Voltage-Gated Channels and Calcium Homeostasis in Mammalian Rod Photoreceptors

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Voltage-gated channels in mammalian rods, freshly dissociated pig rod photoreceptors were recorded with the whole cell patch-clamp technique. Rod cells expressed 1) a hyperpolarization-activated inward-rectifying conductance ($I_h$) sensitive to external Cs$^+$; 2) a sustained outward K$^+$ current ($I_K$) sensitive to tetraethylammonium; 3) a sustained voltage-gated Ca$^{2+}$ current ($I_{Ca}$) sensitive to benzothiazepine (diltiazem) and phenylalkylamine (verapamil) derivatives; 4) a Ca$^{2+}$-activated Cl$^-$ current ($I_{CaCl}$); and 5) a plasma membrane Ca$^{2+}$-ATPase. The Ca$^{2+}$ current showed a range of activation from positive potentials to −60 mV with a maximum between −30 and −20 mV. In contrast to other L-type Ca$^{2+}$ channels, rod Ca$^{2+}$ channels were blocked at similar and relatively high concentrations by the diltiazem isomers and verapamil. The biphasic dose-response for $n$-diltiazem confirmed the low sensitivity of Ca$^{2+}$ channels for the molecule. The ATPase, which was localized at the axon terminal, was found to contribute to Ca$^{2+}$ extrusion. These results suggest that the electrophysiological features of rod photoreceptors had been preserved during evolution from nonmammalian vertebrates to mammals. This work indicates further that mammalian rods express nonclassic L-type Ca$^{2+}$ channels, showing a low sensitivity to the diltiazem isomers used in neuroprotective studies.

INTRODUCTION

Applications of Ca$^{2+}$ channel blockers were recently reported to limit rod photoreceptors degeneration in the rd mouse and the RCS rat, two animal models of retinitis pigmentosa (Frasson et al. 1999; Takano et al. 2004; Yamazaki et al. 2002). Furthermore, patients affected by this inherited retinal dystrophy seemed to have stabilized or improved vision when treated with d-diltiazem, one of these Ca$^{2+}$ channel blockers, administered in conjunction with taurine and vitamin E (Paspantes-Morales et al. 2002). Although diltiazem isomers were known to affect cyclic guanosine 5’-monophosphate (cGMP)-gated channels in membranes of isolated rod photoreceptor outer segments (Koch and Kaupp 1985), no data are currently available on their effects on native rod photoreceptor Ca$^{2+}$ channels.

Since their first original recordings in the 1970s, vertebrate rod photoreceptors were electrophysiologically characterized mainly in nonmammalian vertebrates such as salamanders, because the large size of rods facilitated in these species their recordings. cGMP-gated channels located in the outer segment generate a depolarizing cationic current in dark-adapted photoreceptors (McNaughton 1990). Light activation of the phototransduction cascade results in the cGMP hydrolysis (Stryer 1986) and the consecutive closure of cGMP-gated channels triggered photoreceptor hyperpolarization (Fesenko et al. 1985). The light response is shaped further by ionic conductances generated in the inner segment and in the synaptic terminal (Barnes 1994; Fain and Lisman 1981). A hyperpolarization-activated inward-rectifying current ($I_h$) limits the hyperpolarization due to the cGMP-gated channel closure (Bader et al. 1982; Han et al. 2000; Kawai et al. 2002). Inversely, a voltage-gated K$^+$ current ($I_K$) may counteract the depolarization generated by cGMP-gated channels and thus contribute to the dark resting potential (Beech and Barnes 1989; Han et al. 2000). At the rod terminal, a voltage-gated Ca$^{2+}$ current ($I_{Ca}$) controls the release of the neurotransmitter, glutamate, and thus the transmission of the visual signal (Nachman-Clewner et al. 1999; Schmitz and Witkovsky 1997). Ca$^{2+}$ also activates a Cl$^-$ conductance ($I_{Cl(Ca)}$) that further regulates the voltage-dependent Ca$^{2+}$ channels and thereby the Ca$^{2+}$-dependent glutamate release (Bader et al. 1982; Burkhart et al. 1991). Ca$^{2+}$ homeostasis is not only controlled by these two channel populations but also by a plasma membrane Ca$^{2+}$-ATPase (PMCA) (Krizaj and Copenhagen 1998).

Although the voltage-gated channels and the mechanisms of Ca$^{2+}$ extrusion were extensively investigated in cold-blooded vertebrate rod photoreceptors (Attwell and Wilson 1980; Bader and Bertrand 1984; Bader et al. 1982; Beech and Barnes 1989; Corey et al. 1984; Fain et al. 1978; Kouroumi and Barnes 2000; Krizaj and Copenhagen 1998; Stella et al. 2002; Thoreson et al. 2003; Wollmuth 1995; Wollmuth and Hille 1992), their studies in mammalian cells were limited to $I_h$ and $I_K$ currents (guinea pig: Denmontis et al. 1999; primate: Han et al.)
2000; human: Kawai et al. 2002). Surprisingly, a Na\textsuperscript{+} channel was reported in human rods that were classically considered nonspiking neurons (Kawai et al. 2001).

In this study, we measured ionic currents in freshly dissociated rod cells from the pig retina, and we showed that these cells expressed \( I_{\text{h}}, I_{\text{K}}, I_{\text{Ca}} \) and \( I_{\text{Cl(Ca)}} \). We further characterized the pharmacological properties of \( I_{\text{Ca}} \) by testing the effects of the selected Ca\textsuperscript{2+} channel blockers, diltiazem and verapamil. We also investigated the PMCA implication in the mechanisms of Ca\textsuperscript{2+} extrusion from photoreceptors. These results indicate that physiological features of rod photoreceptors were highly preserved during evolution and that rod Ca\textsuperscript{2+} channels are poorly sensitive to diltiazem isomers.

**METHODS**

**Cell preparation**

Pig eyes were obtained from a local slaughterhouse. Globes were removed from the animals within 5 min after death and were transported at 4°C in CO\textsubscript{2}-independent Dulbecco’s modified eagle’s medium (DMEM/-CO\textsubscript{2}, Gibco, Life Technologies, Cergy-Pontoise, France).

The ionic currents \( I_{\text{h}}, I_{\text{K}}, I_{\text{Ca}} \) and \( I_{\text{Cl(Ca)}} \) were measured in rod photoreceptors that were isolated using a dissociation method described previously (Gaudin et al. 1996). Eyecups were isolated in DMEM/-CO\textsubscript{2} following a circular section below the ora serata and the removal of the anterior chamber. The retina was subsequently isolated and chopped into small fragments. Retinal fragments were washed twice in Ringer solution without Ca\textsuperscript{2+} and supplemented with 0.1 mM EDTA. They were incubated with 0.2% activated papain (Worthington, Freehold, NJ) for 20 min at 37°C. Digestion was stopped by the addition of the same volume of Neurobasal-A medium containing 2% fetal calf serum (Nb-A/FCS, Gibco), 0.1 mg/ml bovine serum albumin (BSA). The fragments were mechanically dissociated by repeated gentle shaking, and cell supernatants were collected, pooled, and centrifuged at 800 rpm for 5 min. After centrifugation, cells were resuspended in Nb-A containing 2% of B27 antioxidant (Gibco), immediately plated, and kept in the incubator at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}-95% air. After 90 min in culture, rods identified with their typical morphology were added to the bath solution without Ca\textsuperscript{2+} and supplemented with 0.1 mM Ca\textsuperscript{2+} before use.

**Electrophysiological recordings**

Recordings were performed as described previously (Picaud et al. 1998). Recording pipettes were pulled from thin-walled borosilicate glass (model TW 150F, World Precision Instruments, Sarasota, FL) using a Brown and Flaming type puller (model P-87, Sutter Instruments, Novato, CA). A patch-clamp amplifier (model RK400, Biologic, Grenoble, France) was used to voltage clamp the recorded cells. Data were filtered at 3 kHz and digitized at 0.250–10 kHz during voltage-step experiments using a data acquisition Labmaster board (Scientific Solutions, Solon, OH) mounted to an IBM-compatible personal computer. Experimental data were acquired and analyzed using the Patchit and Tack software packages, respectively.

**Solutions and drug application**

The standard bathing solution contained (in mM) 135 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 5 HEPES, and was titrated to pH 7.75 with NaOH. When measuring Ba\textsuperscript{2+} currents, the solution contained (in mM) 5 CsCl\textsubscript{2}, 30 tetraethylammonium (TEA), 100 NaCl, 5 KCl, 2 BaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 5 HEPES, and was adjusted to pH 7.75 with NaOH. Blocking Ca\textsuperscript{2+} channels was achieved by substituting 2 mM CoCl\textsubscript{2} for BaCl\textsubscript{2} in the solution. When investigating Ca\textsuperscript{2+} current inhibition and Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents, the bath solution contained (in mM) 5 CsCl\textsubscript{2}, 30 TEA, 100 NaCl, 5 KCl, 5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 5 HEPES, and was adjusted to pH 7.75 with NaOH. Blocking Ca\textsuperscript{2+} currents was achieved by adding 100 µM CdCl\textsubscript{2} to the solution.

**Immunocytochemistry**

Dissociated cells were fixed in 4% (wt/vol) paraformaldehyde in PBS for 2 min at room temperature. Membrane permeabilization and blocking unspecific binding sites were achieved by incubating cells for 1 h at room temperature with PBS (pH 7.4), containing 10% (vol/vol) normal goat serum (Ngs), 1% (wt/vol) BSA, and 0.1% (vol/vol) Triton X-100. Cells were incubated for 2 h at room temperature with the mouse monoclonal antibody directed against an epitope in the highly conserved hinge region on the intracellular loop of the PMCA (Sigma). The antibody was diluted 1:1,000 in PBS supplemented with 3% (vol/vol) Ngs, 1% (wt/vol) BSA, and 0.1% (vol/vol) Tween 20. After several washes, cells were incubated for 1 h at 37°C in the dark with a goat anti-mouse IgG antibody conjugated to Alexa TM 594 (Molecular Probes, Eugene, OR) diluted at 1:500. After mounting, cells were examined with an Olympus microscope coupled to a camera (DP50) allowing to take pictures using the software Soft Imaging System. Figures were prepared using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

**RESULTS**

**Morphological identification of rod cells**

Freshly dissociated rod cells were identified by their typical morphology with the outer/inner segments and the axon terminal (Fig. 1). The morphology of rods was different from that of cones; they were smaller and had narrower inner segments connected by a thin process to the cell body (Fig. 1A). On the contrary, cones possessed a large inner segment in direct apposition to the cell body with a longer and thicker axon (Fig. 1B). Some recorded rods had lost the outer segment but conserved the inner segment and the synaptic terminal.
Response to voltage steps

Figure 2 shows the currents measured in response to voltage steps from freshly dissociated rod photoreceptors of the pig retina. The voltage steps were applied from a holding potential of –70 mV to potentials ranging from –120 to +50 mV in 10-mV increments (Fig. 2A). The current-voltage (I-V) curve is shown in Fig. 2B. At –70 mV, an inward current was observed that decreased with small depolarizations to –60 and –50 mV. The current reversed at –40 mV, and a linear outward current developed when cells were depolarized beyond –20 mV. The I-V curve showed also an inward rectification when the membrane was hyperpolarized below the holding potential of –70 mV. The following sections describe individually several ionic conductances that underlie these responses to the voltage steps.

Inward-rectifying current

Pig rod cells expressed a large inward conductance when the membrane was hyperpolarized to potentials more negative than the holding potential of –70 mV (Fig. 3). This current was completely and reversibly blocked by 5 mM Cs⁺ applied in the bath solution (Fig. 3B). Figure 3C shows the isolated current obtained by subtracting the currents recorded in the presence of Cs⁺ (Fig. 3B) from those in its absence (Fig. 3A). The steady-state I-V relationship, plotted in Fig. 3D, shows the inward rectification of the current. This current was activated at potentials below –50 mV. A slowly increasing inward current was observed at –60, –70, and –80 mV. When cells were hyperpolarized below –80 mV, the inward current became more pronounced, and its amplitude increased continuously with the hyperpolarizing voltage pulses. These current characteristics are consistent with the presence of an inward-rectifying cationic conductance, Iʜ, in pig photoreceptors.

Delayed-rectifier K⁺ current

Rod cells generated a sustained outward current at potentials more positive than –60 mV (Fig. 4). After blocking the inward-rectifying current with 5 mM Cs⁺ (Fig. 4A), this outward current was suppressed by 30 mM TEA⁺ ions (Fig. 4B). The TEA-sensitive current, calculated as the difference between currents shown in Fig. 4, A and B, is shown in Fig. 4C. The steady-state I-V relationship, plotted in Fig. 4D, shows a biphasic shape with slowly increasing outward currents at potentials between –50 and –20 mV and large current amplitudes above –10 mV. This TEA-sensitive outward current is consistent with the presence of a delayed-rectifier K⁺ current, IК, in pig rod photoreceptors.
Other voltage-gated currents, $I_h$ and $I_K$, were suppressed with 5 mM Cs$^+$ and 30 mM TEA$^+$ applied in the bath solution. Ba$^{2+}$ currents flowing through Ca$^{2+}$ channels (Fig. 5C) were calculated by subtracting the currents obtained in the presence of Co$^{2+}$ (Fig. 5B) from those in the presence of Ba$^{2+}$ (Fig. 5A). The steady-state $I-V$ relationship, plotted in Fig. 5D, shows that this current was sustained, evoked at potentials above –60 mV, and reached a maximum between –30 and –20 mV.

The Ca$^{2+}$ current was sensitive to the benzoiazepine derivative, diltiazem, and to the phenylalkylamine derivative, verapamil (Fig. 6). To quantify the effect of these blockers, the whole cell currents were measured in the presence of 5 mM Ca$^{2+}$, 5 mM Cs$^+$, and 30 mM TEA$^+$ in the extracellular solution. The maximum amplitude of Ca$^{2+}$ currents was obtained by fully inactivating the channels with an addition of 100 μM Cd$^{2+}$ to the bath solution. For a voltage step to –20 mV, d-diltiazem, l-diltiazem, and verapamil blocked the Ca$^{2+}$ current by 16.5 ± 3.2 (n = 9), 16.5 ± 2.9 (n = 9), and 14.1 ± 4.3% (SE; n = 13) at 10 μM and by 65.9 ± 6.2 (n = 9), 58.8 ± 4.8 (n = 9), and 73.6 ± 2.3% (n = 13) at 100 μM, respectively.

Figure 6B shows the dose-response plot for the d-diltiazem inhibition of the Ca$^{2+}$ current measured in the presence of 5 mM extracellular Ca$^{2+}$. d-diltiazem inhibited the Ca$^{2+}$ cur-

Voltage-gated Ca$^{2+}$ current

Rod photoreceptors expressed a sustained inward Ca$^{2+}$ current, $I_{Ca}$, which was activated by stepping the cell potential from –70 mV to more positive values (Fig. 5). To minimize Ca$^{2+}$-activated inward currents, currents generated by voltage-gated Ca$^{2+}$ channels were isolated by measuring cell currents in the presence of 2 mM Ba$^{2+}$ substituted for Ca$^{2+}$ (Fig. 5A). Whole cell currents were measured in the presence of 2 mM Co$^{2+}$, substituted for Ba$^{2+}$, to block Ca$^{2+}$ channels (Fig. 5B).

Voltage-gated Ca$^{2+}$ current (l$_{Ca}$). A–C: measurement of the tetraethylammonium (TEA)-sensitive $I_{K}$ currents in a porcine rod. Current responses to voltage steps were measured in the absence (A) or presence (B) of 30 mM TEA in a rod voltage clamped at –70 mV and stepped from –120 to +50 mV in 10-mV increments. C: TEA-sensitive $I_{K}$ currents were calculated by subtracting current responses in the presence of TEA (B) to those in its absence (A). All measurements were obtained in the presence of 5 mM Cs$^+$ in the bath solution. D: averaged $I-V$ curve of the TEA-sensitive $I_{K}$ currents. Measurements were normalized in each cell to the maximum current (at +60 mV) before averaging (means ± SD, n = 6).

FIG. 5. Voltage-gated Ca$^{2+}$ current ($I_{Ca}$). A–C: measurement of Ba$^{2+}$ currents generated by voltage-dependent Ca$^{2+}$ channels in a porcine rod. Current responses were measured in the presence of Ba$^{2+}$ (A) or Co$^{2+}$ (B) in a rod voltage clamped at –70 mV and stepped from –120 to +50 mV in 10-mV increments. C: voltage-dependent Ba$^{2+}$ currents were calculated by subtracting current responses in the presence of Co$^{2+}$ (B) to those in the presence of Ba$^{2+}$ (A). In this experiment, Co$^{2+}$ (2 mM) was substituted for Ba$^{2+}$ in the bath solution, which always contained 5 mM Cs$^+$ and 30 mM TEA. D: averaged $I-V$ curve of the Ba$^{2+}$ currents generated by voltage-gated Ca$^{2+}$ channels. Data are expressed as means ± SE (n = 5).

FIG. 6. Inhibition of voltage-gated Ca$^{2+}$ currents by Ca$^{2+}$ channel blockers, d-diltiazem, l-diltiazem, and verapamil. A: Ca$^{2+}$ currents in control conditions and in the presence of d-diltiazem, l-diltiazem, and verapamil (10 and 100 μM). Ca$^{2+}$ current in control conditions was calculated by subtracting whole cell currents in the presence of Cd$^{2+}$ (100 μM) to those in its absence. Currents remaining in the presence of the Ca$^{2+}$ channel blockers were obtained by subtracting whole cell currents in the presence of Cd$^{2+}$ from those in the presence of the blocker. All measurements were obtained in the presence of 5 mM Cs$^+$ and 30 mM TEA during a voltage step from the holding potential of –70 to –20 mV. B: dose-response plot of Ca$^{2+}$ current inhibition by d-diltiazem. At each concentration (0.3, 1, 3, 10, 30, and 100 μM), suppressed Ca$^{2+}$ current was normalized to maximum inward cell current. Data are expressed as means ± SE.
When D-diltiazem was applied at 30 μM, Ca²⁺ was detected with a large tail current observed when returning to –70 mV. It depolarizations (5 s; Fig. 7). This current was also associated with a large tail current observed when returning to –70 mV. In symmetrical Ca²⁺ concentrations, an inward current was observed at –10 mV (a), no current was induced at 0 mV (b), and an outward current was generated at +10 mV (c). In contrast, when gluconate was substituted for Cl⁻ in the pipette recording to set E_Cl to –31.2 mV (b), the current was already outward at –20 mV. Cl⁻ dependence of current induced by long depolarizations. When Cd²⁺ (100 μM) was applied in the bathing solution to block Ca²⁺ currents, the slowly activating Cl⁻ current and its accompanying tail current were both suppressed (Fig. 7B). These observations indicate that the slowly activating current was carried by Cl⁻ ions. However, when Ca²⁺ currents were blocked by Cd²⁺ (100 μM) applied in the bath solution, this slowly activating Cl⁻ current and its associated tail current were both suppressed (Fig. 7C). Therefore these current characteristics suggest that the slowly activating current is generated in pig rod photoreceptors by a Ca²⁺-activated Cl⁻ conductance, I(Cl(Ca)).

PMCA

The extrusion of intracellular Ca²⁺ from the photoreceptors inner segments was attributed to a PMCA in salamander rods (Krizaj and Copenhagen 1998). In mammalian cones (Morgans et al. 1998), the tail current of the Ca²⁺-activated Cl⁻ current was used to follow the kinetic of the Ca²⁺ extrusion mechanisms. Therefore a similar approach was used in this work to assess the implication of the PMCA in Ca²⁺ extrusion in pig rod photoreceptors. Figure 8Aa shows the Ca²⁺-activated Cl⁻ current (I(Cl(Ca))) measured in the presence of 2 mM extracellular Ca²⁺ during a voltage step to –20 mV from a holding potential of -31.2 mV, the reversal potential of the current shifted toward a more negative potential and an outward current was still observed for a voltage step to –20 mV (Fig. 7B). These observations indicate that the slowly activating current was carried by Cl⁻ ions. However, when Ca²⁺ currents were blocked by Cd²⁺ (100 μM) applied in the bath solution, this slowly activating Cl⁻ current and its associated tail current were both suppressed (Fig. 7C). Therefore these current characteristics suggest that the slowly activating current is generated in pig rod photoreceptors by a Ca²⁺-activated Cl⁻ conductance, I(Cl(Ca)).

Pig rod cells expressed a current that was activated by long depolarizations (5 s; Fig. 7). This current was also associated with a large tail current observed when returning to –70 mV. It was detected when I_k and I_h were blocked by TEA⁺ (30 mM) and Cs⁺ (5 mM), respectively.

Ca²⁺-activated anion current

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of \(-70 \text{ mV}\). When symmetrical Cl\(^-\) solution were used in the bath and pipette solutions, large Ca\(^{2+}\)-activated Cl\(^-\) tail currents were observed on returning to \(-70 \text{ mV}\) (Fig. 8A). When 1 mM Na\(^{+}\)-orthovanadate, a known PMCA blocker (Carafoli 1992; Morgans et al. 1998), was added to the intracellular recording solution, the duration and magnitude of the Ca\(^{2+}\)-activated Cl\(^-\) tail current was greatly increased (Fig. 8B). In control condition, the kinetics of the tail current was well fitted by a first-order exponential decay with a fast time constant of 0.6 \pm 0.2 s (n = 4), whereas in the presence of Na\(^{+}\)-orthovanadate, the kinetics was not as nicely fitted, and the time constant increased to 15.5 \pm 1.0 s (n = 3). This result suggests that Ca\(^{2+}\) extrusion is controlled by the PMCA in pig rod photoreceptors. To further locate the zone of Ca\(^{2+}\) extrusion, photoreceptors were immunolabeled with an antibody identifying the Ca\(^{2+}\)+ATPase. An intense PMCA immunostaining localized in rod spherules with a distribution similar to that observed in cone pedicles (Fig. 8B). In the pedicles of the dissociated cone photoreceptors, the PMCA immunoreactivity exhibited the inverted V-shaped staining (Fig. 8, C–E), previously described in situ (Morgans et al. 1998). These observations indicate that Ca\(^{2+}\) extrusion is controlled by the plasma membrane Ca\(^{2+}\)-ATPase at the rod photoreceptor terminals in the pig retina.

**Discussion**

In this study, we characterized the electrophysiological features of rod photoreceptors freshly dissociated from the pig retina. In our recording conditions, rods expressed the following ionic currents: 1) a hyperpolarization-activated inward-rectifying current (\(I_h\)); 2) a sustained outward K\(^+\) current (\(I_{K}\)); 3) a voltage-gated Ca\(^{2+}\) current (\(I_{Ca}\)); and 4) a Ca\(^{2+}\)-activated Cl\(^-\) current (\(I_{Cl(Ca)}\)). Furthermore, we showed that Ca\(^{2+}\) homeostasis relies on the Ca\(^{2+}\) influx through a nonclassic L-type Ca\(^{2+}\) channel, which can be regulated by the Ca\(^{2+}\)-activated Cl\(^-\) conductance and on the Ca\(^{2+}\) extrusion generated by a PMCA. Verapamil and diltiazem isolated blocked the Ca\(^{2+}\) channels in rod photoreceptors at relatively high concentrations. Our results suggest that mammalian rods operate like nonmammalian vertebrate photoreceptors and that pig rods provide an adequate model to characterize further the pharmacology of ionic conductances in mammalian rod photoreceptors.

**Voltage-gated \(I_p\), \(I_K\), and \(I_{Na}\) currents**

Rod photoreceptor electrophysiological features were characterized in nonmammalian vertebrate species as salamanders or turtles. These neurons expressed different ionic conductances including \(I_h\) (Attwell and Wilson 1980; Bader and Bertrand 1984; Bader et al. 1982; Fain et al. 1978; Wollmuth 1995; Wollmuth and Hille 1992) and \(I_K\) (Attwell and Wilson 1980; Bader et al. 1982; Beech and Barnes 1989). Rod photoreceptors were subsequently recorded in mammal species such as guinea pigs (Demontis et al. 1999), monkeys (Han et al. 2000), and humans (Kawai and Morgan 2002). In our study, we confirmed the presence of \(I_K\) in mammalian rod cells. A slowly developing, inward-rectifying current (\(I_p\)) was recorded in rod cells of pig retina, which was activated by membrane hyperpolarization below \(-60 \text{ mV}\) and blocked by external Cs\(^+\).

Similarly, the outward K\(^+\) current (\(I_K\)) blocked by external TEA application was observed from positive potentials to \(-60 \text{ mV}\). As proposed in the amphibian (Bader et al. 1982; Fain et al. 1978), monkey (Han et al. 2000), and human retina (Kawai and Morgan 2002), the \(I_h\) current may limit the mammalian rod hyperpolarization due to the cGMP-gated channels closure in bright light conditions (Barnes 1994; Demontis et al. 1999), whereas \(I_K\) could counteract the rod depolarization related to the cGMP-gated channel opening.

Even though the presence of a Na\(^+\) current was reported in human rod photoreceptors generating spikes in these neurons (Kawai et al. 2001), no Na\(^+\) currents could be recorded in porcine rod photoreceptors. This difference may result either from a species specificity or from the cell preparation; human rods were indeed recorded in retinal slices and not in dissociated cells.

**Calcium homeostasis**

Voltage-gated Ca\(^{2+}\) currents have been recorded in rod photoreceptors of nonmammalian vertebrates (Bader et al. 1982; Corey et al. 1984; Kourennyi and Barnes 2000; Stella et al. 2002). These recordings showed a very slow current activation during long depolarization, consistent with their role in the sustained glutamate release in depolarized dark-adapted photoreceptors (Schmitz and Witkovsky 1997). Furthermore, these rod Ca\(^{2+}\) currents activated at potentials close to \(-40 \text{ mV}\) and were sensitive to dihydropyridine Ca\(^{2+}\) channel blockers (Bader et al. 1982; Corey et al. 1984; Kourennyi and Barnes 2000; Schmitz and Witkovsky 1997; Stella et al. 2002). Based on these biophysical features and pharmacological criteria, rod voltage-gated Ca\(^{2+}\) channels were classified as L-type (Nowycky et al. 1985). In mammalian cells, Ca\(^{2+}\) channels were reported to be present at high-density in rod pedicles (Krizaj and Copenhagen 1998; Nachman-Clewner et al. 1999) and to be composed of \(\alpha_{1F}\) subunits (Bech-Hansen et al. 1998; Morgans 2001; Strom et al. 1998). These channel subunits were cloned from the mouse and human retina and expressed in HEK293 cells where they can form functional channels (Baumann et al. 2004; McRory et al. 2004). In these cells, the expressed channels showed an activation threshold close to \(-40 \text{ mV}\), a peak amplitude between 0 and 20 mV, and a very slow inactivation. In our study, voltage-gated Ca\(^{2+}\) channels were recorded in freshly isolated pig rod photoreceptors. Ca\(^{2+}\) channels activated at potentials positive to \(-60 \text{ mV}\) and reached a maximum between \(-30 \text{ and } -20 \text{ mV}\). This activation range was more negative than those reported for the cloned mouse and human rod Ca\(^{2+}\) channels (Baumann et al. 2004; McRory et al. 2004), and generally, for L-type Ca\(^{2+}\) channels (Cox and Dunlap 1992). In fact, this dynamic range correlates more closely to the physiological range of rod photoreceptors. A similar dynamic range was reported also in mammalian cone photoreceptors in the monkey and tree shrew retina (Taylor and Morgans 1998; Yagi and Macleish 1994). In the amphibian retina, the rod Ca\(^{2+}\) channels often appeared to activate at a more positive potential (Bader et al. 1982; Corey et al. 1984; Kourennyi and Barnes 2000; Krizaj et al. 1999; Stella et al. 2002), although this was not confirmed in a recent study (Thoreson et al. 2003). The difference in the dynamic range between the native porcine rod Ca\(^{2+}\) channels and the cloned \(\alpha_{1}\) subunit Ca\(^{2+}\) channels may be due to the lack of the \(\beta\) subunit.
subunit. Experimental conditions, pH, and Ca\(^{2+}\) concentrations may also affect this dynamic range of the channels as suggested previously (Thoreson et al. 2003).

The diltiazem isomers, two benzothiazepine derivatives, and verapamil, a phenylalkylamine derivative, blocked rod cloned channels, although their sensitivities were much lower than those reported for the cardiac and smooth muscle channels (Baumann et al. 2004). Using native porcine rod Ca\(^{2+}\) channels, we found similar sensitivities to these Ca\(^{2+}\) channel blockers as those reported in the cloned rod channels (Baumann et al. 2004), confirming the difference of rod Ca\(^{2+}\) blockers as those reported in the cloned rod channels (Bohle 1992; Glossmann et al. 1983) that showed very different sensitivities to the two diltiazem isomers, both native and cloned mammalian rod Ca\(^{2+}\) channels were equally sensitive to these two compounds. Furthermore, in contrast to skeletal muscle Ca\(^{2+}\) channels (Bohle 1992; Glossmann et al. 1983) that showed very different sensitivities to the two diltiazem isomers, both native and cloned mammalian rod Ca\(^{2+}\) channels were equally sensitive to these two compounds. Finally, the dose response to d-diltiazem exhibited a biphasic shape highly reminiscent to that described in amphibian cone photoreceptors (Hart et al. 2003). Mammalian rod photoreceptors appear to express L-type Ca\(^{2+}\) channels with nonclassic kinetic and pharmacological properties.

In amphibian rods, the Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels was shown to generate a Ca\(^{2+}\)-activated Cl\(^{-}\) current ($I_{CaCl}$) (Burkhardt et al. 1991). Our study indicates that this Ca\(^{2+}\)-activated Cl\(^{-}\) current is also present in porcine rod photoreceptors. This Ca\(^{2+}\)-activated Cl\(^{-}\) conductance provides a feedback mechanism to regulate Ca\(^{2+}\) influx, the Cl\(^{-}\) currents modifying the cell membrane potential, and thus the activation of voltage-gated Ca\(^{2+}\) channels. The sign of this feedback would highly depend on the reversal potential for Cl\(^{-}\) in rods. Its role in the prolonged depolarization of amphibian rod photoreceptors would indicate that it can functions as a positive feedback (Burkhardt et al. 1991). However, in the mammalian cone photoreceptor, the Cl\(^{-}\) current has been proposed to oppose the regenerative depolarization of the terminal region that might be caused by the activation of voltage-gated Ca\(^{2+}\) channels (monkey cone: Yagi and Macleish 1994). In cones again, this current is suspected to contribute also to the feedback from horizontal cells (Burkhardt et al. 1988; Kaneko and Tachibana 1986; Thoreson and Burkhardt 1991; Wu 1986, 1991). As well as in cones, this Ca\(^{2+}\)-activated Cl\(^{-}\) conductance may therefore contribute to the rod regulation of glutamate release in the synaptic cleft and to the feedback signal from horizontal cells.

In amphibian rod photoreceptors, the extrusion of intracellular Ca\(^{2+}\) from the inner segments and synaptic terminals was shown to be dependent on a PMCA activity (Krizaj and Copenhagen 1998). In the mammalian retina, immunolabeling studies located the PMCA to the outer plexiform layer of both rod-dominated rat retina and cone-dominated tree shrew retina, suggesting the PMCA expression in both rod and cone terminals (Krizaj et al. 2002; Morgans et al. 1998). The use of specific antibodies indicated further that mouse rod expressed the isoform 1 of the PMCA family (Krizaj et al. 2002). PMCA activity was shown in cones but was not assessed in rods of the tree shrew retina (Morgans et al. 1998). In this work, we have shown not only that mammalian rod photoreceptors do express a physiologically active PMCA, but also that the PMCA was located to the synaptic terminals of both isolated porcine rods and cones. This distribution did not seem affected by the dissociation because the PMCA immunostaining showed the inverted V shape reported in situ by others (Morgans et al. 1998). Ca\(^{2+}\) homeostasis in mammalian rod pedicles may thus be controlled by the voltage-gated channels as well as the Ca\(^{2+}\)-activated Cl\(^{-}\) currents and the PMCA activity.

**Calcium channel blockers and photoreceptor neuroprotection**

The contribution of Ca\(^{2+}\) channels in rod photoreceptor degeneration was suggested by the reduced photoreceptor degeneration observed when injecting Ca\(^{2+}\) channel blockers to animal models of retinal dystrophies (Frasson et al. 1999; Takano et al. 2004; Yamazaki et al. 2002). This was further shown by knocking out a Ca\(^{2+}\) channel subunit in the rd mouse (Read et al. 2002). However, in a pharmacological model of photoreceptor degeneration, a protection was provided only by l-diltiazem and not by d-diltiazem (Fox et al. 2003). The Ca\(^{2+}\) channels, in contrast to cGMP-gated channels, have similar sensitivities to both diltiazem isomers (Fig. 6A). So, this selective neuroprotection by l-diltiazem suggested that the blockage of cGMP-gated channels might also be very important for photoreceptor neuroprotection. In patients, the diltiazem concentration in serum ranges from 0.03 to 2.06 μg/ml (0.06–4.57 μM) (Bloedow et al. 1982), concentrations too low to efficiently block rod Ca\(^{2+}\) channels, suggesting that the diltiazem effect on rod Ca\(^{2+}\) channels may provide a minor contribution to the reported neuroprotection (Pasantes-Morales et al. 2002). Future studies will be necessary to determine the respective effects of photoreceptor neuroprotective molecules on their Ca\(^{2+}\) channels and cGMP-gated channels.

**GRANTS**

This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM); University Pierre and Marie Curie (Paris VI), Assistance Publique-Hôpitaux de Paris (AP-HP), Fédération des Aveugles de France, RETINA-France, Association Française contre les Myopathies (AFM), and the European Economic Community (RETRAINET: HPRN-CT-2000-00098, PRO-AGE-RET: QLK6-2001-00385, PRO-RET: QLK6-2001-00569). D. Cia received fellowships from the Conseil Régional d’Auvergne, the Fédération des Aveugles de France, and the GIS Aventis. A. Bordais received fellowships from RETINA-France, C. Varela received fellowships from the European Economic Community (RETRAINET: HPRN-CT-2000-00098), and S. Picaud received a contrat d’interface between INSERM and Assistance Publique-Hôpitaux de Paris.

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