes were mounted on a cooled stage of a Zeiss Axiovert 35 or 200 M microscope equipped with phase-contrast optics. Patch-clamp pipettes were pulled on a BB-CH-PC (Mecanex S.A., Geneva, Switzerland) or Flaming/Brown P-97 (Sutter Instruments Co. Novato, CA) electrode puller and had a tip diameter of ~ 1µm and a resistance of approximately 10 MΩ. Membrane rupture was achieved by gentle suction applied to the inside of the pipette following seal formation. For most experiments, the composition of the pipette solution was as follows (in mM): KCl 108, NaCl 10, HEPES 10, EGTA 0.05, MgCl2 5, and ATP 1. The pH was adjusted to 7.0-7.2. For a few experiments the pipette EGTA concentration was increased to 5 mM as described in a recent study (Xu and Slaughter, 2005). Also, in some experiments, 75 mM gluconate replaced chloride in the pipette. Since these pipette modifications did not affect the main conclusions of the present study, data were pooled where appropriate. In experiments designed to block K⁺ currents, cesium was substituted for K⁺ in the pipette solution and tetraethylammonium (TEA) chloride was added to the bath. Data acquisition and analysis were carried out using pCLAMP software version 6.0.3 or 9.2 (Axon Instruments, Inc., Union City, CA).

**Intracellular Ca²⁺ measurements:** Intracellular calcium levels were monitored using fura-2 and dual-wavelength stimulation (340/380 nm) while recording at 510 nm. Cells were incubated in 10 µg/ml fura-2 AM (Molecular Probes) for 30 minutes, rinsed three times with amphibian salt solution and kept at 10°C until needed. Light stimulation and data acquisition were performed by a computer controlled system, Stallion 4.1, from Intelligent Imaging Innovations. To reduce the bleaching effects over the long recording
times, cells were stimulated for 10 msec at 340 nm and at 380 nm followed by 500 msec of no stimulation. The camera was a Zeiss AxioCam HSm mounted on a Zeiss Axiovert 200M. The objective was a Zeiss Fluar 40 x oil-immersion NA 1.3. Images were typically binned 4 x 4. Square regions of interest (ROIs) were used as permitted by the software package. For the terminal, the ROI was the smallest square that included the terminal. Background subtraction of the glass coverslip was applied to all cells. The optical recording was externally synchronized to pCLAMP to compare changes in calcium with electrophysiological responses. The optical system was calibrated using standards from Molecular Probes and concentration estimates were obtained using a Kd of 225 nM.

**Results**

The polarized structural features of rod photoreceptors, including outer and inner segment, axon-like projection and terminal enlargement, are readily retained after isolation and attachment to the substrate *in vitro* (MacLeish et al. 1983; MacLeish and Townes-Anderson, 1988; Mandell et al. 1993). In a given culture, however, the dissociation procedure yielded a variable proportion of intact rods, rods with no discernable outer segment, and rods with terminals that were either missing, or in various stages of retraction. In the present study, we also took advantage of our ability to ablate the terminals of selected rods with the tip of a patch pipette, without overt damage to the rest of the cell (for example, Fig. 2A1, A2). Thus, patch-clamp recordings of membrane currents could be obtained from rods that were: (i) morphologically intact (with
prominent terminals); (ii) lacking visible terminals; and (iii) lacking terminals, as a result of mechanical micro-ablation under visual guidance.

**General properties of I\textsubscript{Cl(Ca)} in intact photoreceptors**

A Ca\textsuperscript{2+}-dependent chloride current, I\textsubscript{Cl(Ca)}, has been described in vertebrate photoreceptors (Bader et al. 1982; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Yagi and MacLeish, 1994; Morgans et al. 1998). However, whether or not it is located predominantly in the soma or terminals (or both) is presently unclear. This current is usually observed as an inward tail current, following repolarizations to voltages below E\textsubscript{Cl} from prolonged depolarizing steps that facilitate calcium entry. In the present study, we found that I\textsubscript{Cl(Ca)} could readily be recorded from intact rods with patch pipettes containing either high (Fig. 1A) or low (not shown) chloride concentrations. These currents resembled those recorded from isolated salamander photoreceptors in previous studies (Bader et al. 1982; Barnes and Hille, 1989). With high [KCl] pipettes, the peak amplitude of the inward I\textsubscript{Cl(Ca)} tail current in intact rods varied typically between 100 and 500 pA (mean ± S.E.M. = 268 ±33 pA; n=15); the duration of I\textsubscript{Cl(Ca)}, measured from the beginning to the end of the tail current, showed broad variability (see Fig. 1), lasting typically between 1 and 12 sec (mean = 5.5 ± 0.9 sec; n = 12).

In many cells recorded with high [KCl] pipettes, I\textsubscript{Cl(Ca)} was robust, and its presence was revealed during moderate depolarizing steps to ~ –30 mV by a prominent inward current superimposed on the usual outward K\textsuperscript{+} current at these potentials (e.g. Fig. 1 A; arrow). Consistent with chloride being the main charge carrier, this inward current reversed near 0 mV (i.e. the chloride equilibrium potential), in 3 experiments where
outward K⁺ currents were abolished using CsCl pipettes and bath applied TEA (15 mM; e.g. Fig. 1B). The dependence of I_{Cl(Ca)} on entry of extracellular Ca²⁺ was demonstrated in experiments in which Ca²⁺ entry was substantially reduced or abolished by ‘puffing’ Ca²⁺-free solutions over the cell (n=8), or by recording in the presence of 150 µM cadmium (n = 5), or the L-type Ca²⁺ channel blocker, 10 µM nitrendipine (n= 12). An example of the reversible blockade of I_{Cl(Ca)} (and of outward current) during perfusion with 150 µM cadmium is shown in Fig. 1C1-C3.

**Evidence that I_{Cl(Ca)} requires presence of rod terminal**

The frequency of successful recordings of I_{Cl(Ca)} from morphologically intact rods in normal extracellular medium, with KCl- or K gluconate-filled pipettes, was 100% (n = 125). In contrast, we observed that in the majority (~71%) of cases (n= 40/56), I_{Cl(Ca)} was conspicuously absent in rods without visible terminals, when viewed under phase contrast microscopy. These observations led us to hypothesize that the terminal was required for functional expression of I_{Cl(Ca)} in rod photoreceptors. To test this hypothesis, we carried out repeated recordings from the same rod, one before and another after the terminal was ablated with the tip of a patch pipette. In all such cases (n = 10) I_{Cl(Ca)}, present before terminal ablation, was no longer present after ablation. Further, in 23 additional cells, the terminal was ablated before the initial recording and in each case I_{Cl(Ca)} was not detectable. An example of repeated recordings from the same freshly-isolated rod, where I_{Cl(Ca)} was abolished after terminal ablation, is shown in Fig. 2B1, B2.

We used several criteria to validate that overt cell damage following terminal ablation did not account for the loss of I_{Cl(Ca)} reported above. First, all cells were
discarded when terminal ablation led to morphological changes in the cytoplasm of the soma that signaled loss of integrity, injury, or cell death; this was usually apparent within 45 sec of terminal ablation and occurred in ~30% of the experiments. Second, even when cells appeared ‘healthy’ after terminal ablation, whole-cell recording was discontinued if the holding current at -70 mV was < 50 pA; in the majority of recordings from morphologically-intact rods the holding current varied typically between 10 and 30 pA. Third, we considered acceptable, repeated recordings from the same cell where there was minimal change in magnitude of the voltage-activated outward K⁺ current during voltage steps to 0 mV (see example, Fig. 2D). This voltage represents the chloride equilibrium potential (ECl), where K⁺ outward current is dominant. Data from a group of 5 cells examined in this way are summarized in Fig. 2E, where the mean (± S.E.M) outward current before and after terminal ablation was not statistically different (P>0.05) during voltage steps from -70 mV to 0 mV.

During synapse maturation in vivo and in vitro, nerve cells utilize special targeting mechanisms that result in the accumulation of specific ion channels in presynaptic terminals (Trimmer and Rhodes 2004; Misonou et al. 2006). The terminal localization of ICli(Ca) in acutely-isolated rods, and the fact that the majority of these rods had lost contact with their synaptic partners and other adjacent cells, led us to ask whether ICli(Ca) retained its polarized distribution after short-term culture. As exemplified in Fig 2 C1, C2, ICli(Ca) was still preferentially localized to the terminal even after 56 hr in culture (n = 2), since it was lost after terminal ablation. Thus, the functional polarity of ICli(Ca) in isolated rods can be maintained in culture for >2 days without cell-cell or synaptic contact.
In addition to eliminating $I_{\text{Cl(Ca)}}$, terminal ablation also removes an important source of $\text{Ca}^{2+}$ entry into the photoreceptor via L-type $\text{Ca}^{2+}$ channels located in the terminal (Krizaj and Copenhagen, 1998; Thoreson et al. 2003; Steele et al. 2005). This led us to investigate further whether any residual $I_{\text{Cl(Ca)}}$ could be unmasked in terminal-ablated rods by increasing the calcium load. To address this, we recorded from terminal-ablated rods in the presence of the L-type $\text{Ca}^{2+}$ channel agonist Bay K 8644 (Nowycky et al. 1985). As exemplified in Fig. 3 using ramp depolarizations, $I_{\text{Cl(Ca)}}$, visible in intact rods as a robust inward tail current and producing a small inward current inflection during the ramp (arrow in Fig. 3A), was negligible in rods with ablated terminals even when the $\text{Ca}^{2+}$ load was enhanced with 0.5 $\mu$M Bay K 8644 (Fig. 3B1-B3; n = 7). Thus, our inability to unmask $I_{\text{Cl(Ca)}}$ in terminal ablated rods with a high calcium load points to specific targeting of $I_{\text{Cl(Ca)}}$ to the terminal.

**Intracellular calcium imaging**

We performed calcium imaging experiments on cells filled with fura-2 and compared the time course of the rise and fall of intracellular calcium in different cellular compartments with those of the simultaneously-recorded tail current. An applied voltage step to 0 mV loaded the cell with calcium and, following repolarization to -70 mV, both the intracellular calcium transient and tail current were measured. The maximal amplitude of the tail current differed among the cells as did the maximal activity of intracellular calcium. We did, however, observe a consistent relationship between the duration of the tail current and that of the calcium changes in the terminal. Fig. 4 shows the responses in 3 cells where it is easy to discern the greater similarity and tight correlation between the
time course of the calcium changes in the terminal and time course of the tail current, as compared to calcium changes in the soma. The graph in 4D summarizes the relationship between the duration at half maximal amplitude (t1/2) for terminal and soma calcium concentration changes with that for the tail current. The t1/2 for terminal calcium and for the tail current fell along a 45° line, as expected for the tight correlation between the two variables, while results for the soma showed much scatter and were poorly correlated. These results strengthen the claim that \( I_{\text{Cl(Ca)}} \) is restricted to the terminal.

**Calcium-dependent K⁺ current, \( I_{\text{K(Ca)}} \) is robustly-expressed in terminal-ablated rods**

In contrast to \( I_{\text{Cl(Ca)}} \), the other major Ca\(^{2+}\)-dependent current in rods, \( I_{\text{K(Ca)}} \), was robustly expressed in rods with ablated terminals. At positive step potentials, the outward current in intact rods consists mainly of \( I_{\text{Cl(Ca)}} \), \( I_{\text{K(Ca)}} \), and voltage-activated, delayed rectifier-type \( I_{\text{K(v)}} \) currents. In the absence of selective blockers of \( I_{\text{Cl(Ca)}} \), we studied K⁺ currents in relative isolation using terminal-ablated rods, since these lack \( I_{\text{Cl(Ca)}} \) and the calcium current was small. As illustrated in Fig. 3B1-B3, outward current in terminal-ablated rods was greatly enhanced (>2x) in the presence of Bay K 8644 (n =7), indicating the presence of both dihydropyridine-sensitive L-type Ca\(^{2+}\) and \( I_{\text{K(Ca)}} \) currents in soma and/or inner segment. Additional experiments supported a robust expression of \( I_{\text{K(Ca)}} \) in terminal-ablated rods. First, as exemplified in Fig. 5A1-A3, when nominally Ca\(^{2+}\)-free solutions were applied from a ‘puffer’ pipette positioned close to the soma, outward K⁺ current was strongly and reversibly suppressed (see also Table 1); current suppression by low Ca\(^{2+}\) occurred at all voltages positive to –30 mV. Second, a characteristic ‘hump’ or N-shape
in the I-V relation that is typical of Ca\(^{2+}\)-activated K\(^+\) currents could readily be demonstrated during whole-cell recordings from terminal-ablated rods (n=11, see Fig. 5 A3, A4). In a population of 5 cells with ablated terminals, the mean steady-state current at +30 mV was 1448 pA (± 141 pA, S.E.M.). For three of these cells, the mean steady-state current at +30 mV in calcium-free solution was 316 pA (± 37 pA, S. E.M.) and the recovery current was 1297 pA (± 81 pA). In intact rods, the outward current was also markedly suppressed by 20-60 nM charybdotoxin (ChTx; see also, Xu and Slaughter, 2005), a selective blocker of Ca\(^{2+}\)-activated large-conductance, maxi-K or BK channels (Miller et al., 1985; Galvez et al., 1990). The magnitude of this suppression by ChTx was ~73% (step to +20 mV), a value similar to that seen in Ca\(^{2+}\)-free solutions and in intact rods exposed to 150 µM Cd\(^{2+}\) (Table 1). Taken together, these data indicate that Ca\(^{2+}\)-dependent BK channels are robustly expressed in terminal-ablated rods and contrast with those reported in a recent study (Xu and Slaughter, 2005).

**IK(V) and Ih are functionally expressed in terminal-ablated rods**

Two other voltage-dependent currents have been described in salamander rods, i.e. a Ca\(^{2+}\)-insensitive, delayed-rectifier type current, IK(V) and a non-selective cation current, Ih that is activated by hyperpolarization (Bader et al. 1982). As exemplified in Fig. 5 A2, A5, a detectable voltage-dependent outward current, IK(V), persisted when Ca\(^{2+}\)-dependent K\(^+\) currents were blocked in terminal-ablated rods. In general, this current was considered to represent the major portion of the residual voltage-dependent current evoked during depolarizing steps in the presence of agents designed to block the Ca\(^{2+}\)-dependent K\(^+\) current, e.g. 150 µM Cd\(^{2+}\), nominally Ca\(^{2+}\)-free solution, 10 µM
nitrendipine, or 20-60 nM ChTx. Since all these agents, except ChTx, block Ca\textsuperscript{2+} entry and therefore indirectly block I_{\text{Cl(Ca)}}, they provide an estimate of I_{K(V)} in both terminal-ablated and intact rods. As indicated in Table 1, these treatments reduced the outward current at +20 mV to <200 pA, a value less than ~30% of the total control outward current. The fact that the magnitude of this residual current was similar in rods with and without ablated terminals (Table 1; see also Fig. 2D, E for step to 0 mV), suggests its predominant location was in the soma and/or inner segment. This residual I_{K(V)} current appeared sensitive to 2-3 mM 4-AP (Table 1) and 15 mM TEA (n=2; not shown), known blockers of delayed-rectifier K\textsuperscript{+} currents in rod photoreceptors (see Bader et al. 1982; Barnes and Hille, 1989).

Similarly, comparison of whole-cell currents, before and after terminal ablation, revealed that I_h was located in the soma and/or inner segment. Figure 6A1, A2 shows an example of recordings obtained during hyperpolarizing voltage steps in the same cell before and after terminal ablation. The amplitude and time course of the hyperpolarizing currents were virtually identical to those of I_h reported by Bader et al. (1982). The bar chart (Fig. 6B) summarizes the results for a population of 3 cells and shows that the magnitude of the steady-state I_h current at –120 mV is virtually unchanged after terminal ablation.

Discussion

In the present study we identified a compartmentalization of ion channel function in salamander rod photoreceptors, which represent a class of highly polarized sensory cells. The outer segment is specialized for phototransduction, containing photopigment and the
machinery for the visual cascade that lead to regulation of cGMP-gated cationic channels during the light response. However, though up to five additional ionic conductances have been described, their relative distribution was unclear, with the exception of the voltage-gated L-type Ca\(^{2+}\) channels that appear highly concentrated in the terminal (Morgans et al. 1998; Krizaj and Copenhagen, 2002; Steele et al. 2005). Using a combination of whole-cell recording and micro-ablation procedures, we show that in contrast to the remaining currents, ion channels mediating the Ca\(^{2+}\)-dependent chloride current, I_{Cl(Ca)}, is compartmentalized almost exclusively in the rod terminal. Furthermore, simultaneous measurements of I_{Cl(Ca)} and intracellular Ca\(^{2+}\) transients, using voltage clamp and ratiometric fura-2 spectrofluorimetry respectively, confirmed that intracellular [Ca\(^{2+}\)] rose more steeply in the rod terminal relative to the soma during membrane depolarization. Moreover, they revealed that the time course of decay of I_{Cl(Ca)} followed very closely that of Ca\(^{2+}\) concentration in the terminal, which had a different kinetic profile from the soma. Thus, even though the duration of I_{Cl(Ca)} was highly variable from cell to cell (typically 1-12 sec), the time to half-decay for both I_{Cl(Ca)} and terminal [Ca\(^{2+}\)] was similar within a given cell. Several studies have pointed to the complex control of intracellular Ca\(^{2+}\) dynamics, and indirectly I_{Cl(Ca)}, in the rod synaptic terminal which is endowed with local, specialized mechanisms that regulate intra-terminal calcium ((Krizaj and Copenhagen, 2002; Steele et al. 2005). Since this region is specialized for synaptic transmission to second-order retinal cells, our findings highlight an important conductance that needs consideration in models of the physiological function of the synaptic terminal during the light response (Thoreson et al. 2003; Thoreson and Bryson, 2004).
Evidence for selective targeting of $I_{\text{Cl(Ca)}}$ to salamander rod terminal

The calcium-dependent chloride current, $I_{\text{Cl(Ca)}}$, is a well-described property of vertebrate photoreceptors, and is activated by entry of extracellular Ca$^{2+}$ via dihydropyridine-sensitive L-type calcium channels (Atwell et al. 1982; Bader et al. 1982; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Yagi and MacLeish, 1994; Morgans et al. 1998; Thoreson et al. 2003), or Ca$^{2+}$ release from intracellular stores (see Krizaj and Copenhagen, 2002). In the present study, the most direct evidence for the compartmentalization of $I_{\text{Cl(Ca)}}$ was obtained during repeated whole-cell recordings from the same rod, one before and another after terminal ablation. In all such cases $I_{\text{Cl(Ca)}}$, which was present initially, was no longer detectable after terminal ablation, though several other voltage- and Ca$^{2+}$-dependent currents remained largely intact. Further, attempts to reveal $I_{\text{Cl(Ca)}}$ in terminal-ablated rods, by augmenting Ca$^{2+}$ entry with the L-type Ca$^{2+}$ channel agonist, Bay K 8644, were unsuccessful, though this calcium load was sufficient to enhance the Ca-dependent $K^+$ or BK current. The simplest explanation for these findings is that the chloride channels underlying $I_{\text{Cl(Ca)}}$ are specifically targeted to the photoreceptor terminal. Additional supporting evidence for compartmentalization of $I_{\text{Cl(Ca)}}$ was obtained in comparative studies on its frequency of occurrence in rods with, versus rods without, obvious terminals when viewed under phase contrast microscopy. $I_{\text{Cl(Ca)}}$ was always present in rods with visible terminals, and was usually (~71% of cases), but not always, absent in rods lacking visible terminals. We infer from these studies that failure to observe a visible terminal under phase contrast microscopy is not conclusive evidence for the lack of terminal contribution to the whole-cell response. It is possible for the terminal to be obscured by the soma, for example during substrate
attachment, and this has actually been observed in scanning electron micrographs of isolated rods (P.R. MacLeish & W. Anderson, unpublished observations). Further, after tissue dissociation and plating, rod terminals often display different lengths and shapes, suggestive of varying stages of regression into the cell body. These considerations caution against inferences about channel compartmentalization based on results from cells without obvious terminals.

**Physiological significance of terminal localization of $I_{CI(Ca)}$**

The observed, highly-specific targeting of $I_{CI(Ca)}$ to the salamander rod terminal, a region specialized for synaptic transmission, raises questions about its physiological significance. Clearly, the physiological impact of the Cl\(^{-}\) channels mediating $I_{CI(Ca)}$ depends on the Cl\(^{-}\) equilibrium potential ($E_{Cl}$) which has been estimated to be approximately -20 mV in salamander rods, i.e. positive to the dark resting membrane potential (~ -46 mV; Thoreson et al. 2002). In salamander retinal slices, a feedback interaction between intracellular Ca\(^{2+}\) and these Cl\(^{-}\) channels has been proposed to stimulate Cl\(^{-}\) efflux, which in turn inhibits presynaptic Ca\(^{2+}\) channels involved in regulating neurotransmitter release (Thoreson et al. 2003). Alternatively, the hyperpolarization induced by light stimulus in the rod photoreceptor may reduce voltage-gated Ca\(^{2+}\) influx, causing closure of these Cl\(^{-}\) channels and a positive feedback amplification of the light-evoked response. This mechanism would lead to an enhanced reduction of transmitter release when the light is turned on, as well as a facilitation of release when the light is turned off, thereby increasing the signal-to-noise ratio for the receptor. Interestingly, in olfactory neurons which have an unusually high intracellular
[Cl\textsuperscript-], and hence a relatively depolarized $E_{\text{Cl}}$, activation of $I_{\text{Cl(Ca)}}$ causes Cl\textsuperscript- efflux which is thought to contribute to high-gain, low-noise amplification of the olfactory receptor potential (see Menini, 1999). As proposed for cones, the channels underlying $I_{\text{Cl(Ca)}}$ may also contribute to the stabilization of the membrane potential in salamander rods. Since the estimated $E_{\text{Cl}}$ in these rods is \(~-20\text{ mV}\) (Thoreson et al. 2002), i.e. near membrane voltages that maximize Ca\textsuperscript{2+} entry, stabilization of the membrane potential in the terminal would be facilitated via a positive feedback loop. These considerations, together with our demonstration of the highly-localized distribution of $I_{\text{Cl(Ca)}}$ in the rod terminal, raise the possibility that the soma and terminal regions may not be isopotential, and that this chloride conductance may allow local and autonomous regulation of membrane potential and transmitter release by the photoreceptor terminal.

**Distribution of remaining voltage-gated channels in salamander rods**

Whereas the underlying ion channels that mediated $I_{\text{Cl(Ca)}}$ in rods were preferentially sorted to the rod terminal, those that contributed to most of the remaining ionic currents did so primarily due to their location in the soma and/or inner segment. Though we could not exclude the presence of any of these conductances in the outer segment, this region is generally thought to be relatively devoid of voltage-activated currents (Fain and Lisman, 1981; Barnes and Hille, 1989). In particular, the major K\textsuperscript+ currents, i.e. the Ca\textsuperscript{2+}-sensitive BK current, $I_{\text{K(Ca)}}$, and Ca\textsuperscript{2+}-insensitive, delayed rectifier-like current, $I_{\text{K(V)}}$, as well as the hyperpolarization-activated cation current, $I_{\text{h}}$, were hardly affected after terminal ablation, suggesting that any terminal contribution to the whole-cell current was negligible. Given the small size and surface area of the rod terminal relative to the soma
and inner segment, these data do not allow firm conclusions to be drawn regarding the distribution or density of these channels within the various rod compartments. For example, we estimated that even with a uniform distribution of any particular group of ion channels over the photoreceptor surface, the contribution of the terminal region to the resulting whole-cell current would be < 1%. In this regard, our demonstration that \( I_{K(Ca)} \) remained largely intact in terminal-ablated rods conflicts with a recent study suggesting that this current is polarized preferentially to the salamander rod terminal (Xu and Slaughter, 2005). In the latter study, \( I_{K(Ca)} \) was detectable in rods with, but not those without, visible terminals as viewed under light microscopy (see Discussion above). While our data do not exclude the possibility of BK channel expression in the terminal, such a contribution would be relatively small in our studies. At present we cannot account for this discrepancy in localization of the major component of \( I_{K(Ca)} \) between the two studies, other than methodological differences. Interestingly, though Ca\(^{2+}\)-activated BK channels are often found concentrated in synaptic terminals of central neurons (Trimmer and Rhodes, 2004), they are known to be clustered in the apex of other sensory cells, e.g. mouse inner hair cells, away from the basal synaptic sites (Pyott et al. 2004; see also, Kwon and Guggino, 2004).

Since in our study, \( I_{K(Ca)} \) was robustly expressed in rods with ablated terminals and was dependent on entry of extracellular calcium through voltage-dependent L-type Ca\(^{2+}\) channels, the latter must also be present in the soma and/or inner segment (see also Szikra and Krizaj, 2006). Indeed, in rods with ablated terminals, \( I_{K(Ca)} \) was substantially enhanced by Bay K 8644, a L-type Ca\(^{2+}\) channel agonist (Nowycky et al. 1985). Similar L-type Ca\(^{2+}\) channels also appear to be concentrated in the salamander rod terminal where
they contribute to local ‘hot spots’ for Ca\(^{2+}\) entry following membrane depolarization (Morgans et al. 1998; Nachman-Clewner et al. 1999; Krizaj and Copenhagen, 2002; Steele et al. 2005).

In conclusion, our data indicate that salamander rod photoreceptors are endowed with discrete targeting mechanisms that preferentially sort ion channels mediating the anionic Ca-dependent Cl\(^{-}\) current to the terminal region, which also acts as a local hot spot for calcium entry. The mechanisms that underlie sorting of these channels to this compartment, are currently unknown, but result in a functional polarity that may contribute to stabilization of membrane potential and local regulation of neurotransmitter release at the terminal, as well as enhancement of signal-to-noise ratio for the receptor. Interestingly, this polarity persisted in rods even after isolation for two days in culture, suggesting that cell-cell contact was not essential, at least for its short-term maintenance. It remains to be determined whether the sorting signals are similar to those that direct membrane proteins in other polarized epithelial and neuronal cells (Trimmer, 1999; Trimmer and Rhodes, 2004).

Acknowledgements
We wish to thank Dr. Xiaoming Chen for technical assistance during the course of this study.

Grants
This work was supported by NIH grants NS-35510 and NS-34194 to PRM and RR-07571 (Research Facilities Improvement Grant). We also recognize support from the Keck Foundation. CAN was a visiting scientist on research leave from McMaster University and supported by the Natural Sciences and Engineering Research Council of Canada.
References


**Krizaj D, Copenhagen DR.** Calcium regulation in photoreceptors. *Front Biosci* 7: 2023-2044, 2002.


Figure 1. Expression of $I_{Cl(Ca)}$ in salamander rods. Voltage-clamp currents in response to step depolarizations from -70 mV to select voltages. A – Total membrane current in control medium. $I_{Cl(Ca)}$ is seen as a prominent tail current at the end of the depolarizing steps and as an inward current at -20 mV (smallest step, arrow). B - $I_{Cl(Ca)}$ reverses close to 0 mV. Outward currents were blocked by cesium in the pipette and TEA in the bath. Current responses changed polarity close to 0 mV, the reversal potential for chloride in this experiment. Note prominent tail current for all voltage steps. C - $I_{Cl(Ca)}$ requires external calcium. C1-C3 were obtained from the same cell before, during and after puffing calcium-free solution. $I_{Cl(Ca)}$ disappeared in calcium-free solution along with the Ca$^{2+}$-dependent outward currents during the voltage step.
Figure 2. Effect of terminal ablation. A1, A2 – Phase-contract micrograph of the same cell with an intact terminal (A1) and after ablation (A2). B1, B2 – Membrane current in control solution from same freshly-dissociated rod before (B1) and after (B2) terminal ablation. Note total absence of tail current after ablation and little, if any, change in outward currents. C1, C2 – Responses from the same cell in culture for 56 hrs. Note robust expression of $I_{Cl(Ca)}$ after this time in culture (C1) and selective removal upon terminal ablation (C2). D – Comparison of currents in B1 and B2 at 0 mV, the $I_{Cl(Ca)}$ reversal potential, before and after ablation. Note similarity in amplitude of outward currents in two cases. E- Ablation does not significantly alter outward current at 0 mV. The mean (± S.E.M.) current amplitudes at 0 mV are shown for a population of 5 cells before and after terminal ablation.
Figure 3. Responses to ramp depolarizations. A – Response from fully-intact cell. $I_{\text{Cl(Ca)}}$ is seen as a tail current at the end of the ramp and contributes to the inflection in the total current trace in the range -20 mV to 0 mV. B1- B3 – A different cell from that in A in which the terminal was ablated before recordings were made. B1, B3 – Currents in control medium. Note absence of $I_{\text{Cl(Ca)}}$ at end of ramp. B2 – Response in presence of 0.5 μM Bay K 8644. The presence the L-type calcium channel agonist led to a marked increase in the amplitude of the outward current (due presumably to Ca$^{2+}$-dependent K$^+$ current) during the ramp, indicating an increase of intracellular calcium but no obvious unmasking of a tail current.
Figure 4. Tail current and terminal calcium changes have similar kinetics. A, B and C show simultaneous recording of tail current and changes in intracellular calcium in 3 different fura-containing cells loaded with calcium by depolarization to 0 mV. Following repolarization to -70 mV, the tail current and calcium signals in terminal and soma are shown. The time course of the tail current was similar to that of the calcium changes in the terminal. D shows the time interval at half maximal (t1/2) amplitude for calcium changes in the terminal and soma compared to t1/2 of the tail current. Note the excellent fit along the 45° line when t1/2 for the terminal calcium is plotted against the t1/2 for tail current.
Figure 5. Rods with ablated terminal express robust calcium-dependent outward K\(^+\) current. A1-A3 – Responses from same cell to voltage steps from -60 mV to +60 mV in 20 mV steps before (A1), during (A2) and after (A3) puffing of calcium-free solution. The arrows in A1 and A3 indicate the response to +60 mV depolarizing step. A4- The voltage-corrected, steady-state I-V relationship in the presence of calcium. A5 – The voltage-corrected steady-state I-V relationship in calcium-free solution. The dotted line in A4 is the I-V curve in calcium-free solution. Note superposition of curves below about -30 mV and above +60 mV. Pipette resistance was 8 MΩ.
Figure 6. Expression of $I_h$ is not noticeably altered by terminal ablation. Whole-cell current responses to a series of hyperpolarizing steps from -70 mV to -120 mV. Note prominent time-dependent increases in the amplitude of the inward currents at voltages negative to -100 mV. The responses are from the same cell before (A1) and after (A2) terminal ablation. B – Peak amplitude of the current (± S.E.M.) at -120 mV for a population of three cells before and after terminal ablation.
<table>
<thead>
<tr>
<th>Control</th>
<th>ChTx (20-60 nM)</th>
<th>Calcium free</th>
<th>Cadmium (150 µM)</th>
<th>Nitrendipine (10 µM)</th>
<th>Nitrendipine 1(0 µM) + 4-AP (2.3 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 10</td>
<td>n = 6</td>
<td>n = 8</td>
<td>n = 4</td>
<td>n=6</td>
<td>n=3</td>
</tr>
<tr>
<td>694 ± 92 pA</td>
<td>190 ± 31.4 pA</td>
<td>159.3 ± 17.8 pA</td>
<td>179 ± 31.6 pA</td>
<td>135 ± 7 pA</td>
<td>94 ± 10 pA</td>
</tr>
</tbody>
</table>

Table 1. Mean steady-state outward current (+ SEM) from intact rods at +20 mV from a holding voltage of -70 mV for the conditions shown. ChTx: Chrybdotoxin, 4-AP: 4 amino pyridine