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Multiple Ca^{2+} -Dependent Mechanisms Regulate L-Type Ca^{2+} Current in Retinal Amacrine Cells

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Tekmen M, Gleason E. Multiple Ca^{2+} -dependent mechanisms regulate L-type Ca^{2+} current in retinal amacrine cells. *J Neurophysiol* 104: 1849–1866, 2010. First published August 4, 2010; doi:10.1152/jn.00031.2010. Understanding the regulation of L-type voltage-gated Ca^{2+} current is an important component of elucidating the signaling capabilities of retinal amacrine cells. Here we ask how the cytosolic Ca^{2+} environment and the balance of Ca^{2+} -dependent effectors shape native L-type Ca^{2+} channel function in these cells. To achieve this, whole cell voltage clamp recordings were made from cultured amacrine cells under conditions that address the contribution of mitochondrial Ca^{2+} uptake (MCU), Ca^{2+} /calmodulin (CaM)-dependent channel inactivation (CDI), protein kinase A (PKA), and Ca^{2+} -induced Ca^{2+} release (CICR). Under control conditions, repeated activation of the L-type channels produces a progressive enhancement of the current. Inhibition of MCU causes a reduction in the Ca^{2+} current amplitude that is dependent on Ca^{2+} influx as well as cytosolic Ca^{2+} buffering, consistent with CDI. Including the Ca^{2+} buffer bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) internally can shift the balance between enhancement and inhibition such that inhibition of MCU can enhance the current. Inhibition of PKA can remove the enhancing effect of BAPTA suggesting that cyclic AMP-dependent phosphorylation is involved. Inhibition of CaM suppresses CDI but spares the enhancement, consistent with the substantially higher sensitivity of the Ca^{2+} -sensitive adenylyl cyclase 1 (AC1) to Ca^{2+} /CaM. Inhibition of the ryanodine receptor reduces the current amplitude, suggesting that CICR might normally amplify the activation of AC1 and stimulation of PKA activity. These experiments reveal that the amplitude of L-type Ca^{2+} currents in retinal amacrine cells are both positively and negatively regulated by Ca^{2+} -dependent mechanisms.

INTRODUCTION

Amacrine cells are retinal interneurons that signal extensively in the inner plexiform layer of the retina. The functions of amacrine cells are diverse but include shaping the response properties of ganglion cells, the output cells of the retina (Baccus 2007; Demb 2007; Zhou and Lee 2008). These key players in retinal signal processing often participate in serial (Dowling and Boycott 1966; Dubin 1970; Guiloff et al. 1988; Pollard and Eldred 1990; Zhang et al. 1997) and reciprocal synapses (Hartveit 1999; Shields and Lukasiewicz 2003; Vigh and von Gersdorff 2005), implying that local synaptic environments might be regulated independently of one another. It has been established for several classes of amacrine cells that L-type Ca^{2+} channels are employed at their synapses to control neurotransmitter release (Bieda and Copenhagen 2004; Gleason et al. 1994; Habermann et al. 2003; Vigh and Lasater

2004). Thus the regulation of these channels can play a central role in visual signal processing.

The pore-forming region of the L-type channel is encoded by one of four genes: $\text{Ca}_v1.1$ – 1.4 . $\text{Ca}_v1.1$ is expressed in skeletal muscle, $\text{Ca}_v1.2$ and 1.3 are the dominant L-type channels in the brain (Hell et al. 1993), and $\text{Ca}_v1.4$ is expressed predominately at ribbon synapses in the retina (Strom et al. 1998). L-type Ca^{2+} channels are distinctive in that they can support fairly sustained levels of Ca^{2+} influx. This Ca^{2+} influx can have a variety of effects including regulation of the channel itself via Ca^{2+} /calmodulin (CaM)-dependent inactivation (CDI) that occurs for most Ca_v1 and 2 (non-L-type) Ca^{2+} channels (for review see, Halling et al. 2005).

The molecular players and details of this inactivation have been described by an elegant set of experiments on $\text{Ca}_v1.2$ channels (Dick et al. 2008; Tadross et al. 2008). The efficiency of the inactivation process is optimized by the preassociation of CaM to the channel (Erickson et al. 2001; Pitt et al. 2001). Ca^{2+} entering through the channel binds CaM and inactivation is initiated. In $\text{Ca}_v1.2/1.3$ channels, the CaM sensors detect both local and global Ca^{2+} (Dick et al. 2008). The local concentration of Ca^{2+} eliciting this response is on the order of 100 μM , which only exists within hundreds of angstroms of the channel pore (Neher 1998; Sherman et al. 1990).

Another known regulator of L-type Ca^{2+} channels is protein kinase A (PKA). Phosphorylation of L-type channels by PKA enhances the whole cell current amplitude by increasing the open time of the channels (Bean et al. 1984; Yue et al. 1990b). The level of PKA activity can be regulated by cell surface receptors linked to G proteins that either stimulate (G_s) or inhibit (G_i) adenylyl cyclase (AC). There are nine membrane bound isoforms of AC, all of which can be stimulated by activated G_s (for review, see Willoughby and Cooper 2007). Alternatively, AC1 and AC8 can be directly activated by the Ca^{2+} /CaM complex with AC1 being about five times more sensitive to Ca^{2+} /CaM (K_d s ~ 100 nM, AC1; ~ 500 nM, AC8) (Fagan et al. 1996; Wu et al. 1993). We have previously reported that metabotropic glutamate receptor 5- and phospholipase C-dependent activation of PKA enhances the amplitude of L-type Ca^{2+} currents in retinal amacrine cells, possibly via an AC1-dependent mechanism (Sosa and Gleason 2004).

If these two Ca^{2+} -dependent Ca^{2+} channel regulators (CDI and AC1/8) coexist in amacrine cells, then we would predict that mechanisms regulating cytosolic Ca^{2+} will influence the outcome of L-type Ca^{2+} channel regulation. It has been previously shown that synaptic transmission between retinal amacrine cells is affected by mitochondrial Ca^{2+} uptake (MCU) (Medler and Gleason 2002). This work led us to hypothesize that at least part of the impact of MCU on synaptic transmis-

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sion was in maintaining the L-type Ca^{2+} channels in a relatively noninactivated state. Here we test this hypothesis by examining the effects of disrupting MCU on L-type Ca^{2+} channel function.

Entry of Ca^{2+} through L-type channels is known to elicit Ca^{2+} -induced Ca^{2+} release (CICR) in amacrine cells (Mitra and Slaughter 2002; Warrier et al. 2005). Because this amplification of the Ca^{2+} signal has the potential to affect other Ca^{2+} -dependent processes, we also test the role of ryanodine receptor (RyR) activity in the Ca^{2+} -dependent regulation of these channels.

Using a primary cell culture system consisting of identified GABAergic amacrine cells (Gleason et al. 1993), we have begun to clarify the physiological relationships between L-type Ca^{2+} channel inactivation, MCU, PKA activity, and CICR. Although the molecular details of CDI and PKA-dependent current enhancement have been worked out, most of this work has been done in expression systems or in cardiac myocytes. It remains to be determined how these factors interact in the native environment of retinal amacrine cells; an interneuron critical to shaping the output of the retina. Given the dependence of synaptic transmission on L-type Ca^{2+} channel function in these cells (Gleason et al. 1994), our aim is to investigate the balance of Ca^{2+} -dependent mechanisms regulating L-type Ca^{2+} channel functions in retinal amacrine cells.

METHODS

Cell culture

Primary cell cultures of chick retinal amacrine cells were used in our experiments. The chicken embryos (*Gallus gallus*, Animal Science Department, Louisiana State University, Baton Rouge, LA) were dissected on embryonic day 8, and retinal cells were dissociated and cultured as previously described (Hoffpauir and Gleason 2002). Cell cultures were maintained at 37°C under 5% CO_2 atmosphere until they were ready for experiments, 8–14 days after plating. For electrophysiology experiments, amacrine cells were identified based on their morphology. Cells with large somas (10–15 μm) with two to five primary processes have been previously identified as amacrine cells based on immunocytochemical and physiological criteria (Gleason et al. 1993; Huba and Hofmann 1990, 1991; Huba et al. 1992).

Solutions

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). External solutions consist of the following (in mM): 116.7 NaCl, 20.0 TEACl, 3.0 CaCl_2 , 0.4 MgCl_2 , 5.6 glucose, and 10.0 HEPES. Voltage clamp experiments performed in the perforated-patch configuration employed the following internal solution (in mM): 135.00 CsAc, 10.0 CsCl, 1.0 NaCl, 2.0 MgCl_2 , 0.1 CaCl_2 , 1.1 EGTA, 10.0 HEPES, and 200 $\mu\text{g/ml}$ amphotericin B. Voltage clamp experiments performed in the ruptured-patch configuration used the following internal solution (in mM): 100.00 CsAc, 10.0 CsCl, 2.0 MgCl_2 , 0.1 CaCl_2 , 10.0 HEPES, 3.0 ATP (dipotassium), 1.0 ATP (disodium), 20.0 phosphocreatine, 2.0 GTP, and 50 U/ml creatine phosphokinase. Solutions were adjusted to pH 7.4 with NaOH for external solutions and with CsOH for internal solutions. Two different Ca^{2+} buffers were also included in internal solutions in ruptured-patch recordings: Ethylene glycol-bis (2-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid (EGTA, 1.1 or 14 mM) and 1,2-bis-(*o*-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA, 10 mM, Enzo Life Sciences, Plymouth Meeting, PA).

A pressurized gravity flow perfusion system (1.5–2 ml/min) was used to deliver the external solutions (AutoMate Scientific, Berkeley,

CA). Unless otherwise indicated, the following reagents were purchased from Enzo Life Sciences. Reagents added via the bath included the protonophore carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP, 1 μM), a PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide $\cdot$ 2HCl (H89, 1 μM), an adenylate cyclase (AC) inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine, (SQ 22,536, 200 μM) a general phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 μM), a calcineurin inhibitor cyclosporine A (CsA, 1 μM), and a selective inhibitor of Ca^{2+} /CaM-dependent phosphodiesterase (PDE 1) 8-methoxymethyl-1-methyl-3-(2-methylpropyl) xanthine (8-M-IBMX, 100 μM), inhibitors of the RyR, ryanodine (14 μM) and dantrolene (20 μM , Sigma). CaM inhibitor calmidazolium chloride (CMZ, 10 μM) was added to the pipette solution. In all electrophysiology experiments, (–)-bicuculline methobromide (10 μM , Tocris Bioscience, Ellisville, MO) was included in external solutions to block GABA_A receptor-mediated autaptic currents (Gleason et al. 1993). Tetrodotoxin (TTX, 300 nM, Alomone Labs, Jerusalem, Israel) was included in external solutions to block voltage gated Na^+ currents.

Electrophysiology

Cell culture dishes were mounted on the stage of an Olympus IX70 inverted microscope. A reference Ag/AgCl pellet served to ground the bath. Patch electrodes were pulled from thick-walled borosilicate glass with a filament (1.5 mm OD, 0.86 mm ID; Sutter Instrument, Novato, CA) using a Flaming-brown Micropipette Puller (Sutter Instruments). For electrophysiology experiments, either ruptured- or perforated-patch whole cell recording was performed. For perforated-patch recordings, only cells with stable resistances (changes of <5 M Ω) were used in the experiments. Recordings were made using Axopatch 1D-patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Data were recorded using Clampfit 9.2 and 10.0 software (Molecular Devices). Electrode resistance values were monitored and ranged from 3 to 8 M Ω . Junction potential corrections (–8 mV) were made for the data in Fig. 2, D and E.

Because we were concerned that some experimental manipulations might produce long term changes in the cells, experimental and control recordings were often done in separate groups of cells. We also used separate groups of cells for experiments with different internal solutions. When this sort of protocol was used, the different treatments (or internal solutions) were alternated from cell to cell. As such, comparisons were made between cells from the same culture and often from the same culture dish.

Western blots

Chicken and mouse brains were homogenized in nondenaturing lysis buffer containing a cocktail of protease inhibitors [PMSF (1 mM), leupeptin, (5 $\mu\text{g/ml}$) aprotinin (2.5 $\mu\text{g/ml}$), 1,10 ortho-phenantroline (0.2 $\mu\text{g/ml}$), and pepstatin (0.7 $\mu\text{g/ml}$)]. Samples were spun at 4,000 rpm for 20 min at 4°C. Protein content was determined using the BCA protein assay kit from Pierce (Rockford, IL). Proteins (300 μg) were separated on a 7.5% SDS gel along with 10 μl PAGERuler molecular weight markers (Fermata, Glenburnie, MD). Proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 4% milk, 0.1% Tween 20 in Tris buffered saline. The polyclonal antibody raised against human AC1 (Abcam, Cambridge, MA) was diluted 1:500 in PBS with 1% BSA. Goat anti-rabbit secondary antibody conjugated on horseradish peroxidase (Pierce) was diluted to 1:1,000 in PBS with 1% milk. Membranes were incubated in primary and secondary antibodies for 1.5 h each, at room temperature. Proteins were visualized using the Supersignal Western Pico Reagent (Pierce).

Immunocytochemistry

Adult White Leghorn chickens were killed by intraperitoneal injection of sodium pentobarbital (500 mg/kg, Sigma-Adrich) followed by decapitation. These methods were approved by the Institutional Animal Care and Use Committee, Louisiana State University. The eyes were enucleated and hemisected. After removing the vitreous, 4% paraformaldehyde was added to the eye cups and kept at 4°C for 1 h. Following fixation, eye cups were washed in PBS + 1% glycine. Retinae were then dissected from eye cups and incubated in 15% sucrose for 30 min, 20% sucrose for 1 h, and 30% sucrose solution overnight at 4°C. Retinae were embedded in OCT compound (Sakura Finetek, Torrance, CA) by freezing in dry ice and isopentane. Sections (12–16 μm) were cut on a Leica CM1850 cryostat (Wetzlar, Germany) and mounted on presubbed slides (Southern Biotech, Birmingham, AL).

Cells grown on glass coverslips were fixed in 2% paraformaldehyde for 30 min at 4°C after 8 days in culture. Fixed cells and retinal sections were preincubated for 1 h at room temperature in a blocking solution consisting of dilution solution (see following text) with 10% normal goat serum. Primary polyclonal antibodies raised against human AC1 were diluted at 1:100 in dilution solution (PBS, 1% bovine serum albumin, 0.5% saponin) and applied to either retinal sections or cells for 1 h at room temperature then washed in PBS. Secondary goat-anti-rabbit antibodies conjugated to Cy3 were obtained from Millipore (Temecula, CA) and were diluted 1:1,000 in dilution solution. Cells were incubated in secondary antibodies for 1 h at room temperature. After washing, coverslips and slides were mounted in a medium containing 70% glycerol, 28% PBS and 2% n-propyl gallate. Cells and retinal sections were viewed on an Olympus 1X70 microscope equipped with epifluorescence and images were captured using Slidebook software and hardware (Intelligent Imaging Innovations, Denver, CO).

Data analysis

The Origin 7.5 and 8.0 (OriginLab, Northampton, MA) software package was used to analyze and plot the data. Images in Fig. 8 were adjusted for brightness and contrast in Adobe Photoshop (San Jose, CA). Equivalent adjustments were made for experimental and control images. Figures were assembled in Adobe Illustrator. Statistical analyses were done using the *t*-test and data are presented as mean $\pm$ SE. Maximum *P* value for significance was 0.05.

RESULTS

Ca^{2+} current amplitude increases with repeated depolarizations

Whole cell recordings were made from single isolated amacrine cells in the perforated-patch configuration. It has been previously established that these cells express L-type but not N or P-type Ca^{2+} channels (Gleason et al. 1993). To examine the normal variability in current amplitude over time, Ca^{2+} currents were elicited by depolarizing amacrine cells from -70 to 0 mV either for 1,000 ms, every 60 s, or for 100 ms every 30 s (Fig. 1, A and C, respectively). It is evident, especially for the longer voltage steps (Fig. 1A), that inactivation of the current occurs over the duration of the step. It was also observed that the time course and degree of inactivation could vary from cell to cell. The physiological basis for these differences are not known but could feasibly be due to different expression levels of calmodulin or other effectors (see DISCUSSION). The tail currents after the voltage step (observable in Fig. 1C as well as in subsequent figures) are primarily due to the activity of the plasma membrane Na/Ca exchanger transporting Ca^{2+} back out of the cell. Our ability to identify and to measure electro-

genic Na/Ca exchange activity has been firmly established for these cells (Gleason et al. 1994, 1995; Hurtado et al. 2002; Medler and Gleason 2002).

Although inactivation occurred during the voltage step, the peak current amplitudes tended to increase over time, implying that multiple levels of regulation are occurring. Figure 1, B and D, shows the peak current amplitude for steps (1 s and 100 ms) delivered every 60 and 30 s (respectively) from separate populations of amacrine cells. A progressive increase in the current amplitude was typically observed with both protocols. This increase is not due to changes in series resistance because only cells with stable series resistances (see METHODS) were included in the analysis. The rate of the increase in current amplitude was inherently variable among cells as indicated by the substantial error bars that tended to increase over the duration of the recording. Because the only “treatment” that the cells received in these recordings was the history of voltage steps and the resulting Ca^{2+} influx, we predicted that the primary sources of enhancement were Ca^{2+} dependent. Perhaps then, variability in current amplitude enhancement was related to the Ca^{2+} current density in each amacrine cell. To determine whether the rate of increase in current amplitude was linked to Ca^{2+} current density, these quantities were plotted for a population of cells that had been recorded under the same conditions (step to 0 mV for 100 ms, every 30 s, Fig. 1E). Regression analysis revealed an R^2 value of 0.02 (Fig. 1E) and does not indicate a dependence on current density. This implied that the regulation under these stimulus protocols is not a simple transform based on the amount of Ca^{2+} influx. Instead it suggested that the regulation of these channels has multiple Ca^{2+} -dependent components that vary intrinsically among cells. This suggestion is borne out by much of the data presented in subsequent sections.

Disruption of MCU inhibits the Ca^{2+} current

To test the hypothesis that L-type Ca^{2+} channel inactivation in amacrine cells is regulated by MCU (Medler and Gleason 2002), we used the protonophore FCCP to temporarily collapse the proton gradient across the inner mitochondrial membrane and disrupt MCU via the Ca^{2+} uniporter (Herrington et al. 1996; Werth and Thayer 1994; White and Reynolds 1997). Whole cell current recordings were made in the perforated-patch configuration. Amacrine cells were depolarized from -70 to 0 mV for 1 s, every 60 s. Disrupting MCU had two effects: an increase in the inward current amplitude recorded at -70 mV (before and after the voltage step to 0 mV) and a decrease in the Ca^{2+} current amplitude recorded during the step to 0 mV. We have previously shown that the relatively time-invariant increase in inward current at -70 mV (Fig. 2A, arrow) is due to a persistent FCCP-dependent increase in cytosolic Ca^{2+} that activates the electrogenic plasma membrane Na/Ca exchanger (Medler and Gleason 2002). Furthermore, it has been established that the FCCP-dependent Ca^{2+} increase driving this exchanger activity is due to the RyR-dependent leakage of Ca^{2+} from stores that is normally sequestered by mitochondria via the uniporter (Sen et al. 2007). Importantly, we have also previously demonstrated that Na/Ca exchange activity is negligible at 0 mV and thus does not contribute significantly to the current recorded during the voltage step to 0 mV (Gleason et al. 1995). To simplify the appear-

FIG. 1. The Ca^{2+} current amplitude increases over time in control conditions. *A* and *C*: perforated-patch recordings are shown from 2 different amacrine cells. Cells were depolarized from -70 to 0 mV for 1 s, every 60 s (*A* and *B*), or for 100 ms, every 30 s (*C* and *D*). Current traces are shown from the time points indicated in *B* and *D*. Pairs of traces were selected to show that currents recorded a minute apart are only slightly different in amplitude (*A* and *C*, left). These traces were also selected for comparison because traces from similar time points are depicted in subsequent figures. Traces collected further apart in time show that the differences in amplitude are augmented over a longer time frame (*A* and *C*, right). *B* and *D*: each data point is the mean normalized peak current amplitude elicited by the 2 protocols and plotted over time (30 s $n = 6$; 60 s $n = 4$). *E*: current density (pA/pF) for the current elicited by the 1st voltage step is plotted against its rate of current amplitude increase for each cell. The rate was estimated by drawing a line (fit by eye) through the data at the 1st 3 time points for each cell, then calculating the slopes of those lines. The measurements from all cells depolarized from -70 to 0 mV for 100 ms, every 30 s were included in this analysis. Regression analysis does not reveal a correlation between these 2 quantities ($R^2 = 0.02$).

ance of the data, the FCCP-dependent Na/Ca exchange current at -70 mV has been subtracted from subsequent data (as in Fig. 2*B*). However, we show this current in insets (Figs. 5 and 6) to confirm that this FCCP-dependent Ca^{2+} elevation and exchanger activity persists under some key experimental conditions.

More directly relating to our hypothesis, disrupting MCU also caused a reversible decrease in the Ca^{2+} current amplitude (Fig. 2, *A* and *B*, gray trace). Under these conditions, disrupting MCU significantly reduced the Ca^{2+} current amplitude by $35 \pm 7\%$ ($n = 7$, $P = 0.03$, Fig. 2*C*). A series of control experiments previously established that the effects of FCCP on

depolarization-induced Ca^{2+} elevations in these cells are not due to ATP depletion (Medler and Gleason 2002) (also see DISCUSSION) or changes in pH (Sen et al. 2007). Another possibility was that the decrease in current amplitude was due to an FCCP-dependent shift in the activation range of the channels. To address this, currents were elicited by steps over a range of voltages from -80 to $+10$ mV in 5 mV increments to reveal the voltage of activation. These experiments were done in ruptured-patch because the voltage of activation can be better resolved with the relatively low series resistance recordings achieved in this configuration. These recordings were

FIG. 2. Inhibition of mitochondrial Ca^{2+} uptake (MCU) reduces the Ca^{2+} current amplitude without altering the voltage of activation. *A* and *B*: perforated-patch recordings from an amacrine cell in the absence (black trace) and presence of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP, gray trace). Amacrine cells were depolarized from -70 to 0 mV for 1 s every 60 s. Representative current traces are from the 3rd and 4th voltage steps. The sustained FCCP-dependent inward current at -70 mV (*A*, arrow) is due to Na/Ca exchanger activity and has been subtracted from subsequent data. This current is negligible at 0 mV so no subtraction was done for the data collected during the voltage step (*B*). *C*: normalized mean peak current amplitude is plotted over time ($n = 7$). *D* and *E*: activation range of the channels was revealed by eliciting currents by steps over a range of voltages (-80 to $+10$ mV, in 5 mV increments). Two different internal Ca^{2+} buffering conditions were used (1.1 mM EGTA $n = 5$, or 10 mM BAPTA $n = 4$) in ruptured-patch configuration. No FCCP-dependent shift in activation range was observed under either condition in any of the cells tested.

made with two different internal Ca^{2+} buffering conditions (1.1 mM EGTA, 10 mM BAPTA). Although we observed a difference in the effects of FCCP on Ca^{2+} current amplitude in 10 mM BAPTA (see following text), no FCCP-dependent shift in activation range was observed under either Ca^{2+} buffering condition (Fig. 2, *D* and *E*, respectively). These data are consistent with a role for MCU in limiting the degree of Ca^{2+} /CaM-dependent inactivation for these channels.

Effects of disrupting MCU are dependent on the duration and frequency of the voltage step

To examine whether increases in Ca^{2+} influx enhanced the effects of disrupting MCU, we first altered the duration of the voltage steps. Ca^{2+} currents were recorded in response to voltage steps lasting either 50 ms or 3 s (Fig. 3, *A* and *B*, respectively). Currents from 50 ms steps were reduced by $35 \pm 4\%$ ($n = 7$), whereas currents from 3 s steps were reduced by $60 \pm 5\%$ in the presence of FCCP ($n = 6$, *C*). The significantly ($P = 0.002$) larger effect of FCCP for longer voltage steps supports the possibility that Ca^{2+} -dependent inactivation was being altered by disrupting MCU.

If the level of FCCP-dependent inhibition of L-type channel current increased with prolonged voltage steps, and this was due to the greater Ca^{2+} influx during longer steps, we would also predict that increasing the frequency of depolarization would intensify the effects of inhibiting MCU. To test this, single amacrine cells were depolarized from -70 to 0 mV, for 100 ms, and voltage steps were delivered either every 60 s (Fig. 3*D*) or every 5 s (*E*). Disrupting MCU caused a reduction in the Ca^{2+} current amplitude under both recording condition; however, the FCCP-dependent reduction in current amplitude was significantly larger for higher frequency depolarizations [$61 \pm 4\%$ reduction for every 5 s ($n = 6$), and $35 \pm 4\%$ reduction for every 60 s ($n = 7$), $P = 0.001$, Fig. 3*F*]. These results further support the possibility that inhibition of the Ca^{2+} current amplitude is dependent on cytosolic Ca^{2+} levels.

Effects of MCU are not dependent on calcineurin

The established role of PKA-dependent channel phosphorylation in L-type current enhancement raises the possibility that the effect of inhibiting MCU is to reduce channel phosphorylation. The most likely candidate for such an activity

FIG. 3. The effect of inhibiting MCU is dependent on the duration and frequency of depolarization. *A* and *B*: perforated-patch recordings from representative amacrine cells depolarized from -70 to 0 mV for either 50 ms (*A*) or 3 s (*B*). Steps were delivered every 60 s. Recordings were made in the absence (black traces) and presence (gray traces) of FCCP. *C*: mean normalized peak current amplitudes are plotted against step number for both step durations (50 ms, $n = 7$; 3 s, $n = 6$). *D* and *E*: perforated-patch recordings from individual amacrine cells stepped from -70 to 0 mV for 100 ms. Voltage steps were delivered either every 60 s (*D*) or 5 s (*E*). Currents were recorded in the absence (black traces) and presence (gray traces) of FCCP. *F*: normalized mean peak current amplitude plotted over time for each depolarization frequency (5 s, $n = 6$; 60 s, $n = 7$).

under these conditions would be the Ca^{2+} /CaM-dependent phosphatase, calcineurin. To test for the involvement of calcineurin, we asked whether the potent ($\text{IC}_{50} = 7$ nM) (Fruman et al. 1992) calcineurin inhibitor CsA (for review, see Kunz and Hall 1993) would block the effects of FCCP. Recordings were made using 100 ms steps from -70 to 0 mV, every 30 s. Cells were preincubated for 10–20 min in CsA ($1 \mu\text{M}$) before FCCP was applied. The inhibitory effect of FCCP persisted in the presence of CsA (Fig. 4, *A* and *B*), and CsA alone had no consistent effect on the current (*C*). Negative results require cautious interpretation but it is important to note that with similar exposure times (10–20 min) and concentrations (0.1 – $1 \mu\text{M}$), CsA has been demonstrated to be effective in inhibiting calcineurin in intact neurons (Xu and Krukoff 2007) and smooth muscle cells (Schuhmann et al. 1997). These data therefore suggest that calcineurin activity is not mediating the FCCP-dependent inhibition of the current and may not be a major regulator of L-type Ca^{2+} channel activity in these amacrine cells.

Effects of MCU are Ca^{2+} influx-dependent

Were the effects of disrupting MCU on the current amplitude due to an excess of Ca^{2+} originating from channel entry

or were they due to the FCCP-dependent elevation in cytosolic Ca^{2+} as revealed by the increase in the activity of the Na/Ca exchanger (Fig. 2*A*)? To distinguish between these two possibilities, we replaced external Ca^{2+} with Ba^{2+} . Ba^{2+} is known to carry the current through L-type channels but is a poor substitute for Ca^{2+} with respect to inactivation (Brehm and Eckert 1978; Chad and Eckert 1986; Tillotson 1979; Yue et al. 1990a; Zuhlke et al. 1999). Perforated-patch recordings were made in amacrine cells bathed with either normal external ($3 \text{ mM } \text{Ca}^{2+}$, Fig. 5*A*) or external solution with equimolar Ba^{2+} replacing Ca^{2+} (*B*). Cells were stepped from -70 to 0 mV for 1 s. The currents recorded in Ba^{2+} inactivated relatively little over the course of the 1 s depolarization consistent with minimal Ca^{2+} -dependent inactivation. With Ba^{2+} as the charge carrier, the effect of disrupting MCU was significantly reduced ($18 \pm 3\%$ reduction with Ba^{2+} , $n = 7$; $44 \pm 7\%$ reduction with Ca^{2+} , $n = 7$; $P = 0.007$) indicating that the primary source of the inhibition was from Ca^{2+} crossing the plasma membrane (Fig. 5*C*). The relatively small inhibitory effect of FCCP on Ba^{2+} current amplitude that was observed may be due to either the low level of CaM activation known to occur with Ba^{2+} (Dick et al. 2008) and/or the FCCP-dependent Ca^{2+} elevation (Me-

FIG. 4. Calcineurin is not a major regulator of the Ca^{2+} current. *A*: a representative recording from an amacrine cell showing the Ca^{2+} current recorded in cyclosporin A (CsA, $1 \mu\text{M}$) just prior to the addition of FCCP (black trace) and after 1 min of FCCP exposure (gray trace). Cells were depolarized from -70 to 0 mV for 100 ms once every 30 s. *B*: the time course of normalized Ca^{2+} current amplitude is plotted for 6 cells treated with CsA. *C*: under the same recording conditions as in *A* and *B*, CsA alone had no consistent effect on the Ca^{2+} current ($n = 5$).

dler and Gleason 2002; Set et al. 2007). The persistence of the FCCP-dependent Ca^{2+} elevation in external Ba^{2+} is demonstrated by the presence of the FCCP-dependent Na/Ca exchange current shown in Fig. 5*B*, *inset*. The relatively small effect of inhibiting MCU on Ba^{2+} current amplitude is consistent with the hypothesis that MCU normally sequesters Ca^{2+} entering through L-type Ca^{2+} channels.

Increasing cytosolic Ca^{2+} buffering alters the effects of inhibiting MCU

Different levels of internal Ca^{2+} buffering are known to alter CDI (Brehm and Eckert 1978; Dick et al. 2008; Kalman et al.

1988; Kohr and Mody 1991; Tadross et al. 2008). To further explore the role of CDI in the effects of blocking MCU, experiments were repeated under different Ca^{2+} buffering conditions. Either EGTA (1.1 or 14 mM) or the faster buffer BAPTA (10 mM) (Adler et al. 1991) was included in recording pipette. For these experiments, Ca^{2+} currents were recorded in the ruptured-patch configuration. Currents were recorded before (Figs. 6, *A–C*, black traces) and during application of FCCP (gray traces). First, it should be noted that the different buffering environments differentially affect the two components of the Na/Ca exchange current. The FCCP-dependent component of the exchange current occur (~ 35 pA in each

FIG. 5. The effects of inhibiting MCU are dependent on Ca^{2+} influx. *A* and *B*: voltage steps were delivered from -70 to 0 mV for 1 s, every 60 s in perforated-patch recordings. Recordings were made in external solutions containing either 3 mM Ca^{2+} or 3 mM Ba^{2+} . Currents are shown from the 3rd (black traces) and 4th voltage steps (in FCCP, gray traces). *B*, *inset*: unsubtracted current at -70 mV just prior to the voltage step to 0 mV reveals that the FCCP-dependent Na/Ca exchange current (and thus the FCCP-dependent Ca^{2+} elevation) persists in external Ba^{2+} . *Inset*: scale bar is 20 pA. *C*: mean normalized peak current amplitudes are plotted over time for data collected in both Ca^{2+} and Ba^{2+} ($n = 7$ for each).

FIG. 6. Increasing cytosolic Ca^{2+} buffering alters the effects of inhibiting MCU. A–C: representative Ca^{2+} currents recorded in the ruptured-patch configuration with internal solution containing 1.1 mM EGTA (A), 14 mM EGTA (B), or 10 mM bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA, C). Dashed lines (A–C) define the 0 current level and the end of the voltage step so that tail currents can be compared under the 3 buffering conditions. Currents were elicited by 100 ms voltage steps from -70 to 0 mV delivered every 30 s. Currents shown are from the 2nd voltage step (black traces) or the 4th voltage step (in FCCP, gray traces). A–C, insets: the unsubtracted current recorded at -70 mV (Na/Ca exchange current) demonstrating the presence of the FCCP-dependent Ca^{2+} elevation under all 3 Ca^{2+} buffering conditions. Inset scale bar is 20 pA. D: mean normalized peak current amplitude is plotted over time for the 3 buffering conditions. E: FCCP-dependent changes in current amplitude (comparing data points 2 and 4) are plotted for each buffering condition. Single asterisk, $P < 0.05$; triple asterisk, $P < 0.001$.

cell) under all three Ca^{2+} buffering conditions (Fig. 6, A–C, insets), indicating that Ca^{2+} elevations can persist under these buffering conditions. The Ca^{2+} current-dependent component of the Na/Ca exchange current (visible as tail currents after the voltage step) is more sensitive to Ca^{2+} buffering conditions with the current nearly eliminated in 10 mM BAPTA. With 1.1 mM EGTA, the effect of FCCP was not significantly different from perforated-patch recordings (perforated $45 \pm 4\%$ reduction, $n = 6$; 1.1 mM EGTA $36 \pm 6\%$ reduction, $n = 6$, $P = 0.23$, Fig. 6A, D, and E), suggesting that this level of artificial Ca^{2+} buffering approximates that found in intact amacrine cells. However, with 14 mM EGTA, the effects of disrupting MCU were significantly suppressed [$13 \pm 6\%$ reduction (vs. $36 \pm 6\%$), $n = 6$, $P = 0.02$] and delayed (no Ca^{2+} current reduction observed until a minute of FCCP exposure) when compared with 1.1 mM EGTA (Fig. 6, B, D, and E). Hence more EGTA minimized but did not eliminate the effect of MCU on the Ca^{2+} current amplitude, consistent with the idea that stronger buffering reduces CDI. Overall, blocking MCU in the presence of 10 mM BAPTA produced a $12 \pm 5\%$ ($n = 22$) enhancement in the Ca^{2+} current that was significantly different from the results in 1.1 mM EGTA ($P = 0.0002$, Fig. 6E). The sign of the effect varied from cell to cell with current

enhancement in 12/22 cells ($31 \pm 6\%$ enhancement) or small reductions in the current in 10/22 cells ($0.7 \pm 2\%$, Fig. 6, C, D, and E). These data indicated that strong Ca^{2+} buffering reduced the impact of MCU on channel inactivation. We hypothesize that in BAPTA, CDI is reduced. In some cells, this reveals another Ca^{2+} -dependent process that enhances current amplitude.

Inhibition of PKA and AC decreases the Ca^{2+} current amplitude

It is well known that PKA-dependent phosphorylation can enhance L-type Ca^{2+} currents by increasing the open time of the channels (Bean et al. 1984; Yue et al. 1990b). In amacrine cells, we have previously demonstrated that metabotropic glutamate receptor 5 activation leads to a PKA-dependent enhancement of the L-type current that does not result from changes in voltage sensitivity (Sosa and Gleason 2004). To test for the involvement of PKA in the FCCP-dependent enhancement of the Ca^{2+} current in BAPTA, we examined the effects of the PKA inhibitor, H89 (1 μM) with BAPTA (10 mM) internal. H89 has been shown to be specific for PKA when used at concentrations $< 10 \mu\text{M}$ (Chijiwa et al. 1990). For these experiments, different cells were used for the different treat-

ments to avoid complications due to previous drug exposures (see METHODS). On average, BAPTA-loaded cells tested with FCCP showed enhancement of the Ca^{2+} current ($12 \pm 5\%$, $n = 22$, Fig. 6E). Inhibition of PKA with H89 reduced the Ca^{2+} current amplitude in all cells tested indicating basal PKA activity ($41 \pm 5\%$, $n = 4$, Fig. 7, A and C). When used in combination (H89 + FCCP), the effect on the Ca^{2+} current was generally larger but the difference was not statistically significant ($58 \pm 8\%$ reduction, $n = 6$, $P = 0.17$, Fig. 7, B and C) than with either reagent alone indicating that with reduced PKA activity, inhibition of MCU still contributes to inactivation. Our interpretation of the target of H89 (PKA) is consistent with our previous observation that 8-bromo cAMP enhances the Ca^{2+} current in these cells (Sosa and Gleason 2004).

PKA is activated by cAMP which is generated by the enzymatic activity of AC. To confirm the involvement of this classical pathway, we asked whether inhibition of AC would have the same effects on the Ca^{2+} current as inhibition of PKA. The general AC inhibitor SQ 22,536 (200 μM) was used to inhibit AC (Fabbri et al. 1991). In all cells tested ($84 \pm 4\%$ reduction, $n = 7$), SQ 22,536 exposure reduced the amplitude of the Ca^{2+} current (Fig. 7, D and E), consistent with basal AC activity producing cAMP and driving basal PKA activity.

Expression of AC1

Because the PKA activity appeared to be independent of cell surface receptor activation in these experiments, we postulated that the enzyme is stimulated by cAMP that has been generated through the activity of the Ca^{2+} /CaM-dependent adenylate cyclase AC1. The K_d of AC1 for Ca^{2+} 100 nM (Fagan et al. 1996; Wu et al. 1993) is near resting cytosolic Ca^{2+} levels in these cells (50–100 nM, Hurtado et al. 2002) making this enzyme a good candidate for mediating both basal PKA activity as well as enhanced activity due to Ca^{2+} influx via L-type V-gated Ca^{2+} channels. We have previously demonstrated AC1-like immunoreactivity in cultured amacrine cells (Sosa and Gleason 2004). Here we further examine the expression of AC1 using a different, and more fully characterized, polyclonal antibody raised against human AC1. The specificity of this antibody was confirmed in Western blots using homogenates of both chicken and mouse brain (Fig. 8A). Single bands near the predicted molecular weight of AC1 (130 kDa) were detected for both chicken and mouse brain homogenate, indicating that the antibody recognizes the avian form of AC1. On sections of chicken retina, the anti-AC1 antibody labeled photoreceptors most strongly (Fig. 8C), but labeling was also strong in cell bodies in the ganglion cell layer. Cells at the inner border of the inner nuclear layer (most likely amacrine cells) were also labeled in a distinctly punctate pattern. Pro-

FIG. 7. Protein kinase A (PKA) and adenylate cyclase (AC) are both involved in regulating the Ca^{2+} current amplitude. A and B: representative Ca^{2+} currents recorded in the ruptured-patch configuration with internal solution containing 10 mM BAPTA. Current records are from 2 different cells. Currents were elicited by 100 ms voltage steps from -70 to 0 mV delivered every 30 s. Currents shown were elicited from the 2nd (black trace) and 4th (during the treatment, gray trace) voltage steps. Cells were exposed to either the PKA inhibitor *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide $\cdot$ 2HCl (H89, A) or H89 + FCCP (B) for 1 min. C: normalized peak current amplitude data are plotted over time. The vertical gray bar indicates the time frame of the 3 treatments. FCCP data from Fig. 6D are re-plotted here for comparison. D: a representative recording from an amacrine cell showing the inhibitory effect of 9-(tetrahydro-2-furanyl)-9H-purin-6-amine, (SQ 22,536) on the Ca^{2+} current amplitude. E: normalized peak current amplitude data are plotted over time.

FIG. 8. AC1 is expressed in the retina and in cultured retinal cells. *A*: image of a Western blot showing that the anti-AC1 antibody binds a band at the predicted molecular weight (130 kDa) in both chicken and mouse brain tissue. *B–D*: fluorescent images of frozen sections of chicken retina treated with Cy3-conjugated secondary antibodies only (*B*) and the anti-AC1 primary antibody plus the secondary antibody (*C* and *D*). The secondary-only controls show low nonspecific binding of the secondary antibody. In *C*, strong AC1 immunoreactivity is observed in the photoreceptor layer. Also labeled are cells at the inner border of the inner nuclear layer (INL) and in the ganglion cell layer. The labeling pattern is punctate and is most prominent in cell bodies. *D*: higher magnification view of 2 cells at the inner border of the INL shows that labeling is found on processes extending down into the inner plexiform layer (◄). *E–G*: fluorescent images of retinal neurons in culture. *E*: a field of cells labeled with the Cy3-conjugated secondary antibody only shows low nonspecific binding of the secondary antibody. *F*: a field of retinal neurons is shown, most of which are amacrine cells (◄, for example). A strongly labeled cone photoreceptor is also shown (◄). *G*: a higher magnification image of 3 amacrine cells. As in retinal sections, the labeling pattern of the anti-AC1 antibodies is punctate and appears in cell bodies and processes. All scale bars are 10 μm .

cesses could be observed extending from these cells down into the inner plexiform layer where amacrine cell synapses form (Fig. 8*D*). In culture, cone photoreceptors were usually the most strongly labeled cells. The intensity of anti-AC1 labeling was variable among amacrine cells but all amacrine cells showed some level of AC1 expression (Fig. 8*F*). Punctate AC1 expression was detected both in cell bodies and processes of amacrine cells in culture (Fig. 8*G*). It is important to note that the AC inhibitor SQ 22,536 (Fig. 7) has been demonstrated to be an effective inhibitor of AC1 in neurons at the concentration used in our experiments (Liauw et al. 2005). These results support the hypothesis that AC1 can be involved in L-type Ca^{2+} channel regulation in retinal amacrine cells.

Inhibition of CaM primarily affects inactivation

From the results presented thus far, a scenario emerges where at least two Ca^{2+} /CaM-dependent mechanisms might collaborate to regulate the function of L-type Ca^{2+} channels in retinal amacrine cells: first, the Ca^{2+} /CaM-dependent inactivation

that is sensitive to Ca^{2+} from 5 to 100 μM (Tadross et al. 2008) and second, the Ca^{2+} /CaM-dependent activity of AC1 that is more Ca^{2+} -sensitive ($K_d \sim 100$ nM). If these suggestions are valid, then we would predict that inhibition of CaM activity would be least effective in blocking the effects of AC1 activity. To test this, we looked at the effects of the calmodulin inhibitor calmidazolium (CMZ, 10 μM) (Weiss et al. 1982) on the FCCP-dependent alterations in current amplitude. Ruptured-patch recordings were made with either EGTA (1.1 mM) and CMZ in the pipette (Fig. 9*A*) or BAPTA (10 mM) and CMZ in the pipette (Fig. 9*B*) and then tested the effects of blocking MCU with FCCP. With inhibition of CaM, suppression of MCU produced an enhancement of the Ca^{2+} current under either buffering condition (EGTA + CMZ $36 \pm 12\%$ enhancement, $n = 6$, $P = 0.0003$; BAPTA + CMZ $19 \pm 9\%$ enhancement, $n = 6$, $P = 0.5$; Fig. 9, *C* and *D*). Recall that in the absence of CMZ, inhibition of MCU caused a decrease in current amplitude (presumably due to increased CDI) with 1.1 mM EGTA internally (EGTA $36 \pm 6\%$ reduction, $n = 6$, Fig. 6*A*). The switch in the sign of the response to FCCP when

FIG. 9. The effects of inhibition of calmodulin depend on the Ca^{2+} buffering environment. *A* and *B*: representative ruptured-patch recordings from amacrine cells loaded with calmidazolium chloride (CMZ) via the patch pipette. Recordings were made with either 1.1 mM EGTA+CMZ (*A*) or 10 mM BAPTA+CMZ (*B*) in the pipette. Current are shown from the 3rd voltage step (black trace) and the 5th voltage step (in FCCP, gray trace). *C*: mean normalized peak current amplitudes are plotted over time for both recording conditions. *D*: percent change in Ca^{2+} current amplitude was determined by comparing the current from the voltage step delivered just before FCCP to that elicited by the voltage step delivered 1 min later (in FCCP). CMZ-free data are re-plotted from Fig. 6*E* for comparison purposes. Triple asterisk, $P < 0.001$. Other pair-wise comparisons were not statistically different.

calmodulin is partially inhibited is consistent with a shift in the balance toward AC1 activation. The enhancement tended to be larger in EGTA than BAPTA however this difference was not statistically significant.

Phosphodiesterases play a role in regulating Ca^{2+} current amplitude

If the enhancement is due to the generation of cAMP via AC1 and subsequent activation of PKA, then phosphodiesterase (PDE) activity could affect the current amplitude. To test the involvement of PDEs, we employed the general PDE inhibitor IBMX (100 μM) (Beavo et al. 1970). Ca^{2+} current

recordings were made in the perforated-patch configuration. At the outset, we reasoned that if we boosted cAMP levels by inhibiting its degradative enzyme, then the current amplitude should be enhanced. Our results, however, did not conform to expectations in that the effect of IBMX on the current amplitude varied from cell to cell. Twenty six percent (Fig. 10, *A* and *B*) of amacrine cells tested ($n = 23$) responded with the expected increase in current amplitude ($75 \pm 29\%$ enhancement $P = 0.04$). However, IBMX produced a decrease in the current amplitude in 26% of cells (Fig. 10, *C* and *D*, $13 \pm 2\%$ reduction, $P = 0.00002$) and no change (less than $\pm 5\%$) in 48% of cells (*E* and *F*). Some of the variability in responses

FIG. 10. Phosphodiesterase activity can regulate the Ca^{2+} current amplitude. *A, C, E, and G*: representative perforated-patch recordings from 100 ms voltage steps from -70 to 0 mV delivered every 30 s. Both pairs of recordings in each panel (*A, C, E, and G*) were made from the same amacrine cell. Control traces on the left were obtained from the 1st (black) and 2nd voltage steps (gray). *Right*: the control current (black) was recorded just prior to the application of 3-isobutyl-1-methylxanthine (IBMX, 2nd voltage step, *A, C, and E*) or 8-methoxymethyl-1-methyl-3-(2-methylpropyl) xanthine (8-M-IBMX, *G*). IBMX or 8-M-IBMX traces (gray) were recorded after 30 s of exposure to the reagent (3rd voltage step). Cells were separated into 3 groups based on the effect of IBMX on the Ca^{2+} current amplitude: cells exhibiting current amplitude increases (*A and B, n = 6*), cells exhibiting current amplitude decreases (*C and D, n = 6*) and cells the Ca^{2+} current of which was unaffected ($\pm 5\%$ change) by IBMX (*E and F, n = 11*). *G*: representative recordings from a single amacrine cell using the same protocol as for *A, C, and E* but with the PDE1 selective inhibitor 8-M-IBMX. 8-M-IBMX produced a small inhibition in all cells examined ($n = 12$ *G and H*). *B, D, F, and H*: mean percent changes in Ca^{2+} current amplitude are plotted for each group of cells (comparing time points depicted in *A, C, E, and G*). Single asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$.

could be due to the diversity of PDEs that can be inhibited by IBMX. To address this possibility we used 8-M-IBMX (100 μM), an inhibitor that is specific for PDE1 a Ca^{2+} /CaM-dependent phosphodiesterase (Fig. 10G) (Wells and Miller 1988). All cells ($n = 12$) tested responded to 8-M-IBMX with small, consistent decrease in the current amplitude ($4 \pm 1\%$ reduction, $P = 0.004$, Fig. 10H). These results imply that relatively high levels of cAMP generated in an environment with reduced PDE activity can have an inhibitory effect on the L-type channels (Ishikawa et al. 1993).

Ca^{2+} -induced Ca^{2+} release functions to enhance the L-type calcium channel current

Thus far our data suggest that influx of Ca^{2+} can regulate L-type channels in at least two ways: by activating Ca^{2+} /CaM-

dependent inactivation and by activating AC1 and ultimately PKA. Ca^{2+} released from internal stores might also have regulatory effects on L-type channels. It is established that in amacrine cells, activation of L-type Ca^{2+} channels leads to CICR (Mitra and Slaughter 2002; Warrier et al. 2005). To examine the role of CICR, we looked at the effects of inhibiting the RyRs using a blocking concentration of ryanodine (14 μM) (Meissner 1986) and the RyR inhibitor dantrolene (20 μM) (Nelson et al. 1996). If CICR normally contributes to channel inactivation, then blocking CICR should enhance the current. If CICR normally contributes to activation of AC1 and ultimately stimulation of PKA, then inhibition of RyRs would cause a decrease in current amplitude. We found that inhibition of the RyRs with either ryanodine (Fig. 11A) or dantrolene (B) consistently produced suppression of the Ca^{2+} current (ryan-

FIG. 11. Ca^{2+} -induced Ca^{2+} release (CICR) normally contributes to Ca^{2+} current enhancement. A and B: the perforated-patch configuration was used to record Ca^{2+} current from individual amacrine cells with a similar voltage protocol as in Fig. 10. The three pairs of traces in each panel (A and B) are all from the same amacrine cell. Control traces were recorded 30 s apart (A and B, left). Either ryanodine (14 μM , A, middle) or dantrolene (20 μM , B, middle) was used to inhibit activation of the ryanodine receptor. Co-application ryanodine and 8-M-IBMX (A, right) and dantrolene and 8-M-IBMX (B, right) are also shown. C and D, mean percent change in current amplitude (comparing time points depicted in A and B) is plotted for both inhibitors (ryanodine $n = 3$, dantrolene $n = 5$). *, $P < 0.05$; ***, $P < 0.001$.

odine $25 \pm 4\%$, $n = 3$, $P = 0.002$, Fig. 11C; dantrolene $11 \pm 3\%$, $n = 7$, $P = 0.0002$, D). These results were consistent with the hypothesis that CICR normally functions to enhance the L-type Ca^{2+} current, possibly by increasing the activation of AC1. Interestingly, 8-M-IBMX tended to block the effects of these RYRs inhibitors although this effect was not statistically significant for dantrolene (ryanodine + 8-M-IBMX $P = 0.002$; dantrolene + 8-M-IBMX $P = 0.2$). This observation implied that in the absence of PDE activity, increased cAMP levels can compensate for the lower level of AC1 activation during CICR suppression.

DISCUSSION

We find that in retinal amacrine cells, L-type Ca^{2+} channels are regulated by multiple Ca^{2+} -dependent processes (Fig. 12). Under control conditions, Ca^{2+} currents tend to increase in current amplitude over time under our recording conditions. This increase in amplitude may represent the balance of PKA-dependent enhancement via Ca^{2+} /CaM-dependent AC1 activity and CDI, which is largely mediated by Ca^{2+} entering through the Ca^{2+} channels. Interestingly, we observe an enhanced rate of Ba^{2+} current amplitude increase before FCCP application for cells recorded in external Ba^{2+} (Fig. 5C, 1st 3 data points). This enhanced rate of increase might represent the smaller contribution from CDI relative to basal AC1 activity. Disruption of MCU reduces the Ca^{2+} current amplitude in a Ca^{2+} -dependent manner, suggesting that under normal conditions, mitochondria function to limit CDI and thus maximize the availability of L-type Ca^{2+} channels for signaling. We provide evidence that the reduction of the inhibitory effects of blocking MCU in BAPTA reveals the enhancing effect of PKA activity that is most likely due to the Ca^{2+} /CaM-dependent activation of AC1. Inhibition of RyRs reduces the Ca^{2+} current amplitude suggesting that internal Ca^{2+} stores normally con-

tribute to Ca^{2+} current enhancement. We propose that the link between the PKA-dependent enhancement of the Ca^{2+} current and the enhancing effects of CICR might also be the activity of AC1. Together, these studies indicate that L-type Ca^{2+} channels in amacrine cells are regulated by Ca^{2+} via complex and interacting mechanisms.

Variability in cultured GABAergic amacrine cells

Throughout this work there is variability observable in our data. One example appears in Fig. 1, B and D. Under control conditions the current amplitude increases over time, but as indicated by the large error bars, different levels of this effect in different cells becomes apparent over time. The origin of this variability is not known, but if the enhancement is due to AC1 activity (as we have suggested), it is perhaps relevant that our AC1 antibody labeling intensity varies among amacrine cells in culture implying differing AC1 expression levels among these cells (Fig. 8F). Another example of variability would be in the time course of inactivation during the voltage steps. We have not yet investigated the source of this variability except to confirm that cells that are different in this regard to not have distinctive response properties in our experiments. These examples of variability (and others) raise the question of whether some of the variability is the representation of the multiple amacrine cell types known to exist in the vertebrate retina. It is important to consider, however, that our culture conditions have apparently narrowed the range of possible amacrine cell fates. In the retina, different classes of amacrine cells can release GABA, glycine, acetylcholine, or dopamine at their synapses. In our cultures, however, we have only observed GABAergic synaptic transmission from these cells. We have viewed this as a benefit of the system that allows us to study a specific subset of amacrine cells that represent a substantial fraction of amacrine cells in the retina. Amacrine cells in the vertebrate retina can be further categorized by their morphology including their lamination pattern in the inner plexiform layer (MacNeil and Masland 1998). In culture, however, this information is lost. There appear to be a few morphological groups of amacrine cells identifiable in the two dimensions of the culture dish, but we have not made a systematic attempt to relate these morphological groups to those found in the intact retina. We considered the possibility that different levels of AC1 expression might correspond to different morphological types of amacrine cell in culture. On examination, this seemed unlikely because it was clear that cells with similar morphologies had different levels of AC1 antibody labeling.

L-type Ca^{2+} channels expressed by amacrine cells

The molecular identity of the L-type Ca^{2+} channels expressed by amacrine cells is not fully determined. However, it has been established that $\text{Ca}_v1.3$ mRNA is expressed by AII amacrine cells in the mouse retina (Habermann et al. 2003). In the chicken retina, an immunohistochemistry study demonstrated amacrine cell expression of $\text{Ca}_v1.3$ and possibly $\text{Ca}_v1.4$ but not $\text{Ca}_v1.2$ (Firth et al. 2001). In cultures of chick retinal neurons enriched for photoreceptors, other neuronal cells (presumably including amacrine cells) are reported to also express $\text{Ca}_v1.3$ (Ko et al. 2007). Interestingly the characteris-

FIG. 12. Working model of the Ca^{2+} -dependent regulation of L-type Ca^{2+} channels in amacrine cells. A: when the cell is depolarized, L-type voltage-gated Ca^{2+} channels are opened and Ca^{2+} influx occurs. B: some fraction of entering Ca^{2+} is rapidly moved into nearby mitochondria via the uniporter. C: another fraction of the entering Ca^{2+} binds the channel-associated calmodulin and Ca^{2+} /calmodulin (CaM)-dependent channel inactivation (CDI) is initiated. D: Ca^{2+} may also bind nonchannel-associated calmodulin that can activate AC1 leading to PKA activity and current amplitude enhancement. E: another target of Ca^{2+} is the ryanodine receptor, which can lead to CICR and possibly contribute to the Ca^{2+} /CaM-dependent activation of AC1.

tics of CDI differ between $\text{Ca}_v1.3$ and $\text{Ca}_v1.4$ in that $\text{Ca}_v1.3$ contains sequence bestowing local as well as global Ca^{2+} sensing, whereas $\text{Ca}_v1.4$ should only sense global Ca^{2+} levels (Dick et al. 2008). This combination would be consistent with our data showing that 10 mM BAPTA removes a variable fraction of CDI but not all of it.

Effects of FCCP in amacrine cells

Our interpretation of the results in FCCP are based on the assumption that the primary effect of FCCP in amacrine cells is to reduce the proton gradient across the inner mitochondrial membrane and thus inhibit Ca^{2+} uptake via the uniporter. Several pieces of evidence from our previous work indicate that this is a fair assumption. It is well known that in the absence of a proton gradient, the ATP synthase can consume ATP. We have previously established, however, that at the same FCCP concentration (1 μM) and time frame of exposure used here (1 min), the interruption of ATP synthesis does not alter the amplitude or time course of depolarization-dependent Ca^{2+} elevations (Medler and Gleason 2002). This is consistent with reports from other neuronal cell types indicating that the glycolytic pathway can maintain ATP levels for ten's of minutes (Kauppinen and Nicholls 1986; Peng 1998; Werth and Thayer 1994; White and Reynolds 1995). Another potential complication would be if FCCP (as a protonophore) altered cytosolic pH. However, using SNARF-1 pH imaging we have established that under similar conditions, cytosolic pH is unperturbed by FCCP in amacrine cells (Sen et al. 2007). Furthermore, we have demonstrated that the effects of FCCP on cytosolic Ca^{2+} in amacrine cells are localized to within $\sim 10 \mu\text{m}$ of a mitochondrion indicating that the effects of FCCP are mitochondria-associated (Sen et al. 2007).

Mitochondrial Ca^{2+} uptake regulates Ca^{2+} -dependent inactivation

We know that the inhibition of uniporter activity causes an increase in basal (un-stimulated) cytosolic Ca^{2+} levels. This Ca^{2+} elevation is independent of extracellular Ca^{2+} (Medler and Gleason 2002) but is dependent on internal Ca^{2+} stores (Sen et al. 2007). As such, we cannot rule out the possibility that some fraction of the effects we see in FCCP are due to the increase in basal Ca^{2+} levels. Nonetheless we propose that when the uniporter is inhibited, the primary effect is that normally sequestered Ca^{2+} now initiates additional CDI. What is our evidence that CDI is being enhanced when the uniporter is inhibited? The activity dependence of the effect of FCCP is consistent with a Ca^{2+} -dependent inhibition of the current such as CDI in that both increasing the duration of the voltage steps and the frequency of the voltage steps intensified the effects of FCCP. Furthermore, replacement of extracellular Ca^{2+} with Ba^{2+} reduces the effects of FCCP and Ba^{2+} is known to substitute poorly for Ca^{2+} in calmodulin binding and activation (Dick et al. 2008).

Interestingly, the sign of the effect of FCCP on the Ca^{2+} current amplitude is often inverted when BAPTA is present internally. Because BAPTA is estimated to bind Ca^{2+} ~ 400 times faster than EGTA (Adler et al. 1991), the differential effects of the two Ca^{2+} buffers can provide information about the spatial relationships between sources of Ca^{2+} and Ca^{2+}

targets. The greater effectiveness of BAPTA over EGTA in reducing MCU-sensitive CDI suggests that mitochondria are in close proximity to the L-type Ca^{2+} channels, on the order of tens of nanometers (Burrone et al. 2002).

L-type Ca^{2+} channel CDI has also been shown to be regulated by MCU in chromaffin cells (Hernandez-Guido et al. 2001). In these cells, however, 14 mM EGTA internally eliminated the inhibitory effect of disrupting MCU on CDI. In amacrine cells, FCCP-dependent effects on CDI were clearly observed in 14 mM EGTA suggesting a more intimate association between mitochondria and L-type Ca^{2+} channels in amacrine cells than in chromaffin cells. Ca^{2+} -dependent inactivation has also been demonstrated for store-operated CRAC channels (Parekh 1998; Zweifach and Lewis 1995), and this inactivation is also minimized by MCU in T lymphocytes and basophilic leukemia cells (Gilibert and Parekh 2000; Hoth et al. 2000; for review, see Gilbert and Parekh 2000). These related observations in a neuron, a secretory cell, and cells of the immune system suggests that a widely expressed function of mitochondria is their ability to maintain the availability of Ca^{2+} influx pathways.

PKA-dependent regulation of L-type Ca^{2+} channels

PKA is known to phosphorylate L-type Ca^{2+} channels and to increase their open time (Bean et al. 1984; Yue et al. 1990b). PKA and the Ca^{2+} /CaM-activated phosphatase calcineurin are known to be localized to $\text{Ca}_v1.2$ channels by the anchoring protein AKAP79/150 in neurons (Oliveria et al. 2007; for review, see Dai et al. 2009). Our evidence that calcineurin is not a major regulator of L-type Ca^{2+} current in amacrine cells is consistent with the report that amacrine cells do not express $\text{Ca}_v1.2$ (Firth et al. 2001). Comparatively little is known about the regulation of $\text{Ca}_v1.3$, but consistent with our observations, it has been demonstrated that PKA-dependent phosphorylation of $\text{Ca}_v1.3$ leads to current enhancement (Liang and Tavalin 2007; Qu et al. 2005). Current enhancement for $\text{Ca}_v1.4$ is apparently voltage dependent rather than phosphorylation dependent (Kourennyi and Barnes 2000).

AC1 in the retina

Intriguingly, expression of AC1 mRNA is enriched in the retina in comparison to brain and spinal cord (Xia et al. 1993). In the developing mouse retina, an in situ hybridization study demonstrated AC1 mRNA in photoreceptors and ganglion cells (Nicol et al. 2006). An immunocytochemistry study in the mouse retina found AC1 expression in the inner nuclear layer. In the chicken retina, we find AC1 protein strongly expressed in photoreceptors, in nearly all cells in the ganglion cell layer, and in amacrine cells. This labeling pattern is consistent with what we find in culture where AC1 expression was detected in amacrine cells and quite strongly in cone photoreceptors. AC1 expression was not determined for cultured ganglion cells because they do not persist under our culture conditions (Hyndman and Adler 1982).

Ca^{2+} -dependent AC activation

It has been established that AC1 and AC8 can be activated by Ca^{2+} entering the cell across the plasma membrane. The strongest evidence for influx-dependent AC1/8 activation is for

capacitative Ca^{2+} entry (CCE), but it is clear that entry via voltage-gated Ca^{2+} channels is effective as well (for review, see Willoughby and Cooper 2007). In rat cerebellar granule cells, depolarization-dependent Ca^{2+} influx was shown to promote cAMP accumulation (Cooper et al. 1998). An expression study with AC8 in a pituitary cell line has demonstrated that both CCE and voltage-gated Ca^{2+} channel activity were effective in stimulating the enzyme (Fagan et al. 2000). Interestingly, although release of Ca^{2+} from stores was substantial in this preparation, it was not effective in stimulating AC8 activity. In immortalized gonadotropin-releasing hormone neurons derived from the rat hypothalamus, it was also demonstrated that AC1 was activated by voltage-dependent Ca^{2+} influx but not by release of Ca^{2+} from stores (Krsmanovic et al. 2001). Here we provide evidence that in amacrine cells, both Ca^{2+} influx and release from stores enhances the L-type Ca^{2+} current amplitude, and we propose that the effect of store release could be due to further stimulation of AC1. It might be that the structure and organization of amacrine cells differ from the cells discussed in the preceding text such that release of Ca^{2+} from stores has an impact on AC1 activity. The spatial relationship between RyRs, L-type Ca^{2+} channels and AC1 is not known. The resistance of AC1 activity to 10 mM BAPTA could imply that AC1 is intimately associated with both channel types. The importance of localization is also made relevant in light of our previous work showing that the metabotropic glutamate receptor 5 (coupled to the IP_3 pathway)-dependent enhancement of this same Ca^{2+} current can be suppressed by 5 mM BAPTA internally. This receptor-mediated enhancement of the current is also thought to involve AC1, but the source of the activating Ca^{2+} here is presumably IP_3 receptors rather than RyRs (Sosa and Gleason 2004). An additional consideration is the high affinity of AC1 for Ca^{2+} that is about five times that of BAPTA [100 nM AC1 (Fagan et al. 1996; Wu et al. 1993) vs. 500 nM BAPTA (Adler et al. 1991)].

It has been shown in amacrine cells that RyR-mediated CICR can activate Ca^{2+} -sensitive K^+ currents (Mitra and Slaughter 2002). A previous study on cultured amacrine cells demonstrated a role of CICR in enhancing synaptic GABA release, but IP_3 receptors rather than RyRs were involved (Warrier et al. 2005). Although RyRs and IP_3 receptors are expressed throughout the cell bodies and dendrites of cultured amacrine cells (Warrier et al. 2005; Sen et al. 2007), they might be differentially regulated in the two regions. If this is case, then nonsynaptic L-type Ca^{2+} channels might be the primary target of the regulatory mechanism involving CICR and RyR demonstrated here.

In the retina, bipolar cells can support sustained release of glutamate (for review, see Heidelberger et al. 2005), and amacrine cells are one of their postsynaptic targets. Although some amacrine cells are known to feedback onto bipolar cells, limiting the duration of excitation (Chavez et al. 2006; Dong and Hare 2003; Hartveit 1999; Singer and Diamond 2003), others may be subjected to prolonged depolarization and potentially significant elevations in cytosolic Ca^{2+} . On the whole, the mechanisms described here (MCU, PKA activity, and CICR-dependent enhancement) tend to promote and preserve the activity of the L-type channels in amacrine cells. This may be important in maintaining signaling capabilities of amacrine cells at their cell bodies or at their synapses or, in some cases, both.

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DISCLOSURES

There are no disclosures to be made.

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