Mechanism linking NMDA receptor activation to modulation of voltage-gated sodium current in distal retina

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Davis, Scott F. and Cindy L. Linn. Mechanism linking NMDA receptor activation to modulation of voltage-gated sodium current in distal retina. Am J Physiol Cell Physiol 284: C1193–C1204, 2003. First published January 29, 2003; 10.1152/ajpcell.00256.2002.—In this study, we investigated the mechanism that links activation of N-methyl-D-aspartate (NMDA) receptors to inhibition of voltage-gated sodium channels in isolated catfish cone horizontal cells. NMDA channels were activated in voltage-clamped cells incubated in low-calcium saline or dialyzed with the calcium chelator BAPTA to determine that calcium influx through NMDA channels is required for sodium channel modulation. To determine whether calcium influx through NMDA channels triggers calcium-induced calcium release (CICR), cells were loaded with the calcium-sensitive dye calcium green 2 and changes in relative fluorescence were measured in response to NMDA. Responses were compared with measurements obtained when caffeine depleted stores. Voltage-clamp studies demonstrated that CICR modulated sodium channels in a manner similar to that of NMDA. Blocking NMDA receptors with AP-7, blocking CICR with ruthenium red, depleting stores with caffeine, or dialyzing cells with calmodulin antagonists W-5 or peptide 290–309 all prevented sodium channel modulation. These results support the hypothesis that NMDA modulation of voltage-gated sodium channels in horizontal cells requires CICR and activation of a calmodulin-dependent signaling pathway.

in intracellular calcium stores; voltage-gated sodium channels

REGULATION OF CYTOPLASMIC free calcium concentrations is an important mechanism by which neurons modulate their excitability. Understanding how neurons regulate intracellular calcium levels can have important physiological consequences for all areas of neurobiology. Most neurons contain two nonmitochondrial intracellular calcium stores located in the smooth endoplasmic reticulum (33, 34). One is sensitive to the phosphatidylinositol 4,5-bisphosphate metabolite, inositol 1,4,5-trisphosphate (IP3). IP3 is generated when phosphatidylinositol 4,5-bisphosphate metabolite, inositol 1,4,5-trisphosphate (IP3). IP3 is generated when phosphatidylinositol 4,5-bisphosphate is metabolized by the phospholipase C (PLC) pathway. PLC is activated by a ligand binding to G protein-coupled receptors. The binding of IP3 receptors on the surface of the endoplasmic reticulum is coupled to calcium release into the cytoplasm (4).

The other type of nonmitochondrial calcium store found in most neurons is sensitive to increased cytoplasmic calcium and agents such as caffeine and ryanodine (33, 48). Typically, increased cytoplasmic calcium occurs through activation of ligand-gated receptors and/or by opening of voltage-gated calcium channels. The resulting increase in intracellular free calcium concentration ([Ca2+]i) causes release of calcium from the ryanodine-sensitive intracellular store. This mechanism, called calcium-induced calcium release (CICR), has been shown to increase cytoplasmic calcium concentrations from resting levels of ~100 nM to over 1 μM (53, 63). Calcium entry through ligand-gated receptors and voltage-gated channels results in short increases in cytoplasmic calcium concentration and brief changes in neuronal membrane potential (4). Increases in intracellular calcium through CICR result in a larger and more persistent change in intracellular calcium levels, which can activate various signal transduction pathways and affect greater changes in cellular physiology such as modulation of neuronal excitability, transmitter release, and gene expression. Both IP3- and ryanodine-sensitive intracellular stores have been demonstrated in catfish cone horizontal cells (30–32).

Catfish cone horizontal cells are second-order neurons in the retina that receive synaptic input from photoreceptors and interplexiform cells (62, 68) and make synaptic connections onto bipolar cells and photoreceptors (2, 68). Horizontal cells are electrically coupled by gap junctions (43) and are responsible for the lateral spread of information in the distal retina (42, 68) and the surround inhibition recorded from bipolar cells and retinal ganglion cells (2, 68) to affect contrast detection and visual acuity in the retina. Voltage-clamp studies have identified several categories of voltage-dependent channels (58, 59) and numerous types of neurotransmitter-gated receptors (23, 47, 48, 51) on catfish cone horizontal cells. The voltage-gated ion channels include three types of channels that are activated in the physiological operating range of the horizontal cells. These include the classic delayed rectifier, the transient tetrodotoxin (TTX)-sensitive sodium channel, and the transient naught rectifier.

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channel (26, 58, 59), and a long-lasting, sustained dihydropyridine-sensitive (L-type) calcium channel (58, 59). All of these channels activate between −70 and −20 mV, although in the catfish the classic delayed rectifier is unusually small in this range of membrane potentials. It has been proposed that the other two voltage-gated channels activated between −70 and −20 mV, the voltage-gated sodium and calcium channels, contribute to shaping the physiological response to light and help maintain the cell’s membrane potential in the dark (1, 59, 60, 71). In addition, modulation of these channels would have considerable physiological implications as to the type of information conveyed through the retina.

Glutamate is the major excitatory neurotransmitter released from teleost photoreceptors onto the second-order horizontal cells (18, 37, 50). Glutamate receptors have been classified into two major classes, metabotropic and ionotropic. Metabotropic glutamate receptors are G protein-coupled receptors linked to a variety of signal transduction pathways, whereas ionotropic glutamate receptors directly couple glutamate binding to opening of a nonspecific cation channel. Ionotropic glutamate receptors have been further subdivided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors on the basis of their pharmacological and electrophysiological characteristics. The NMDA receptor is characterized by its voltage dependence due to a magnesium block and its high permeability to calcium (36, 38). The presence of the NMDA-type glutamate receptor has been pharmacologically and electrophysiologically characterized in isolated catfish cone horizontal cells (47, 48). However, the function of the NMDA receptor in the outer retina is unclear.

In our recently published paper (11), we described how NMDA receptor activation modulates both the voltage-gated sodium and calcium channels in catfish cone horizontal cells and provided the first step toward understanding the physiological implications of NMDA’s effects on visual processing in the distal retina. Previous studies demonstrated that an increase of intracellular calcium through calcium-permeable channels induces CICR from calcium-sensitive intracellular stores and modulates the activity of voltage-gated calcium currents (31, 32). However, the mechanism by which NMDA receptor activation leads to modulation of voltage-gated sodium channels has yet to be described. In the present study, we explore this mechanism and provide evidence that NMDA modulation of voltage-gated ion channel activity in catfish cone horizontal cells involves CICR and a calmodulin-dependent signaling pathway.

**MATERIALS AND METHODS**

**Animals.** Channel catfish (*Ictalurus punctatus*) were obtained from a local catfish farm. Experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**Experimental solutions.** All voltage-clamp experiments in which the voltage-gated sodium current was studied were performed in catfish saline containing (in mM) 126 NaCl, 4 KCl, 3 CaCl2, 15 dextrose, 2 HEPES, and 10 4-aminopyridine (4-AP), to block the outwardly rectifying potassium current, with the L-type calcium channel dihydropyridine antagonist nitrendipine (10 μM), to block the L-type calcium channel. Magnesium blocks the pore of the NMDA receptor at membrane potentials hyperpolarized from −30 mV (38) and was therefore omitted from all extracellular solutions.

Application of control and experimental solutions was achieved via a gravity-fed perfusion system. The complete exchange of solutions occurred within 500 ms. In this study, 100 μM NMDA was used because dose-response studies demonstrated that this was the minimal dose yielding a maximal effect (11). In some experiments, catfish saline containing 10 mM caffeine to release and deplete intracellular calcium stores from ryanodine-sensitive intracellular stores. This release was blocked when 2 μM ruthenium red was allowed to diffuse into the cells through the recording pipette solution. The calmodulin antagonists N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-HCl (W-5; 50 μM) and W13 M kinase II fragment 290–309 (peptide 290–309; 100 μM), both obtained from Sigma, were added to the recording pipette solution for other experiments. Agonist and antagonist concentrations used were determined from dose-response experiments (11). The minimal dosage that provided a maximum effect was used throughout the study.

**Horizontal cell isolation.** Isolated catfish cone horizontal cells were prepared as previously described (32). Briefly, dark-adapted channel catfish were anesthetized with tricaine methanosulfonate (10 mg/ml). When the animal no longer responded to tactile stimulation, it was pithed and both eyes were removed under dim red light. After removal of the cornea and lens, the remaining eyecups were placed in magnesium-free, low-calcium catfish saline (0.3 mM calcium compared with 3 mM in control saline) containing hyaluronidase (0.1 mg/ml) for 4 min to digest the vitreous humor (pH adjusted to 7.4 with NaOH). The eyecups were then transferred to fresh low-calcium catfish saline containing cysteine-activated papain (0.7 mg/ml) for another 4 min. The papain-treated tissue was rinsed, and the retina was manually peeled off the eyecup. Retinas were placed in fresh papain containing low-calcium catfish saline for another 4 min, rinsed well, and cut into 8–10 pieces. Retinal pieces were stored in normal catfish saline containing 1 mg/ml bovine serum albumin. Consistent recordings can be obtained from these retinal pieces for 48 h (48). Before recording, a piece of retina was further dissociated to yield isolated cone horizontal cells. This was accomplished by manual trituration of the retina through a series of progressively smaller-tipped Pasteur pipettes. Once retinal pieces were broken down into isolated cells, a sample of cells was transferred to a recording chamber mounted on the stage of an inverted Nikon Diaphot 300 microscope. Cells were allowed to settle for 5 min before an experiment began. Individual cells were viewed with Hoffman contrast and were easily identified on the basis of characteristic morphology (41).

**Electrophysiology.** Cells were voltage clamped as described by Hamill et al. (19). Patch pipettes were pulled from borosilicate glass by a Narishige (Tokyo, Japan) vertical microelectrode puller. Electrodes used in these experiments were uncoated and unpolished and contained (in mM) 1 CaCl2, 10 HEPES, 140 potassium gluconate or 140 cesium chloride, 2 MgCl2, and 11 EGTA. Electrode resistance was measured in normal catfish saline. Electrodes with resistances measuring between 3 and 8 MΩ were used in this study.

Once a cell was voltage clamped, the membrane potential was changed in either a rampwise or a stepwise manner to...
evoke voltage-gated ion currents. The rampwise stimulus paradigm consisted of changing the membrane potential of a voltage-clamped catfish cone horizontal cell between −60 and +50 mV over a 500-ms period. This created a current-voltage (I-V) relationship for the cell. The stepwise stimulus paradigm consisted of stepping the membrane potential of a voltage-clamped cell from the resting membrane potential to various depolarized membrane potentials. To elicit peak voltage-gated sodium currents, membrane potentials were changed from a holding potential of −60 mV to 10 mV in normal saline. From the current traces obtained, the following four parameters were routinely measured: 1) peak current amplitude, 2) membrane potential corresponding to the peak current amplitude, 3) membrane potential corresponding to where a detectable inward current was measured, and 4) membrane potential corresponding to where the current reversed. A paired t-test was done on all data to determine whether experimental values differed significantly from controls.

Recordings were obtained with an Axon Instruments Axopatch 200A amplifier (Foster City, CA). Series resistance and capacitive artifacts were compensated for by using amplifier controls. No data were collected from cells with leakage currents >0.02 nA. Data collection was controlled by a computer with a Digidata 1200 data acquisition board. Digitization and analysis were performed with the pCLAMP program (Axon Instruments). Data were filtered at 1 kHz and sampled at 10 kHz.

Calcium imaging. Isolated catfish cone horizontal cells were loaded with the membrane-permeant, calcium-sensitive dye calcium green 2 (Molecular Probes; Eugene, OR). Calcium green 2 has a high calcium affinity ($K_d = 190$ nM) and is useful for detecting low calcium signals. After cells were loaded for 20 min at room temperature, they were washed and transferred to the stage of an inverted microscope (Nikon Diaphot) equipped for fluorescence measurements in magnesium-free saline containing the L-type calcium channel dihydropyridine antagonist nitrrendipine (10 μM), to block the voltage-gated calcium current. The cell’s membrane potential was changed in a rampwise fashion from −60 mV to +50 mV under control conditions, 1 min after application of 100 μM NMDA (gray trace) and 2 min after NMDA was washed out (recovery). Traces were superimposed for comparison. To generate these current traces, the extracellular bathing solution contained no pharmacological ion channel blockers and the intracellular pipette solution contained 140 mM potassium gluconate instead of cesium chloride to prevent blocking of voltage-gated potassium channels intracellularly. All subsequent experiments examining voltage-gated sodium current activity or calcium imaging used bathing saline containing pharmacological blockers of the voltage-gated potassium and calcium channels, and pipette electrodes contained 140 mM cesium chloride instead of 140 mM potassium chloride to block any possible contribution of voltage-gated potassium channels.

RESULTS

NMDS receptor activation linked to modulation of voltage-gated sodium channels. Figure 1 demonstrates the effect of NMDA on the I-V relationship recorded from a voltage-clamped catfish cone horizontal cell in saline containing pharmacological blockers of the voltage-gated sodium and calcium currents. Current traces were obtained under control conditions, 1 min after application of 100 μM NMDA, and 2 min after NMDA was washed out (recovery). NMDA at 100 μM induced an inward current from −60 mV to 0 mV and an outward current when the cell was depolarized beyond 0 mV. Besides inducing current that reversed near 0 mV, activation of NMDA receptors in isolated catfish cone horizontal cells also modulated voltage-gated sodium channels by reducing the peak amplitude of the induced voltage-gated sodium current (Fig. 2). As shown in Fig. 2, A and B, peak voltage-gated sodium current was elicited under control conditions and 1 min after NMDA was applied to a voltage-clamped catfish cone horizontal cell. Peak sodium current activity was elicited with the stepwise stimulus paradigm and superimposed for comparison. In the presence of NMDA, the amplitude of the voltage-gated sodium current decreased by 75% compared with control current amplitude (Fig. 2A); however, 1 min after NMDA application the NMDA-induced current was still apparent. Therefore, the resulting decrease of input resistance and opening of agonist-gated cation channels could be sufficient to explain the decrease of sodium channel activity. However, in our previous paper (11), we provided evidence that modulation of the voltage-gated sodium and calcium channels in catfish cone horizontal cell is not solely due to the NMDA-induced change of input resistance. This evidence is based on the finding that NMDA-induced modulation of the voltage-gated sodium and calcium channels persisted significantly longer than the NMDA-induced current and the return of input resistance to control conditions. Combined with evidence that NMDA does not modify the delayed rectifier in catfish cone horizontal cells and that voltage-clamp conditions were optimal, these results suggested that NMDA’s modulatory effects on voltage-gated sodium channel activity may be processed through a
second messenger system in catfish cone horizontal cells. The evidence provided here supports the hypothesis that the modulatory effect of NMDA on voltage-gated sodium channels is likely due to activation of intracellular signaling pathways that alter ion channel activity.

Figure 2B demonstrates another current trace obtained from the same voltage-clamped catfish cone horizontal cell 3 min after application of 100 μM NMDA, when the NMDA-induced current had completely recovered. Under these conditions, the amplitude of the peak voltage-gated sodium current was decreased by 35% (±3.2%) compared with control conditions. Full recovery of NMDA’s modulatory effect on the voltage-gated sodium current took 10 min. These results were typical of data obtained from six other voltage-clamped cells, in which NMDA caused a mean decrease of voltage-gated sodium current amplitude by 36.2% compared with control conditions (±5.2%; P < 0.01). NMDA had no effect on the membrane potential associated with the peak current. Because these results illustrate the effect of NMDA minus the NMDA-induced current, all subsequent sodium current traces were obtained under control conditions or after full recovery of the NMDA-induced current and recovery of input resistance.

Figure 2C demonstrates the effect of NMDA on the I-V relationship obtained when the same cell was stimulated with the rampwise stimulus paradigm instead of the stepwise paradigm. The two current traces were obtained under control conditions and 3 min after application of 100 μM NMDA, after the NMDA-induced current had completely recovered. NMDA significantly reduced the amplitude of the voltage-gated sodium current at all activated membrane potentials from −30 mV to +28 mV in this cell, as well as 11 other voltage-clamped horizontal cells (P < 0.01). Full recovery of the NMDA-induced modulation of the voltage-gated sodium current took 10 min in all cells.

**NMDA-induced changes of intracellular calcium.** The finding that NMDA’s effect on voltage-gated channel activity lasted significantly longer than the NMDA-induced current suggests that NMDA’s modulation of channel activity is not directly due to current through the NMDA receptor. Because NMDA receptor channels are highly permeable to calcium influx (36, 38) and calcium permeation through agonist-gated channels was previously found to be involved in modulation of voltage-gated calcium currents (31, 32), we tested the hypothesis that calcium ions act as a second messenger to link activation of NMDA receptors and modulation of the voltage-gated ion sodium channels in catfish cone horizontal cells. To determine whether changes in [Ca²⁺], are associated with activation of the NMDA receptor, isolated cells were loaded with the calcium-sensitive dye calcium green 2 in saline containing the dihydropyridine antagonist nitrendipine, to block activity of the L-type voltage-gated calcium channels. Changes in relative fluorescence, demonstrating the fluctuations in [Ca²⁺], were monitored before and after NMDA application (Fig. 3A). Although concentration changes could not be measured directly with calcium green 2, an increase of the relative fluorescence signal from baseline represents an increase of intracellular calcium. Application of 100 μM NMDA to a calcium green 2-loaded catfish cone horizontal cell elicited a relative increase of [Ca²⁺]; persisting until NMDA was washed out. This response could be repeated at intervals of 1 min after NMDA washout, which likely corresponds to the time required for intracellular stores to refill. Similar changes of fluorescence were obtained from 10 other catfish cone horizontal cells loaded with calcium green 2 in the presence of 100 μM NMDA. When NMDA was applied in the presence of the competitive NMDA antagonist AP-7 (100 μM), NMDA failed to elicit any increase of relative fluorescence (Fig. 3B, n = 6). AP-7 at 100 μM blocked the effect of NMDA by 100%. To demonstrate that the NMDA-induced increase of [Ca²⁺] was initiated by extracellular calcium permeating the NMDA channel, experiments were repeated in saline containing low (0.3 mM) extracellular calcium (Fig. 3C) and 10 μM nitrendipine. In low-calcium saline, NMDA’s fluorescence signal was reduced by 88% compared with the fluorescence signal obtained with NMDA under control conditions. This significant difference was recorded in seven other cone horizontal cells, in which low calcium decreased the relative fluorescence signal by a mean of 85% (±5.2%; P < 0.01). Likewise, when loaded cells were incu-
bated in the membrane-permeant calcium chelator BAPTA-AM (5 mM), the effect of NMDA on relative fluorescence was reduced by a mean of 78% (±3.5%) compared with control relative fluorescence signals and represents a significant decrease \( (n = 5; P < 0.01) \). These results support the hypothesis that calcium permeation through the NMDA receptor causes a significant increase in \([Ca^{2+}]_i\), which may link receptor activation to calcium-dependent signaling pathways and subsequent modulation of ion channel proteins.

**Release of calcium from intracellular stores.** Increased intracellular calcium can link receptor activation to modulation of channel activity in several different ways. In one scenario, calcium could directly affect channel activity. A rise in free calcium concentration could also activate a multitude of calcium-dependent enzymes, which may ultimately affect channel activity. The question remains as to whether the influx of calcium through the NMDA receptor is enough to trigger these effects or whether this calcium signal may be amplified through the process of CICR. The existence of CICR from intracellular calcium stores in catfish cone horizontal cells has been well documented. (31, 32). Previous studies demonstrated that these intracellular stores release calcium in response to calcium and agents such as 10 mM caffeine (44) or 10 μM ryanodine. In addition, release of calcium from these stores can be depleted with caffeine and directly inhibited with 2 μM ruthenium red (31, 32).

Figure 4 illustrates relative fluorescence measurements obtained from a voltage-clamped catfish cone horizontal cell loaded with the calcium-sensitive dye calcium green 2 for 20 min at room temperature. A: typical increase of relative fluorescence measured when 100 μM NMDA was applied to loaded cells. NMDA was applied for 15 s at 3 different times to the cell. Between NMDA applications, NMDA was rinsed with normal saline for ~1 min. B: NMDA was applied 3 times to a catfish cone horizontal cell loaded with calcium green with 100 μM AP-7 in the bathing saline. C: NMDA was again applied 3 times to a calcium green-loaded cell. In this instance, 10 mM calcium in the bathing saline was replaced with 0.3 mM calcium. D: another catfish cone horizontal cell was loaded with calcium green 2 for 20 min in saline containing 5 mM BAPTA-AM. After incubation in BAPTA-AM, NMDA was applied 3 consecutive times and changes of relative fluorescence were recorded.
horizontal cell before, during, and after application of NMDA. The cell was held at its resting membrane potential in saline containing zero magnesium and 10 μM nitrendipine. Caffeine (10 mM) and NMDA (100 μM) each elicited a large increase in fluorescence signal. Similar changes of relative fluorescence were obtained from eight other catfish cone horizontal cells. However, when ruthenium red was allowed to dialyze into the cell before application of caffeine or NMDA, the increase in fluorescence signal was diminished between 70% and 90% compared with fluorescence signals obtained under control conditions (Fig. 4, B and D; n = 5). Together, these results suggest that a majority of the caffeine-induced and the NMDA-induced increase of fluorescence signal is the result of calcium release from intracellular stores.

Evidence for CICR. Figure 5 provides evidence for NMDA-induced CICR. As shown in Fig. 5A, an increase in relative fluorescence was measured after application of 100 μM NMDA to a calcium green 2-loaded catfish cone horizontal cell. After a prolonged washout, application of 10 mM caffeine produced a similar increase in relative fluorescence in the cell. After another prolonged washout, 100 μM NMDA was applied again, immediately followed by a second dose of 10 mM caffeine. There was no prolonged washout between the second application of NMDA and the second application of caffeine. The subsequent application of caffeine elicited a response in relative fluorescence that was 80% smaller than the first application. This suggests that NMDA receptor activation caused near-depletion of an intracellular calcium store and that both NMDA and caffeine affect the same intracellular store. This was the typical response recorded from a total of six isolated catfish cone horizontal cells loaded with calcium green 2 when caffeine was applied to a loaded voltage-clamped catfish cone horizontal cell immediately after application of NMDA. Under these conditions, the second caffeine fluorescence response was reduced by an average of 78% (±5.1%) compared with the first control caffeine response (P < 0.01).

As shown in Fig. 5B, the reverse experiment was performed, which provided further evidence of NMDA receptor-induced calcium release from intracellular stores. In this instance, caffeine was applied first to deplete ryanodine-sensitive intracellular stores. Caffeine was applied first, followed by application of 100 μM NMDA. After a prolonged washout, caffeine was applied again, followed by a second dose of NMDA. The second caffeine response was smaller than the first response, suggesting that ryanodine-sensitive intracellular stores were depleted by the first dose of caffeine. This was the typical response recorded from a total of six isolated catfish cone horizontal cells loaded with calcium green 2 when caffeine was applied to a loaded voltage-clamped catfish cone horizontal cell immediately after application of NMDA. Under these conditions, the second caffeine fluorescence response was reduced by an average of 78% (±5.1%) compared with the first control caffeine response (P < 0.01).

Fig. 4. Caffeine and NMDA cause an increase of intracellular calcium in catfish cone horizontal cells. A: a catfish cone horizontal cell was loaded with calcium green 2 for 20 min and voltage clamped at −60 mV before application of 10 mM caffeine elicited a similar response in relative fluorescence. B: the change of relative fluorescence caused by caffeine was significantly reduced when 2 μM ruthenium red was allowed to diffuse into voltage-clamped cells before application of 10 mM caffeine. C: 100 μM NMDA responses obtained from a voltage-clamped catfish cone horizontal cell loaded with calcium green 2. D: in the presence of 2 μM ruthenium red, NMDA elicited significantly reduced relative fluorescence responses compared to control responses.

Fig. 5. Evidence of calcium-induced calcium release (CICR) in catfish cone horizontal cells. A: the 2 increases of relative fluorescence on left, recorded in a loaded catfish cone horizontal cell, correspond to application of 100 μM NMDA and a subsequent application of 10 mM caffeine after washout. After another prolonged washout, 10 mM caffeine was applied on the tail of a second NMDA application. The second caffeine response was much smaller when on the tail of the NMDA response. B: 10 mM caffeine was applied to deplete intracellular calcium stores. In the presence of caffeine, 100 μM NMDA failed to elicit a further change in relative fluorescence.
feine (10 mM) application was immediately followed with perfusion of NMDA (100 μM). As can be seen from Fig. 5B, when intracellular stores were depleted with caffeine, NMDA had little effect. Similar results were obtained from five other isolated catfish cone horizontal cells loaded with calcium green 2. In the presence of caffeine, NMDA is unable to generate an increase in fluorescence signal.

**NMDA modulation of voltage-gated ion channels is mediated by CICR.** If CICR is involved in linking NMDA receptor activation to modulation of voltage-gated ion channels in catfish cone horizontal cells, caffeine should mimic NMDA’s effect on these channels. Figure 6 illustrates a side-by-side comparison of the effect of NMDA and caffeine on the voltage-gated sodium channel. Voltage-gated sodium currents were elicited with the stepwise stimulus paradigm before and after NMDA application (Fig. 6A) and caffeine application (Fig. 6B). Caffeine reduced the peak amplitude of the sodium current $I_{Na}$ in a manner that mimicked NMDA’s effect. As can be seen in Fig. 6A NMDA reduced the peak amplitude of $I_{Na}$ by 35%, whereas as shown in Fig. 6B caffeine reduced the peak amplitude of $I_{Na}$ by 58%. These results were replicated in eight other voltage-clamped cells, in which caffeine reduced the peak amplitude of $I_{Na}$ by a mean of 56% (±5.1%) compared with control conditions. Caffeine always produced a larger decrease of $I_{Na}$ amplitude. We propose that this is due to the extent of calcium release from intracellular stores caused by caffeine compared with release of calcium caused by 100 μM NMDA. As evident from data obtained in other studies (32), 10 mM caffeine produces near-complete depletion of calcium from intracellular stores in catfish cone horizontal cells for a specified period of time. However, 100 μM NMDA does not deplete stores to the same degree that 10 mM caffeine does.

To further test the hypothesis that NMDA modulates voltage-gated sodium channels in horizontal cells via a CICR-dependent mechanism, we applied NMDA to isolated voltage-clamped cells in the presence of ruthenium red. When present in the recording pipette, ruthenium red significantly reduced modulation of the voltage-gated sodium channels by NMDA (Fig. 7). Under control conditions, 100 μM NMDA reduced the voltage-gated sodium current amplitude by an average of 35% (±3.2%; n = 9). When 2 μM ruthenium red was present in the recording pipette solution, NMDA reduced peak sodium current amplitude by a mean of only 5.5% (±3.7%; n = 6) compared with control conditions. These results lend further support to the proposition that NMDA modulates voltage-gated sodium channels in catfish cone horizontal cells through the process of CICR.

Fig. 7. The effect of NMDA on voltage-gated sodium channels is dependent on CICR and calmodulin. Percent change in sodium current amplitude from control values for voltage-clamped isolated horizontal cells treated with NMDA alone or treated with NMDA in the presence of agents to block CICR or calmodulin is shown. Error bars represent SE. Numbers above each bar represent the number of times each experiment was performed. *Significant difference from control (P < 0.01).

**Fig. 6.** Caffeine mimics the effect of NMDA on voltage-gated sodium current activity. A: a voltage-clamped isolated catfish cone horizontal cell was stepped from a holding potential of −60 mV to elicit peak sodium current activity. Application of NMDA caused a substantial reduction in current amplitude. B: another voltage-clamped cell showing that application of 10 mM caffeine mimics NMDA’s effect on the sodium current amplitude.
dependent signal transduction pathway was demonstrated previously in other systems (31, 73). Therefore, we tested the hypothesis that modulation of voltage-gated sodium channels in catfish cone horizontal cells by NMDA is CaM dependent.

The effect of NMDA on the voltage-gated sodium channels was measured in the presence of one of two CaM antagonists, W-5 and peptide 290–309. Each of these agents was allowed to dialyze into voltage-clamped cells through the recording pipette solution. Both CaM antagonists significantly reduced NMDA’s effect on voltage-gated sodium current amplitude. W-5 (50 μM) reduced the NMDA-induced reduction of peak calcium current amplitude by only 8% (±1.0%; n = 5) compared with the 35% reduction obtained under control conditions (Fig. 7). Peptide 290–309 (20 μM) also reduced NMDA’s effect to an average reduction of only 3% (±0.9%; n = 5) compared with control conditions (Fig. 7). Both of these CaM antagonists significantly affected NMDA’s effect on channel activity (P < 0.01). These results suggest that NMDA receptor modulation of voltage-gated sodium channels in catfish cone horizontal cells may be dependent on CaM activation.

Previous electrophysiological and calcium-imaging studies have identified non-NMDA as well as NMDA channels on catfish cone horizontal cells. The non-NMDA channels have been pharmacologically classified as dl-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA)/kainic acid (KA) channels that are permeable to calcium. In catfish, permeation of calcium through these non-NMDA channels has been shown to induce CICR (32). In light of the current finding that calcium permeation through NMDA channels also induces CICR in catfish cone horizontal cells, it seems likely that activation of AMPA/KA receptors would modulate the voltage-gated sodium channels in a similar manner as activation of NMDA receptors. Figure 8 summarizes the effect of 20 μM KA on peak voltage-gated sodium current activity. In the presence of 20 μM KA, peak current activity decreased by a mean of 55% (±5.2%; n = 7) compared with control conditions, even after the KA-induced current had completely recovered. This represents a significantly larger reduction than the 36.2% mean reduction in current caused by NMDA (P < 0.01) and correlates with greater calcium permeability through catfish AMPA/KA channels compared with catfish NMDA channels (32). The effect of KA modulation of voltage-gated sodium channel activity was significantly reduced when experiments were performed in low extracellular calcium, when the KA competitive antagonist CNQX (10 μM) was present, when cells were loaded with 5 mM BAPTA-AM before application of KA, when the intracellular stores were depleted with 10 mM caffeine before application of KA, or when 50 μM W-5 or 100 nM peptide 290–309 was dialyzed into the cell from the recording pipette solution before KA application (Fig. 8; P < 0.01). Together, these results suggest that activation of non-NMDA as well as NMDA receptors on catfish cone horizontal cells induces CICR and acts to modify activity of voltage-gated sodium channels.

**DISCUSSION**

In our earlier report (11), we described modulation of voltage-gated sodium and calcium conductances by NMDA receptor activation in retinal horizontal cells of the catfish. Evidence from the current paper demonstrates that voltage-clamped sodium and calcium currents were dramatically reduced for a relatively long period of time after application of NMDA. Both channels have activation ranges well within the physiological operating range of the horizontal cell, and evidence was presented that both voltage-gated channels could contribute to help shape the physiological response to light and that the L-type sustained calcium current could help maintain the membrane potential in the dark. The fact that these currents could play a role in determining neuronal excitability and maintaining membrane potential means that their modulation would have considerable physiological implications as to the type of information conveyed throughout the
retina in terms of the on- and off-center receptive fields.

Previous studies in this laboratory (31, 32) have focused on the mechanism of voltage-gated L-type calcium channel modulation in catfish cone horizontal cells. These studies demonstrated that an increase of intracellular calcium through agonist-induced CICR linked activation of glutamate receptor subtypes to modulation of the voltage-gated calcium currents recorded in these cells. In the present study, we determined that a similar mechanism links NMDA receptor activation to modulation of voltage-gated sodium channels via a calmodulin-dependent pathway. This mechanism acts to provide a prolonged reduction of the voltage-gated sodium current.

Calcium ions were explored as a possible second messenger linking NMDA receptor activation to modulation of voltage-gated ion channels because of the NMDA receptor’s permeability to calcium. We found that calcium influx through both NMDA and non-NMDA receptors triggered CICR from intracellular stores and that CICR is necessary for sodium channel modulation to occur. Besides catfish, glutamate receptor activation in other systems has also been linked to CICR (13, 27). At rest, the intracellular concentration of calcium is near 100 nM. Release of calcium from intracellular stores has been shown to increase this concentration to >1 μM for transient periods of time (32). This type of increase in intracellular calcium concentration can result in the activation of a myriad of calcium signaling pathways. Calcium ions can directly modulate voltage-gated ion channels, resulting in changes in neuronal activity (8, 57), or calcium may combine with the ubiquitous intracellular calcium protein CaM to initiate intracellular signaling events. Activation of CaM-dependent signaling pathways by NMDA has been demonstrated in a variety of systems (16, 25, 73). Ca²⁺/CaM exerts its effects by binding with various cellular proteins. One of the most common is the CaM kinase II. CaM kinase II phosphorylates other cellular proteins, including ion channels, thereby regulating their activity (61, 72). CaM kinase II is associated with the NMDA receptor near the plasma membrane (28) and has been shown to regulate NMDA receptor activity in the retina (25). Once activated by Ca²⁺/CaM, CaM kinase II can remain active long after the initial signaling event is over because of autophosphorylation of the enzyme until the autophosphorylation is reversed by phosphatases in the cytoplasm. This ability of CaM kinase II to remain active long after the initial signaling event is over has been proposed to be an important mechanism in some types of learning and memory (3, 16, 54). In catfish cone horizontal cells, this mechanism may be an important mechanism during light adaptation (5). Therefore, we tested the hypothesis that NMDA modulation of voltage-gated sodium and calcium conductances was dependent on CaM activation and found that two CaM antagonists blocked NMDA’s modulatory effect on voltage-gated sodium channels. Thus it is likely that Ca²⁺/CaM activates CaM kinase II to directly or indirectly phosphorylate the voltage-gated ion channels, resulting in their modulation.

Previous biochemical and functional studies have examined sodium channel phosphorylation (29). Sodium channel proteins in the mammalian brain consist of α- and β-subunits (6, 7). Expression of α-subunits alone is sufficient for the formation of functional sodium channels (17, 45, 56), but β-subunits are required to give the characteristic kinetic properties and voltage dependence of sodium channel activation and inactivation (22, 40). Biochemical studies of purified brain sodium channels show that α-subunits are exceptionally good substrates for phosphorylation by cAMP-dependent protein kinase (PKA; Ref. 9) and by protein kinase C (PKC; Ref. 10), whereas no phosphorylation of the β-subunits occurs. Similar findings have been identified in synaptosomes (9) and in intact brain neurons.

Fig. 9. Proposed mechanism linking NMDA receptor activation with modulation of voltage-gated sodium and calcium channels in catfish cone horizontal cells. Activation of NMDA receptors and/or non-NMDA receptors by glutamate causes calcium influx through receptor channels and initiates CICR from calcium-sensitive intracellular stores. Increased free intracellular calcium binds to calcium-dependent calmodulin, forming a calcium/calmodulin (CaM) complex. The CaM complex likely activates type 2 calcium/calmodulin-dependent protein kinase (CaMKII), which can directly or indirectly phosphorylate voltage-gated ion channels to affect channel activity. NMDAR, NMDA receptor; KA R, kainate/dl-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) receptor; V-G, voltage gated; P, phosphate group.
in cell culture (46, 55, 69). Although activation of PKA and PKC may be a common mechanism associated with sodium channel phosphorylation in some systems, other signaling pathways may also be directly or indirectly important in neuronal development and function. For example, phosphorylation and dephosphorylation of sodium channels on tyrosine residues has been found to modulate sodium channel inactivation (21, 52) and direct interaction of sodium channels with G proteins may regulate voltage-dependence gating of the sodium channel (34, 35). Few neuronal studies, however, have linked the calcium-dependent CaM pathway to regulation of sodium channel activity, although the calcium-dependent CaM pathway was found to modulate sodium channel in an isoform-specific manner via direct interaction with skeletal muscle sodium channels (12).

In the present study, the effect of the CaM pathway on sodium channel activity in catfish cone horizontal cells may be direct or indirect. Because a PKII site has not been identified on voltage-gated sodium channels in biochemical studies of other systems, it is likely that the CaM-PKII action is indirect, working on a cAMP- or diacylglycerol-dependent pathway, although we cannot rule out the possibility that the voltage-gated sodium channel in catfish is unique and contains a PKII phosphorylation site. However, the interaction between two second messenger systems exists in rat olfactory neurons, in which odorant-evoked elevations in cAMP activate cyclic nucleotide-gated channels, leading to external calcium influx, while at the same time, the excitatory action of calcium activates the calcium-dependent CaM to activate PKII and inhibit adenylate cyclase (66, 67). In this olfactory system, the inhibition of adenylate cyclase may contribute to termination of olfactory signaling (69).

Besides calcium-dependent protein kinases, it is well known that activated CaM also binds to neuronal nitric oxide (NO) synthase and can stimulate guanylyl cyclase to form cGMP (65). NO by itself can serve as an intracellular or intercellular messenger (14), or cGMP can effect a diversity of biological responses including modulation of agonist-gated currents in retinal neurons (39, 70). In addition, there are several other calcium-dependent processes described in the literature that can ultimately lead to modulation of voltage-gated channel activity in neurons (15, 49, 64). Therefore, although the results of this study provide evidence that CaM is involved in the pathway linking NMDA receptor activation to modulation of voltage-gated sodium channels in catfish cone horizontal cells, the influence of other calcium-dependent pathways contributing to this modulation is certainly a possibility.

Together these results provide insight into a potential mechanism for the NMDA receptor-mediated modulation of voltage-gated sodium channels in catfish cone horizontal cells (Fig. 9). We conclude that activation of both NMDA and non-NMDA receptors present on catfish cone horizontal cells results in calcium permeation through agonist-gated channels and induces CICR from calcium-sensitive intracellular stores. Modulation of the voltage-sensitive sodium conductance is dependent on this and the activation of the calcium-binding protein CaM. NMDA receptor activation has been linked to CICR and CaM activation in other systems, most notably in hippocampal models of long-term potentiation (3, 16, 54). Our studies showing that NMDA receptor activation results in modulation of voltage-gated sodium conductance via CICR and CaM provide new insight into the role of the NMDA receptor in synaptic plasticity.

Catfish cone horizontal cells as well as cells in other systems coexpress both NMDA- and non-NMDA glutamate receptors. The question still remains as to how the role of the NMDA receptor differs from the role of the non-NMDA glutamate receptors in synaptic plasticity. At rest, it is likely that only non-NMDA glutamate receptors are activated, because of NMDA receptor channel blockage by physiological concentrations of magnesium. Activation of the non-NMDA glutamate receptors, however, would depolarize the cell and release the voltage-dependent magnesium block of the NMDA channels. Therefore, the presence of non-NMDA glutamate receptors on catfish cone horizontal cells may mediate fast excitatory responses that depolarize the cell and allow activation of NMDA channels. AMPA receptors rapidly desensitize, but we have demonstrated that NMDA activation of the voltage-gated sodium channel leads to relatively long-term reduction of the voltage-gated sodium channel. A previous study demonstrated that modulation of the voltage-gated sodium channel in salamander retina plays a role in contrast adaptation at the level of the ganglion cells (24). Approximately one-half of the contrast adaptation to variable light stimuli in the salamander retina can be attributed to a change in the input-output relationship of the retinal ganglion cells correlated with a change in sodium channel function (24). At the horizontal cell level, it is likely that modulation of voltage-gated ion channels would directly affect the input-output relationship of the cell and directly contribute to physiological processes associated with horizontal cell function, such as light and dark adaptation and surround inhibition.

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