The leading edge of the a-wave of the ERG is generally believed to accurately reflect the changes in the circulating current through the cGMP-gated channels in the outer segment plasma membrane of rods and cones. The aspartate-isolated mammalian electroretinogram (ERG) to a rod-saturating flash contains a fast "nose"-like wave temporally overlapping with the a-wave. We characterize the nature of this nose, investigate the membrane current mechanisms involved in the nose mechanism, and propose a model that can explain the generation of the nose component in the rod inner segment. On the basis of pharmacological treatments and perfusate ion composition alterations we rule out the possible role of most of the known rod membrane current mechanisms that might participate in the generation of the ERG nose component and we propose that the nose is generated by the interplay of voltage-dependent Kx and h channels together with the Na+/K+ ATPase. Our results strengthen the view that the kinetics of the leading edge of the ERG photoresponses should correspond to that of the outer segment light-sensitive current.

Keywords: electroretinogram, a-wave, photoreceptors, nose, HCN, Kx channel, Cs+, ZD7288

and that directly shows when a mechanism in the source/sink pair is affected.

When signal transmission from photoreceptors to second- and higher order retinal neurons is completely blocked, at least four hypothetical ways for the generation of the nose can be seen: First, the nose could originate in cones. The photoresponse kinetics of cones is faster than that of rods and therefore, although the rods outnumber the cones by a factor of more than 30 in the mouse retina (Carter-Dawson & LaVail, 1979), the nose could be formed either (at least partly) by a cone response proper, or indirectly by some mechanism activated by the possible spreading of hyperpolarization from cones to rods via gap junctions. Second, the origin of the nose could be in rods and the nose could be generated by some conductance mechanism(s) other than the cGMP-gated channels in the outer segment. Third, the nose might be generated in Müller cells via concentration changes of ions in the extracellular space. This, however, is very unlikely since Ba2+ that is used to abolish the glial component from the mouse fast PII still leaves the nose intact (Nymark et al., 2005). Fourth, the recently discovered intrinsically photosensitive retinal ganglion cells, ipRGCs (Berson, Dunn, & Takao, 2002) might generate extracellular currents in response to light and thereby generate the nose. Yet, the sparse population of ipRGCs (Hattar, Liao, Takao, Berson, & Yau, 2002) and the very low sensitivity of these cells (Do et al., 2009) exclude the possibility that the nose is generated in ipRGCs.

The objective of this study was to elucidate the molecular mechanisms of the current dipole(s) generating the nose. We start by showing convincing evidence that the nose is generated in the photoreceptor layer and that it is of rod origin. Then we demonstrate that the generation of the nose requires calcium. Yet, we conclude that neither the Na+ /Ca2+–K+ exchanger, the L-type Ca2+ channel nor the Ca2+-activated chloride and potassium channels are essential in the generation of the nose. Additionally we show that the action of Ca2+ in the generation of the nose is not a surface charge screening effect. Our results strongly suggest that voltage-dependent h channels, which are activated by membrane hyperpolarization (Fain et al., 1978), participate in the nose generating mechanism and that the calcium dependence of the nose comes from the role of h channels. Our data indicate that a K+ conductance, most probably the Kx channel, is involved in the generation of the nose. Finally, we present a model that can explain the generation of a transient cornea-negative wave (the nose) in saturated or nearly saturated rod ERG photoresponses.

Materials and methods

Preparation, recording, and light stimulation

Ethical approval

The use and handling of all the animals in this study were in accordance with the Finnish Act on Animal Experimentation (62/2006) with guidelines of the Animal Experimentation Committee of Finland.

The ERG experiments

Pigmented mice (C57Bl/6) were dark-adapted over-night. The animals were sacrificed by CO2 inhalation and decapitation, the eyes were enucleated and bisected along the equator, and the retinas were detached in cooled Ringer under dim red light. The isolated retina was placed in a specimen holder (Donner, Hemilaä, & Koskelainen, 1988) with an active recording area of 1.2 mm or in later experiments 0.5 mm (diameter) at the flat-mounted central retina. The upper (photoreceptor) side was superfused with a constant flow (ca. 1.5 ml/min when retinal temperature was 25°C or 3–4 ml/min in the experiments that were conducted at 37°C) of Ringer’s solution. In the experiments at 25°C the Ringer contained (mM): Na+, 133.9; K+, 3.3; Mg2+, 2.0; Ca2+, 1.0; Cl−, 143.2; glucose, 10.0; EDTA, 0.01; HEPES, 12.0, buffered to pH 7.5 (at room temperature) with 5.8 mM NaOH. Sodium l-aspartate (2 mM) was added to block synaptic transmission to second-order neurons. In the experiments at 37°C the Ringer contained (mM): Na+, 124.3; K+, 3.3; Mg2+, 2.0; Ca2+, 1.0; Cl−, 133.6; glucose, 10.0; EDTA, 0.01; HEPES 10.0; NaOH, 4.8 mM; NaHCO3, 20 mM. dl-2-Amino-4-phosphonobutyric acid (APB; 50 μM) was added to block on-bipolar cell signals. The Ringer was pre-heated to ca. 37°C and bubbled with a mixture of 95% O2 and 5% CO2. Leibovitz culture medium L-15 (Sigma), 0.72 mg/ml, was added to improve the viability of the retina in all experiments. In addition, BaCl2 (10 mM) was added in the lower electrode space, from where it would diffuse to the retina to suppress glial currents by blocking potassium channels located mainly at the endfeet of Müller cells (Bolnick, Walter, & Sillman, 1979; Nymark et al., 2005). In some experiments, instead, 50 μM BaCl2 was added to the perfusate, which also served to block the glial component. Pharmacological manipulations of the standard solution are explained below. The temperature was controlled by a heat exchanger below the specimen holder and monitored with a thermistor in the bath close to the retina.

Recording and light stimulation

The transretinal potential was recorded with two Ag/AgCl pellet electrodes, one in the subretinal space and the other in chloride solution connected to the perfusion Ringer through a porous plug. The DC signal was sampled at 200–10,000 Hz with a voltage resolution of 0.25 μV.

The flashes used to produce short flash stimuli with homogeneous full-field illumination to the distal side of the retina were provided by a dual-beam optical system adapted from the setup used by Donner et al. (1988). In brief, 20-ms light pulses were generated with a 543.5-nm HeNe laser (Melles Griot 05 LGR 173, 0.8 mW) or with a

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532-nm laser (Power Technology IQ5C(532-100)L74, ~130 mW) and a Compur shutter for both laser paths, the midpoint of the flash indicating the zero time for the recordings. In experiments at 37°C or when focusing on the study of the rising phase of photoresponses we replaced the Compur shutter with a faster Oriel shutter (model 76992) producing 2-ms flashes. The Gaussian profile of the laser beam was flattened by conducting the beam through a light guide with mixing fibers. The uniformity of the beam at the level of the retina was confirmed with a small aperture photodiode. The light intensity of each source was controlled separately with calibrated neutral density filters and wedges.

The absolute intensity of the unattenuated laser beam (photons mm⁻² s⁻¹) incident on the retina was measured in each experiment with a calibrated photodiode (EG&G HUV-1000B; calibration by the National Standards Laboratory of Finland). The amount of isomerizations (Rh*) produced by the stimulating flash light in individual rods was calculated as described in Heikkinen et al. (2008).

Chemicals and pharmacological manipulations

Pharmacological manipulations of our Ringer solution (see above) used are explained in each case in the Results section. All the chemicals, except tetrodotoxin (TTX, purchased from Tocris), BAPTA-AM (Invitrogen), and dimethonium (see below), were purchased from Sigma-Aldrich. Very low [Ca²⁺]_free (25 nM) solutions were prepared by using EGTA and the free [Ca²⁺] was calculated using an “EGTA calculator” (Portzehl, Caldwell, & Rueegg, 1964) taking into account 2 mM [Mg²⁺] present in our Ringer’s solution. In BAPTA experiments cells were loaded with BAPTA by perfusing the retina for 30 to 40 min in Ringer containing 50 μM cell permeable BAPTA-AM.

Dimethonium was prepared as described in Shindo, Takahashi, and Nakajima (1971) by reacting N, N'-tetramethylethlenediamine with methyl bromide in acetone/bromide, followed by recrystallization from isobutanol/methanol. The chemical structure and purity was confirmed by proton NMR at 200 MHz.

Response analysis

The Michaelis function is given as

\[ r(t_p) = \frac{Q_F}{Q_F + Q_{F,1/2}}, \]

in which \(r(t_p)\) is the photoresponse amplitude at the time of the peak (\(t_p\)) and \(r_{sat}\) is the amplitude of the saturated response. \(Q_F\) is the flash strength (as a number of isomerized pigments per cell, Rh*) and \(Q_{F,1/2}\) is the half-saturating flash strength.

Results

Dark-adapted ERG photoresponses recorded across the isolated mouse retina

Figure 1 characterizes dark-adapted mouse ERG photoresponses to flashes of light recorded across the isolated retina in conditions where the glial component (“the slow PIII”) and the signal components from higher order

![Figure 1](http://jov.arvojournals.org/pdfaccess.ashx?url=/data/journals/jov/932859/)
neurons were pharmacologically blocked. Figure 1A presents a family of averaged responses to flashes covering the stimulus strength ($Q_{F}$) range from 1 to 506,700 Rh* per rod at 37°C in Ringer containing 50 μM APB to eliminate the b-wave and 50 μM BaCl2 to block glial contribution (see Materials and methods section). The responses to weak flashes are generated by rod activity, while the responses to stronger flashes, however, appear to contain several components of both rod and cone origin. In the responses to strongest flashes the most striking of these is a fast negative “nose-like” component, the origin of which will be characterized in the following. The nose is followed by a relatively small positive wave, also observed in the responses of Nyxnob mice (Purdue, McCall, LaVail, Gregg, & Peachey, 1998), before the response settles to a long-lasting plateau.

In order to maximize the stability of the retina and to ensure full recovery from the treatments intended to probe the molecular mechanisms generating the nose, we chose to conduct our experiments at a lowered temperature (25°C). One further advantage of the lowered temperature is that according to our experience the general glutamate receptor agonist l-aspartate can completely eliminate the b-wave at 25°C in HEPES–Ringer (but not at 37°C in bicarbonate–Ringer) as well as other components based on glutamate-mediated synaptic transmission, thereby reducing the number of different components present in the mouse ERG. These differences in the response waveforms are illustrated in Figures 1A and 1B (two different retinas): Besides the general deceleration due to lower temperature, the waveforms of the responses in Figure 1B are less complex than those in Figure 1A. The character of the nose, however, is not affected by the changes in temperature and the composition of the Ringer solution.

### Mouse rod ERG photoresponses

The mouse retina contains both rods and cones, the cones constituting about 3% of the photoreceptors (Carter-Dawson & LaVail, 1979). To isolate rod activity, the cone component in each response was determined by a double flash technique (Heikkinen et al., 2008; Koskelainen, Hemilä, & Donner, 1994) as shown in Figure 2A: A rod-saturating preflash (strength $Q_{PF}$; constant during each experiment) was followed by a test flash of the strength $Q_{F}$ at time when the cones had fully recovered from the preflash, but the rods still remained saturated. During the maintained saturation of rods the response to the test flash originates only in cones. This pure cone response was then subtracted from the ERG photoresponse obtained with equal stimulus ($Q_{F}$) to give a cone-free rod ERG response.

Figures 2B and 2C show the early part of “mixed” responses (black traces) and the cone-free rod ERG photoresponses (red traces) “purified” by the double-flash method from these mixed responses at 37°C and 25°C, respectively. The intention of this figure is to show that the effect of removal of the cone component is qualitatively similar at both temperatures: The activation phase of the cone response is slower than that of rods, and therefore the leading edge of the mixed response is of purely rod origin up to 80–90% of its peak. The removal of the cone component slightly reduces the time to response peak. It is evident that at 37°C the nose amplitude is not yet saturated even with our strongest possible stimulus (507,000 Rh*), while all the responses in the stimulus strength range 1010–507,000 Rh* reach the plateau level ($n = 2$). At 25°C the behavior is qualitatively similar except that in the 2 experiments where the highest possible stimulus strengths were used the nose amplitude saturated at around 51,000 Rh* in the experiment shown in Figure 2E but not until 250,000 Rh* in the other experiment. Figures 2D and 2E show the respective intensity–response (I–R) data with the amplitude measured either at the plateau (filled symbols) or at the peak of the nose (open symbols). The I–R function measured at the plateau covers a stimulus strength range of approximately 2 log units, which is a typical dynamic range of dark-adapted vertebrate photoreceptors. The I–R data corresponding to the nose is, however, much wider, extending up to 4 log units of stimulus strengths. Additionally, it is not possible to fit the Michaelis or exponential saturation function to the peak amplitude data neither at 37°C nor at 25°C.

### Evidence that other neurons than rods are not required for the generation of the “nose”

The “purified” (cone component subtracted) ERG flash response contains now a “pure” nose component that inevitably has to be generated in rods. The nose could, in principle, be induced in rods by a synaptic feedback signal from second-order neurons in the retina. This feedback, however, is unlikely, since in our experiments the forward synaptic transmission from photoreceptors to bipolar and horizontal cells was blocked by aspartate (Attwell & Wilson, 1980; Sillman, Ito, & Tomita, 1969), supported by the observation that there was no indication of b-wave (and thus ON bipolar cell activity) in the ERG. Without light-induced activity in the second-order neurons there hardly can be any light-induced feedback signaling to rods from these cells. Instead, a possible explanation for the origin of the nose might be found in the electrical connections between photoreceptors; mouse rods are connected to cones via gap junctions (Tsukamoto, Morigiwa, Ueda, & Sterling, 2001). Mouse cones have faster photoresponse kinetics but are less sensitive than rods (Heikkinen et al., 2008). Therefore with strong stimuli cones might be hyperpolarized before rods, and this hyperpolarization is conveyed into rods via gap junctions producing the nose by some, possibly a voltage-sensitive mechanism. To test this hypothesis we
perfused the retina with Ringer containing 0.1 mM carbenoxolone, generally used to block gap junctions in the retina (Hornstein, Verweij, Li, & Schnapf, 2005; Vaney, Nelson, & Pow, 1998). The effect of carbenoxolone on cone-free ERG for one rod-saturating stimulus is shown in Figure 3. Carbenoxolone did not affect the nose amplitude though the activation phase was slightly slower and the recovery phase of the nose was substantially retarded. Since the nose could not be abolished by carbenoxolone, we conclude that the rod nose is not generated by the hyperpolarization of cones coming over into rods via gap junctions, and that the nose has to be generated by the rods themselves. We note here that carbenoxolone impinges on at least one of the mechanisms creating the nose, and thus makes the nose recovery slower. The effect of carbenoxolone was similar in all three retinas tested and did not depend on stimulus strength.

Figure 2. “Pure” mouse rod fast PIII photoresponses. (A) Photoresponse to a double-flash stimulus (black trace): Two identical 20-ms flashes delivered at $t = 0\ s$ (preflash) and $t = 1.7\ s$ ($Q = 64,000\ Rh^*$). The pure “cone response” elicited by the second flash is subtracted from the “mixed response” elicited by the preflash in order to yield the pure rod photoresponse (red). (B) Black traces show the same photoresponses as in Figure 1A in a shorter time scale. Red traces plot the six largest responses after subtraction of cone contribution. (C) Black traces plot the photoresponses to 2-ms flashes at $25\ ^\circ\ C$ in HEPES-buffered Ringer with 2 mM aspartate. Flash strengths: 1, 2, 5, 16, 51, 160, 507, 1600, 5100, 16,000, 50,700, 160,200, 506,700 $Rh^*$. Red traces plot the seven largest responses after subtraction of cone contribution. (D) Rod photoresponse amplitudes read at the plateau ($\bullet$) and at the peak ($\Lambda$) of responses from (B). The smooth curve is the fit to the data with Equation 1 ($Q_{F,1/2} = 18\ Rh^*$). (E) Rod photoresponse amplitudes read at the plateau ($\bullet$) and at the peak ($\Lambda$) of responses from the responses shown in (C). The smooth curve is the fit to the data with Equation 1 ($Q_{F,1/2} = 14\ Rh^*$).
Generation of the nose requires calcium

Figure 4A shows normalized saturated “cone-free” rod photoresponses ($Q_{PF} = Q_F = 55,000$ Rh*) in normal Ringer (1 mM [Ca$^{2+}$]) and in EGTA-buffered solution with low 25 nM free Ca$^{2+}$ concentration. Since lowered extracellular calcium greatly increases the saturated response amplitude, the same steady-state responses are shown without normalization in the inset. In 5 out of 7 experiments the nose was observed to fully disappear in 25 nM [Ca$^{2+}$]free, and in the other two experiments the removal was almost complete. It might be argued that in low Ca the state of rods is unphysiological, with elevated intracellular [cGMP], strong membrane potential depolarization, and a partial collapse of the sodium gradient across the cell membrane, and that the loss of nose may be due to these changes. These changes can be overridden by strong background lights that decrease [cGMP], hyperpolarize the rods, and decrease the cGMP-gated conductance of the outer segment leading to recovery of the sodium gradient. In our experiments with continuous strong backgrounds the nose was present at all background intensities tested up to 1700 Rh*/s in normal calcium (data not shown) while in 25 nM [Ca$^{2+}$]free the nose did not recover even with strongest backgrounds where the rods were still responsive. Therefore we are convinced that the loss of the nose in low Ca is not due to the above-mentioned changes in the physiological state of the rods but instead due to a mechanism that is sensitive to calcium. In the following we investigate the role of known Ca$^{2+}$-dependent mechanisms of the rod plasma membrane that could participate in the generation of the nose.

Screening of negative surface charge by Ca$^{2+}$ does not explain the Ca$^{2+}$ dependency of the nose

Divalent cations, such as Ca$^{2+}$, are known to strongly affect the electrostatic potential adjacent to cell membranes and thereby affecting the voltage dependence of voltage-sensitive channels (Beech & Barnes, 1989; Chandler, Hodgkin, & Meves, 1965; Frankenheuser & Hodgkin, 1957; Gilbert & Ehrenstein, 1969; Hille, 1968; Hille, Woodhull, & Shapiro, 1975). This effect can basically emerge from two mechanisms: (1) Ca$^{2+}$ can be adsorbed to negatively charged sites at the outer edge of the membrane (as suggested by A. F. Huxley in Frankenheuser and Hodgkin, 1957) or (2) Ca$^{2+}$ can stay free in the solution but form a positively charged layer close to the negative surface charge, thereby screening it (McLaughlin, Szabo, & Eisenman, 1971). To test whether the latter mechanism has any role in the generation of the nose, we replaced calcium with the divalent cation dimethonium. Dimethonium is a large organic cation that does not adsorb to the negative surface charges and exerts only a screening effect on phospholipid bilayer membranes (McLaughlin, Eng, Vaio, Wilson, & McLaughlin, 1983). Figure 4A presents the cone-free saturated rod photoresponses in normal Ringer (black), in low calcium solution (25 nM [Ca$^{2+}$]free, red), and in the low calcium solution with 1 mM dimethonium added (blue). The nose disappeared in low Ca$^{2+}$ and the introduction of dimethonium was unable to recover it. A similar result was obtained in all three experiments with dimethonium. This result shows that the role of calcium in the generation of the nose does not come from the screening of the negative surface charge of the rod plasma membrane. Since the effect of Ca$^{2+}$ on the nose does not come via screening of the plasma membrane surface charge, we next investigated the possible role of the known Ca$^{2+}$-dependent conductance mechanisms of the rod processes.

L-type Ca$^{2+}$ channels, Ca$^{2+}$-activated K$^+$, and Ca$^{2+}$-activated Cl$^-$ channels or Na$^+$/Ca$^{2+}$–K$^+$ exchanger are not involved in nose generation

Both amphibian and mammalian rods are shown to have voltage-gated L-type calcium channels at their synaptic terminals where these channels control synaptic transmission from rods to bipolar and horizontal cells (Bader, Bertrand, & Schwartz, 1982; Cia et al., 2005; Nachman-Clewner, St, & Townes-Anderson, 1999). Light-induced
hyperpolarization of the rod membrane closes these channels and hence reduces Ca\(^{2+}\) influx through them. The role of the L-type Ca\(^{2+}\) channels in the nose process can be probed by Cd\(^{2+}\) or Co\(^{2+}\) that effectively block these channels. Figure 4B illustrates the effect of 0.1 mM [Cd\(^{2+}\)] on the normalized saturated “cone-free” rod photoresponse (Cd\(^{2+}\) had no effect on the absolute plateau amplitude). The amplitude of the nose is only slightly reduced by the application of Cd\(^{2+}\) but invariant compared to the control photoresponse recorded in normal Ringer after Cd\(^{2+}\) application. Similar results were obtained in all 3 experiments with Cd\(^{2+}\). Introduction of 2 mM [Co\(^{2+}\)] also did not have any significant effect on the nose (n = 3, data not shown). These results show that the L-type Ca\(^{2+}\) channels are not involved in the generation of the nose.

Vertebrate rod photoreceptors contain large-conductance calcium-activated K\(^{+}\) (BK) channels (Bader et al., 1982; Baylor, Matthews, & Nunn, 1984) that in salamanders are localized at rod terminals (Xu & Slaughter, 2005) or in the inner segment/soma region (MacLeish & Nurse, 2007). The BK current in salamander rods in darkness is \(\sim 10\text{–}20\) pA and in physiological conditions the current is always outward (positive). Moriondo, Pelucchi, and Rispoli (2001) have shown that the BK current can be blocked by the application of 0.1 mM [Cd\(^{2+}\)]. In our experiments Cd\(^{2+}\) did not have any significant effect on the nose component (Figure 4B) showing that the BK channels do not participate in the generation of the nose.

In addition to calcium-activated potassium channels vertebrate rod inner segments contain calcium-activated...
chloride channels (salamander: Bader et al., 1982, porcine: Cia et al., 2005). These channels have been reported to be concentrated at rod terminals (MacLeish & Nurse, 2007). We tested the role of the Ca\(^{2+}\)-activated chloride channels in the generation of the nose by replacing most of the chloride in the perfusate with methyl sulfate (CH\(_3\)SO\(_4\)) that cannot permeate the Ca\(^{2+}\)-activated chloride channels. Our hypothesis was that if the Ca\(^{2+}\)-activated chloride channels were involved in the generation of the nose, removal of most of the current carrier should decrease the size of the nose. Reduction of external chloride inevitably shifts the chloride Nernst potential toward much more positive values, and thus leads to a depolarization of the membrane potential. The depolarization is, however, transient, since under these circumstances the strong outward driving force for chloride must produce a highly enhanced chloride efflux. Indeed, Thoreson, Nitzan, and Miller (2000) have shown that perfusion of salamander rods with low chloride medium depletes intracellular chloride in a time scale of several tens of seconds. In the much smaller mouse rods the kinetics of chloride depletion is expected to be faster rather than slower compared to the large salamander rods. In toad rods a change in external [Cl\(^-\)] from 120.6 to 10.6 mM caused a transient depolarization of around 5 mV that was over in 5 min after the depolarization peak was reached (Stella & Thoreson, 2000). These data suggest that the steady-state chloride Nernst potential in low chloride do not substantially differ from that in normal chloride. If the driving force for chloride in lowered external chloride is close to that in normal external chloride, and while the number of chloride carriers is remarkably decreased (e.g., by more than 1 log unit), the chloride current should be substantially decreased in steady state in low chloride. Figure 4C shows the result when the external chloride concentration was lowered from 143 mM to 6 mM and a steady state was achieved (recordings were made 20–30 min after the solution change). Although most of the current carriers for \(I_{Cl(Ca)}\) had been removed, the amplitude of the normalized nose component was increased and its kinetics was faster compared to controls in normal Ringer before and after the low chloride exposure in all three of this kind of experiment (low Cl had only minor effects on saturated response plateau amplitude). Therefore we conclude that the chloride current through the Ca\(^{2+}\)-activated CI\(^-\) channels does not generate the nose. This interpretation is supported by our observation that externally applied 0.1 mM [Cd\(^{2+}\)] does not attenuate the nose (Figure 4B) although it has been shown to significantly suppress \(I_{Cl(Ca)}\) (Cia et al., 2005).

In the rod outer segment intracellular [Ca\(^{2+}\)] is maintained at low level by Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchange (Schnektamp, 1986; Yau & Nakatani, 1984). To test whether the Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchanger is involved in the generation of the nose, we loaded rods (and the other cells in the retina) with the calcium buffer BAPTA by perfusing the retina with a Ringer containing 50 \(\mu\)M of the cell permeable BAPTA-AM. The introduction of BAPTA slowed down the shut-off of small-stimulus responses (not shown), indicating that rods were successfully loaded. If the current driven by the Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchange were involved in the generation of the nose, the kinetics of the nose should get slower. Figure 4D shows the effect of BAPTA on normalized cone-free saturated rod photoresponses (BAPTA had no effect on steady-state absolute saturated response amplitudes). Control responses were first recorded in normal Ringer (black trace). Then the retina was perfused for 28 min with Ringer containing BAPTA-AM followed by a return to normal Ringer. Responses recorded at 2 min (red) and 40 min (blue) after return to normal Ringer are shown. Two minutes after the loading was stopped the nose was identical to the control, and 38 min later the nose amplitude was only slightly reduced while the nose kinetics was not retarded. Similar results were obtained in all three experiments with BAPTA. Our results indicate that the Na\(^+\)/Ca\(^{2+}\)–K\(^+\) exchange current does not generate the nose.

### Evidence for h channel contribution to the nose

Until now we have shown data on experiments tailored to test the possible role of the known Ca\(^{2+}\)-dependent conductance mechanisms in vertebrate rods that could be involved in the generation of the nose. Still no explanation for the Ca\(^{2+}\) dependency of the nose component was found. Although in a previous section we concluded that the effect of Ca\(^{2+}\) does not occur via screening (without adsorption of Ca\(^{2+}\)) of the negative surface charge, it is plausible that more or less specific binding of Ca\(^{2+}\) to negative surface charges does occur. This may induce shifts in the voltage dependence of voltage-gated ion channel activation, as first observed by Frankenenhaeuser and Hodgkin (1957) in squid axons. In rods a decrease in extracellular calcium is accompanied by a depolarization of the membrane potential, e.g., in toad rods the \(V_m\) is shifted to more positive values by ca. 40 mV when extracellular [Ca\(^{2+}\)]\(_{free}\) is reduced from 1.8 mM to concentrations below 100 nM (Bastian & Fain, 1982). This depolarization, however, does not change the saturation level of \(V_m\). Therefore it seems reasonable to assume that also in our low Ca\(^{2+}\) experiments the resting rod membrane potential is also strongly depolarized in comparison to that in normal Ringer, but during saturated photoresponses the membrane potential can reach as negative values as in normal Ringer.

One voltage-dependent conductance mechanism in the vertebrate rod is the hyperpolarization-activated h channel (Cia et al., 2005; Demontis, Gargini, Paoli, & Cervetto, 2009; Demontis, Longoni, Barcaro, & Cervetto, 1999; Fain et al., 1978). The channel isoform HCN1 is also found in mouse rods and the channels are distributed...
along the whole rod inner segments from cilium to axon terminals (Knop et al., 2008; Moosmang et al., 2001). In normal Ringer most of the h channels are closed at the resting $V_m$ (Bader & Bertrand, 1984; Demontis et al., 1999), while hyperpolarization increases the open probability of these. The h channels can selectively be blocked by Cs$^+$ (in mM range) or by ZD7288 (Satoh & Yamada, 2000). The h channels are responsible for the “peak-plateau” behavior of rod membrane potential at saturating stimulus intensities, i.e., the initial hyperpolarization is followed by a relaxation to a more depolarized plateau level. When h channels are blocked (e.g., by Cs$^+$) bright light hyperpolarizes the membrane potential close to the equilibrium potential for potassium (Fain et al., 1978).

In order to test whether the h channels are involved in the generation of the nose we added 2 to 5 mM Cs$^+$ or 50 $\mu$M ZD7288 in Ringer to block the h channels. The effects of Cs$^+$ and ZD7288 on cone-free rod photoresponses are shown in Figures 5A and 5B, respectively. Both blockers removed the nose although the removal by Cs$^+$ was not always complete. The plateau amplitude of the saturated responses was increased in all experiments (Cs$^+$, $n = 12$; ZD7288, $n = 3$). The magnitude of the increase of the saturated response plateau amplitude varied between experiments: In half of the experiments the plateau level of saturated responses grew larger than the amplitude of the original nose peak (Figure 5A), while in half of the experiments the plateau amplitude stayed smaller than that of the original nose peak. The effect of Cs$^+$ was completely reversible while the effect of ZD7288 was irreversible, even though long exposures (30 min or more) to Cs$^+$ decelerated the leading edge of the photoresponses at all stimulus intensities and decreased sensitivity. ZD7288 had similar effects in all 3 experiments, even in case we used only a short (â€”4 min) “pulse” of ZD7288 solution to the retina. We did three experiments with 3 mM Cs$^+$ in which the exposure time of Cs$^+$ was minimized. In all these experiments the first part of the leading edge of the responses was not affected at all, while a significant increase in the amplitude of half-maximal responses as well as in the plateau amplitude of saturated responses was observed (Figure 5C). The very smallest

![Figure 5](http://jov.arvojournals.org/pdfaccess.ashx?url=/data/journals/jov/932859/)

Figure 5. Comparison of photoresponses in normal Ringer and in the presence of h channel blockers. (A) Response families in normal Ringer (black traces) and in 3 mM Cs$^+$ (red traces) after reaching a steady state. Flash strengths: 4, 22, 140, 1400, and 14,000 Rh$^+$ in both solutions. Inset shows the same responses in a shorter time scale. (B) Response families in normal Ringer (black traces) and in 50 $\mu$M ZD7288 (red traces) after reaching a steady state. Flash strengths: 3.8, 15, 150, and 15,000 Rh$^+$ in both solutions. Inset shows the same responses in a shorter time scale. (C) Families of photoresponses in normal Ringer (black traces) and in 3 mM Cs$^+$ (red traces) recorded within 30 min since the introduction of Cs$^+$. Flash strengths: 3.7, 15, 150, and 15,000 Rh$^+$ in both solutions. Different retinas in (A)–(C).
responses were practically unaffected by short exposures to Cs⁺.

**Voltage-gated Na⁺ channel is not involved in the generation of the nose**

Recently it was shown that human rod photoreceptors contain voltage-gated sodium channels that might have physiological relevance in some conditions (Kawai et al., 2005). To test whether these channels have any role in the generation of the nose we recorded photoresponses in Ringer solution containing the Na⁺ channel blocker tetrodotoxin (TTX). Figure 6 shows normalized cone-free rod photoresponses to saturating flashes in normal Ringer (black trace) and in 1 μM TTX (gray trace). TTX did not have any effect on the nose component. These data confirm that the voltage-activated Na⁺ channels are not involved in the generation of the nose.

**Effects of external [K⁺] and lowered K⁺ conductance on the nose**

Potassium is one of the major membrane current carriers in vertebrate rod photoreceptors. The reversal potential of potassium in vertebrate rods appears to be below −70 mV. Therefore, the currents carried in physiological conditions by potassium are always outward, and potassium channels could serve as a current source in the nose process.

Two major potassium currents have been described in rods: the calcium-activated $I_{K(Ca)}$ treated already, and the hyperpolarization deactivated $I_{Kx}$ (Attwell & Wilson, 1980; Bader et al., 1982; Beech & Barnes, 1989; Cia et al., 2005; Demontis et al., 1999; MacLeish & Nurse, 2007). Both of the potassium conductances are located in the inner segment, and possibly in the synaptic region (MacLeish & Nurse, 2007). The $I_{Kx}$ channel gates in the voltage range from −70 to −30 mV (Beech & Barnes, 1989). In darkness, the resting membrane potential of vertebrate rods is about −30 to −40 mV and most of the $I_{Kx}$ channels are open creating a large standing outward current. This outward potassium current is believed to largely balance the inward potassium current of the Na⁺/K⁺ ATPase associated with the circulating sodium current through the cGMP-gated channels (and the Na⁺/Ca²⁺–K⁺ exchanger) into the outer segment and out of the rods via the Na⁺/K⁺ ATPase. At large hyperpolarizations (to below −60 mV) the $I_{Kx}$ disappears, because both the $I_{Kx}$ conductance and the driving force for potassium ($V_m$ is close to the potassium Nernst potential $E_K$) are low.

To investigate the contribution of the $I_{Kx}$ conductance to the nose we first tested the effects of changes in external [K⁺]. Figure 7A shows that raising $[K⁺]_0$ from 3.3 mM (black trace) to 10 mM (red trace) almost completely removed the nose. Return to 3.3 mM $[K⁺]_0$ completely recovered it (blue trace). Raising the extracellular [K⁺] depolarizes the rod membrane in darkness (Bader et al., 1982; Brown & Pinto, 1974; Capovilla, Cervetto, & Torre, 1980; Owen & Torre, 1983) and at the same time produces a positive shift in $E_K$ that is inevitably more pronounced than the change in $V_m$. Thus, with elevated $[K⁺]_0$ the driving force ($V_m - E_K$) for the outward potassium current $I_K$ is reduced. This reduction of the driving force of $I_K$, however, can hardly explain the nearly complete removal of the nose, because the depolarization due to raised $[K⁺]_0$ increases the open probability of the $I_{Kx}$ channels, thereby compensating the effect of decreased driving force on $I_K$. Instead, we conclude that the disappearance of the nose most probably results from raised $E_K$: the light-induced hyperpolarization cannot reach values negative enough to activate h channels. This is in agreement with the observation that in toads the “peak-plateau” behavior of rod membrane voltage is strongly reduced in 10 mM $[K⁺]_0$, indicating diminished activation of h channels in these conditions (Capovilla et al., 1980). This conclusion is further supported by our result that a decrease in external [K⁺] from 3.3 to 1 mM increased the nose amplitude (Figure 7B).

The voltage-gated potassium conductances can be blocked by tetraethylammonium (TEA) or Ba²⁺ ions in many preparations. In vertebrate rod photoreceptors the $I_{Kx}$ channels are moderately blocked by 10 mM TEA but effectively eliminated by 5 mM Ba²⁺ (Beech & Barnes, 1989). The “block” by Ba²⁺, however, appears not to take place by “plugging” the pore of the $I_{Kx}$ channel but instead by a shift in the activation range of $I_{Kx}$ towards positive $V_m$ (Beech & Barnes, 1989). We used both Ba²⁺ and TEA to probe the contribution of voltage-gated potassium conductances on the nose. Figure 7C and the inset show the effect of 10 mM [Ba²⁺] while Figure 7D presents the effect of 20 mM TEA on the nose component, respectively.
Because Ba$^{2+}$ strongly affected the saturated photoresponse amplitude, we show here the unscaled photoresponses (inset in Figure 7C) and the same responses normalized with regards to the plateau level (Figure 7C). The inset shows that Ba$^{2+}$ strongly decreased the nose amplitude. Although the plateau amplitude of saturated photoresponses was reduced to 56% of that in Ringer solution, the nose amplitude was decreased much more. The relative decrease in the nose amplitude is given in Figure 7C. Ba$^{2+}$ also slowed down the nose kinetics. TEA (20 mM) had qualitatively similar but weaker effects (Figure 7D). These results are consistent with the hypothesis that voltage-gated K$^+$ channels, especially the K$_x$ channels, participate in the generation of the nose. The interpretation of these data is, however, complicated by several factors and will be treated in the Discussion section.

**Discussion**

Information on the origin of various ERG components can be obtained by several experimental methods, e.g., by recording the ERG across different layers of the retina as used by Arden (1976) or by the current-source density analysis technique used by Hagins, Penn, and Yoshikami (1970) in their classical work, and later more extensively by Karwoski et al. (for methodological considerations, see Karwoski, Xu, & Yu, 1996). These methods, however, require the penetration of the recording electrode or electrodes into the retina, which inevitably causes tissue damage in the region of potential recording. In this study we instead chose to use the non-invasive ERG recording across the isolated mouse retina combined with pharmacological manipulations intended to remove desired ERG components in a controlled way, thereby “purifying” the components of interest. Using this approach we were able to gain convincing evidence that the fast nose component in the mouse ERG is generated by rods. On the basis of our pharmacological treatments and perfusate ion composition alterations we have been able to rule out the possible role of most of the known rod membrane current mechanisms that might participate in the generation of the ERG nose component. We propose that the nose mainly originates from the currents of h and K$_x$ channels.

**Figure 7.** Effects of modulations of the K$^+$ current mechanisms in rods. (A) Normalized saturated rod responses in normal Ringer with 3.3 mM K$^+$ (black trace: before and blue trace: after elevated [K$^+$]) and in Ringer containing 10 mM K$^+$ (red). Flash strength: 300,000 Rh*. (B) Normalized saturated rod responses in Ringer (black trace: before and blue trace: after lowered [K$^+$]), and in Ringer with low 1.1 mM K$^+$ (red). Flash strength: 55,000 Rh*. (C) Normalized rod responses in Ringer (black trace: before and blue trace: after exposure to Ba$^{2+}$) and in Ringer with 10 mM Ba$^{2+}$ added (red). The inset shows the same responses without normalization. Flash strength: 300,000 Rh*. (D) Normalized saturated rod responses in Ringer (black trace: before and blue trace: after exposure to 20 mM [TEA]) and in Ringer with 20 mM [TEA] added (red). Flash strength: 63,000 Rh*.
Role of calcium in the nose process

As already observed by Arden (1976), decreasing extracellular [Ca$^{2+}$] to levels below 100 nM removes the nose in saturated murine rod photoresponses. It might seem that calcium served as a current carrier in the nose process. Our data, however, suggest the opposite. The reasoning behind this argument is the following: In toad rods, moderate lowering of [Ca$^{2+}$]$_0$ (up to 10$^{-5}$ M) is shown to depolarize the resting membrane potential while leaving the shape and the peak potential of the saturated response unchanged (Brown & Pinto, 1974; Owen & Torre, 1981). In our experiments the reduction of [Ca$^{2+}$]$_0$ to 10$^{-5}$–10$^{-6}$ M decreased the nose amplitude only slightly (not shown). If calcium were a current carrier in the nose process, this reduction in [Ca$^{2+}$]$_0$ would decrease the number of current carriers by a factor of 100 to a level that can be expected to lower the nose amplitude substantially. Since the nose amplitude was not affected much, ions other than calcium are likely to carry currents in the nose generating process. Additionally, our results showed that the main Ca$^{2+}$ conductance mechanisms of rods, the L-type Ca$^{2+}$ channels and the Na$^+$/Ca$^{2+}$–K$^+$ exchanger, were not related to the nose mechanism.

The very low levels of [Ca$^{2+}$]$_0$ (50 nM or below), which completely abolished the nose in our experiments, have also been shown to eliminate the peak-plateau process of the saturated membrane potential responses of toad rods (Bastian & Fain, 1982; Lipton, Ostroy, & Dowling, 1977). Therefore, it seems plausible that these effects have a common origin, which we suggest to be the h channel. In low [Ca$^{2+}$]$_0$ the resting $V_m$ is strongly depolarized, but the most negative $V_m$ values of saturated photoresponses were only slightly less negative than those in normal Ringer ([Ca$^{2+}$]$_0$ 1.6 mM in Lipton et al., 1977, and 2.0 mM in Bastian & Fain, 1982), thereby reaching the normal activation range of h channels. Therefore the membrane depolarization most probably cannot explain the disappearance of the nose in low Ca$^{2+}$. The gating of h channels, however, has been shown to depend on external calcium (Malcolm, Kourenyi, & Barnes, 2003). They lowered [Ca$^{2+}$]$_0$ from 10 mM to 0.5 mM and observed a negative shift of 5 mV or more in the activation curve of $I_h$ in salamander rods. With our very low [Ca$^{2+}$]$_0$ the activation curve for $I_h$ can be expected to shift even more. Therefore it is likely that low [Ca$^{2+}$]$_0$ shifts the activation range of the h channels beyond the range of negative membrane potentials that can be achieved in those conditions, and this leads to the disappearance of the nose.

Evidence that h and K$_x$ channels generate the nose

In this study we gained multitude evidence supporting the conclusion that the h channels are indispensable for the generation of the nose. Firstly, the introduction of Cs$^+$ and ZD7288, the well-known blockers of h channels, eliminated the nose. Secondly, the disappearance of the nose in very low [Ca$^{2+}$]$_0$ can be explained by a shift of the activation curve of the h channels toward more negative membrane potentials (see above). Further, high external potassium (26 mM) depolarized the resting potential by about 20 mV and the peak level of saturated responses even more in toad rods (Capovilla et al., 1980). This depolarization was accompanied by a loss of the peak-plateau process of saturated voltage responses. Therefore the elimination of the nose by tripling the external [K$^+$] to 10 mM in our mouse experiments was most probably due to the depolarization of the membrane potential beyond the voltage range of the activation of the h channels. In addition, the nose amplitude was increased when [K$^+$]$_0$ was lowered threefold. We interpret this to be due to hyperpolarization of the resting $V_m$ and especially due to more negative $E_K$. In low [K$^+$] the number of open h channels is expected to be slightly increased in the resting state but substantially increased during the saturated photoresponse. The role of the h channels in shaping the ERG signal might have some interest also from the clinical viewpoint. It has been recently shown (Demontis et al., 2009) that the h channels in mouse rods are inhibited by ivabradine, a potential drug molecule for treating angina pectoris (DiFrancesco & Borer, 2007).

It proved complicated to investigate the role of K$^+$ currents in the nose process. Firstly, the potassium currents probably create a substantial portion of the fast PIII. Therefore if the K$_x$ conductance were involved in the generation of the nose, manipulations intended to modify the K$_x$ current component of the nose process inevitably will affect the K$_x$ current generating the fast PIII proper in the same proportion. Secondly, the K$^+$ conductances in the inner segment largely determine the membrane potential of the rod, especially during photoresponses when the cGMP-gated channels are closed. Thus, modulation of the K$^+$ conductances tend to affect the behavior of h channels (see above), thereby producing effects that are not directly related to the potassium currents. The experiments with K$^+$ channel blockers Ba$^{2+}$ and TEA, however, showed a reduction in the fractional nose amplitude, which suggests that K$^+$ ions, most probably mainly through K$_x$ channels, are current carriers in the nose mechanism.

Current dipoles involved in the generation of the nose

In this study we were able to rule out practically all the known conductance mechanisms that might be generating the nose except the h and K$_x$ channels, and we propose the following model for the nose mechanism that largely follows the ionic model of frog rods proposed by Zuckerman (1973). In the model rods have two major current loops in
darkness (Figure 8): (1) A primary “sodium loop” that circulates through the light-sensitive channels in the outer segment and the Na⁺/K⁺ ATPases located at the distal part of the inner segment (Schneider, Shyjan, & Levenson, 1991; Zuckerman, 1973), and (2) a “potassium loop,” which is formed by the Na⁺/K⁺ ATPase and the Kᵦ channels distributed more proximally along the inner segment. In darkness the extracellular currents of these loops flow in the direction from the inner toward the outer segment, i.e., from the proximal to the distal retina. The fast PIII is mainly caused by changes in the currents of these two loops. The fractional significance of each loop depends on the spatial distribution of the membrane current mechanisms (the cGMP-gated channels, the potassium channels, and the Na⁺/K⁺ ATPase) as well as the resistance profile of the extracellular space along the rod photoreceptors. A rod-saturating light drives the current of the sodium loop to zero. The behavior of the potassium loop current, however, is more complicated. Membrane hyperpolarization decreases both the driving force for K⁺ as well as the open probability of the Kᵦ channels, and these factors together decrease the current of the potassium loop. In responses to strong stimuli the potassium current follows the “peak-plateau” process behavior of the membrane potential, i.e., the potassium current first goes to near zero and then partly recovers to a more or less stationary value, which is maintained until the cGMP-gated channels of the outer segment start to open. Hence, this potassium loop forms a transient cornea-negative ERG component, which can be observed as a nose component in the fast PIII.

In addition to the light-induced changes in the Kᵦ current, there is another mechanism possibly contributing to the nose. In mouse rods the h channels are distributed along the whole inner segment including somata, axons, and axon terminals (Knop et al., 2008). As sodium ions are the main current carriers through the h channels under physiological circumstances, a third current loop, here termed as the secondary sodium loop, is formed between the Na⁺/K⁺ ATPase and the h channels as shown in Figure 8. The direction of this extracellular current flow is from distal to proximal, and thus opposite to the extracellular currents in the “primary” sodium loop and the potassium loop. In response to saturating flashes a substantial proportion of the hyperpolarization-activated h channels first open, leading to a partial recovery of the membrane potential, and thus to the closure of most of the h channels. Therefore this secondary sodium loop has a transient character, and most probably it also contributes to the nose.

The relation between ERG signal and light-sensitive current

The leading edge of the a-wave of the ERG is generally believed to accurately reflect the kinetics of the photocurrent (Breton et al., 1994; Hood & Birch, 1990, 1993;...
Robson et al., 2003; Smith & Lamb, 1997) thereby giving information on the amplification of the phototransduction machinery in rods and cones (Lamb & Pugh, 1992; Pugh & Lamb, 1993). Our results supported the view by showing that the h current does not affect the leading edge of the fast PIII of mouse rods. However, the noise component may affect the interpretation of the data of the paired-flash methods revealing the photoreceptor contribution to the ERG in humans and other mammalian rods in vivo. In these methods two flashes are given sequentially, the first flash (“test flash”) produces a response, the amplitude and kinetics of which is to be determined in a “point-by-point” way by giving a second, saturating flash (“probe flash”) at varying times after the test flash (Pepperberg, Birch, & Hood, 1997; Silva, Hetling, & Pepperberg, 2001). In these methods the amplitude of the response to the probe flash is normally measured at a time close to the peak of the nose. If the test flash in the paired-flash experiments is strong enough to open the slowly gated h channels (which most probably is the case except for the very small responses), then the open h channels oppose light-induced hyperpolarization and the response to the probe flash cannot reach as a negative peak value as when no h channels are open before the probe flash. Therefore, the measured amplitude of the probe flash response is expected to become smaller toward more negative membrane potentials during the time of the probe flash.

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