Positive Allosteric Modulators of AMPA Receptors Reduce Proton-Induced Receptor Desensitization in Rat Hippocampal Neurons

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Received 30 June 2000; accepted in final form 30 January 2001

Lei, Saobo, Beverley A. Orser, Gregory R. L. Thatcher, James N. Reynolds, and John F. MacDonald. Positive allosteric modulators of AMPA receptors reduce proton-induced receptor desensitization in rat hippocampal neurons. J Neurophysiol 85: 2030–2038, 2001. Whole-cell or outside-out patch recordings were used to investigate the effects of protons and positive modulators of a-aminom-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptors on the desensitization of glutamate-evoked AMPA receptor currents in isolated hippocampal CA1 neurons. Protons inhibited glutamate-evoked currents (IC50 of 6.2 pH units) but also enhanced the apparent rate and extent of AMPA receptor desensitization. The proton-induced enhancement of desensitization could not be attributed to a reduction in the rate of recovery from desensitization or to a change in the kinetics of deactivation. Non-stationary variance analysis indicated that protons reduced maximum open probability without changing the conductance of AMPA channels. The positive modulators of AMPA receptor desensitization, cyclothiazide and GT-21-005 (an organic nitrate), reduced the proton sensitivity of AMPA receptor desensitization, which suggests that they interact with protons to diminish desensitization. In contrast, the effects of wheat germ agglutinin and aniracetam on AMPA receptor desensitization were independent of pH. These results demonstrate that a reduction in the proton sensitivity of receptor desensitization contributes to the mechanism of action of some positive modulators of AMPA receptors.

INTRODUCTION

Protons modulate the properties of many ion channels, including glutamate receptors. The activity of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is negatively regulated by protons within the physiological range of extracellular pH (Tang et al. 1990; Traynelis and Cull-Candy 1990; Vylicky et al. 1990). Polyamines such as spermine (Johnson 1996; McBain and Mayer 1994; Williams 1997) and neomycin (Lu et al. 1998) potentiate NMDA receptor function by enhancing receptor desensitization; this is a novel mechanism that has not previously been identified. We also tested the hypothesis that positive modulators of AMPA receptors act by reducing this proton-mediated receptor desensitization. Our results indicate that the effects of CTZ and GT-21-005 (an organic nitrate), but not those of aniracetam and WGA, are in part contributed by a proton-mediated modulation of AMPA receptor desensitization.

METHODS

Preparation of acutely isolated hippocampal CA1 neurons

All the animals used in this work were handled in accordance with the regulations of the Medical Research Council of Canada. CA1 hippocampal pyramidal neurons were acutely isolated using the modified procedures of Wang and MacDonald (1995) except that protease (type XIV, Sigma) instead of papain was used. Briefly, Wistar rats 2–3 wk old were placed under halothane anesthesia and decapitated with a guillotine. Hippocampi were quickly removed and placed in a dish to modulating NMDA receptor function, protons have also been reported to inhibit a-aminom-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptor activity (Traynelis and Cull-Candy 1991). However, unlike NMDA receptors, a detailed mechanism of proton-mediated modulation of AMPA receptor function has not been elucidated.

AMPA receptors undergo rapid desensitization that could contribute to the decay of excitatory synaptic currents and perhaps play an important role in modulating synaptic plasticity (Jones and Westbrook 1996). Several reagents commonly described as positive modulators of AMPA receptor function, including cyclothiazide (CTZ) (Partin et al. 1994; Patneau et al. 1993; Yamada and Tang 1993), 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide (PEPA) (Sekiguchi et al. 1997), aniracetam (Ito et al. 1990; Vylicky et al. 1991), organic nitrates (Toong et al. 1998), and wheat germ agglutinin (WGA) (Vylicky et al. 1991) are known to reduce or block AMPA receptor desensitization. However, the mechanism by which these drugs modulate receptor desensitization is poorly understood.

In the present study, we investigated the effects of protons on AMPA receptor function in acutely isolated hippocampal pyramidal neurons. We found that protons inhibited AMPA receptor function by enhancing receptor desensitization; this is a novel mechanism that has not previously been identified. We also tested the hypothesis that positive modulators of AMPA receptors act by reducing this proton-mediated receptor desensitization. Our results indicate that the effects of CTZ and GT-21-005 (an organic nitrate), but not those of aniracetam and WGA, are in part contributed by a proton-mediated modulation of AMPA receptor desensitization.
FIG. 1. Protons inhibit α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function. A: glutamate-evoked whole-cell currents of AMPA receptors at different values of pH. Note that decreasing pH reduced steady-state (\(I_{ss}\)) as well as peak (\(I_p\)) glutamate-evoked currents. B: plot of \(I_p\) vs. pH from 19 neurons. The curve was fitted using the Hill equation. The 50% reduction in current (\(IC_{50}\)) value of pH was 6.1 ± 0.1 and the Hill coefficient was 1.7 ± 0.1. C: fitting of \(I_{ss}\) vs. pH with the Hill equation (\(n = 19\) neurons). The \(IC_{50}\) value was 6.2 ± 0.1 and the Hill coefficient was 1.2 ± 0.1. D: \(I_{ss}/I_p\) values at pH 6.0 and pH 8.0 normalized to the \(I_{ss}/I_p\) value at pH 7.5 (\(n = 19\) neurons).

FIG. 2. Protons reduced AMPA receptor desensitization. A: AMPA receptor currents evoked by glutamate at pH 8.0 and pH 6.0 in an outside-out patch. Desensitization was well fitted by a single exponential. Bottom: normalized currents. The desensitization time constant was reduced by changing pH from 8.0 to 6.0. B: desensitization time constants pooled from 6 outside-out patches. **, \(P < 0.01\) by paired t-test. C: current responses evoked by two different concentrations of glutamate at pH 8.0 and pH 6.0 from the same neuron. Note that the currents evoked by the test concentration (3 mM) decreased more rapidly at pH 6.0. D: pooled data from 8 neurons. Peak currents of the responses to test concentrations were normalized after subtracting steady-state currents evoked by conditioning concentrations. Note that protons enhanced pre-desensitization of the currents.
containing cold oxygenated external solution consisting of (in mM) 140 NaCl, 1.3 CaCl$_2$, 5.4 KCl, 25 HEPES, 33 glucose, 1 MgCl$_2$, and 0.0003 TTX (pH 7.4, osmolarity 320–335 mosmol l$^{-1}$). The hippocampi were cut into slices 300–500 µm thick by hand with a razor blade. The hippocampal slices were digested at room temperature of pH was unnecessary. Some hydrophobic drugs were initially added to the control and to the glutamate-containing solutions (Fig. 6).

WHOLE-CELL RECORDING. Whole-cell recordings were performed with an Axopatch-1B amplifier (Axon Instruments) in voltage-clamp mode. Recording electrodes with resistances of 3–5 MΩ were constructed from thin-walled borosilicate glass (1.5 mm diameter, WPI) using a two-stage puller (PP83, Narishige). Data were digitized, filtered (5kHZ), and acquired online using the pClamp6 program (Axon Instruments). The standard internal solution for the recording electrodes consisted of (in mM) 140 CsF, 35 CsOH, 10 HEPES, 2 MgCl$_2$, 2 tetraethylammonium, 11 EGTA, and 4 Na$_2$ATP (pH 7.3, osmolarity 300 mosmol l$^{-1}$). The standard external solution was the same as that already described except that Mg$^{2+}$ (3 mM) and AP5 (50 µM) were included to block NMDA receptors. After the whole-cell configuration was formed, the recorded cells were voltage-clamped at −60 mV and lifted into the stream of solution. Saturating concentrations of glutamate (3–10 mM) were applied to the neurons for 2 s to evoke responses. Under these recording conditions, the currents evoked by glutamate were completely blocked by GYKI 53655, which confirmed their identity as AMPA receptor responses.

OUTSIDE-OUT PATCH RECORDING. Outside-out patch recordings were carried out as described in Bai et al. (1999). Glutamate was applied by using theta tubing connected to a piezoelectric translator (PZS-100 driven by PZ-150; Burleigh, Fishers, NY). For solution exchange, $\tau$ was <200 µs as determined by measuring the open-tip junction potentials. Currents were filtered at 5 kHz and digitized at 50 kHz.

NON-STATIONARY VARIANCE ANALYSIS. Non-stationary variance analysis was used to estimate conductance and the open probability of the channels at the peak of the response (Sigworth 1980). Generally, 60 responses, evoked by applications of 10 mM glutamate for 100 ms at 5-s intervals, were recorded for each outside-out patch. During an epoch of 60 responses, an average of 10% rundown was observed. Data from patches showing >20% rundown were not included for analysis. Responses from each patch were divided into 10 to 12 groups (5 or 6 for each patch). After each of the five or six responses to the peak was aligned, the local means of each group were calculated to minimize distortion originating from rundown. Each individual response was subtracted from the local mean of the group so that the variance could be computed. Current responses of 90 ms from the peak were selected for analysis. The mean current was divided into 100 equally sized bins and the corresponding variances were pooled. The binned variance versus the mean current was plotted and fit with the equation $\hat{\delta} = I - I_{\text{mean}} + \delta_{\text{base}}$, where $\hat{\delta}$ is the variance, $I$ is the mean current, $N$ is the number of channels activated at the peak, $i$ is the single channel current, and $\delta_{\text{base}}$ is the background variance. Open probability at the peak, $P_{O,\text{Peak}}$, was calculated by $P_{O,\text{Peak}} = I_{\text{peak}}/I(N)$, where $I_{\text{peak}}$ is the peak current; then, the single channel conductance was measured from $\gamma = i(E - E_{\text{rev}})$, where $E$ is the holding potential and $E_{\text{rev}}$ is the reversal potential (0 mV under our recording conditions) (Fig. 6).

SOLUTIONS. Solutions with different pH values were prepared each day by adding 6N HCl or 10N NaOH, as appropriate, to the external solution. A series of solutions with pH values ranging from 8.0 to 5.0 was prepared. Solutions of individual pH values were divided into four tubes so that the following four sets of solutions could be prepared by adding glutamate or drugs. Set 1, control bathing solution; Set 2, glutamate-containing solution; Set 3, control bath solution + drug; Set 4, glutamate-containing solution + drug. The pH was rechecked and readjusted, if necessary, after the addition of drugs to ensure that the pH of the four sets of solutions was the same; generally, readjustment of pH was unnecessary. Some hydrophobic drugs were initially dissolved in DMSO and then diluted to the desired concentration in the external solution. The final concentration of DMSO that was applied to the cells was usually <0.1% except for solutions containing CTZ (0.5%) and aniracetam (1%). In these cases, the same amount of DMSO was added to the control and to the glutamate-containing solutions.

DATA ANALYSIS. Data were expressed as means ± SE. Values in parentheses refer to the number of cells used for statistical analysis. Concentration-response curves were fitted by the Hill equation $I = I_{\text{max}} \times ((1 + (EC_{50} / [\text{ligand}]^{n}))$, where $I_{\text{max}}$ is the maximum response, $EC_{50}$ is the concentration of ligand that produces a half-maximal response, and $n$ is the Hill coefficient. Statistical analyses were performed using either Student’s t-test or two-way ANOVA. $P$ values <0.05 were taken as an indication of significant difference.
RESULTS

Protons inhibit whole-cell AMPA receptor currents in isolated CA1 neurons

We initially used whole-cell recordings to examine the effect of protons on AMPA receptor currents that were evoked by a saturating concentration of glutamate in isolated CA1 neurons. The peak ($I_p$) and steady-state ($I_{ss}$) currents both were significantly depressed with increasing concentrations of protons (Fig. 1A). At pH 5.0, both $I_p$ and $I_{ss}$ were almost completely blocked (Fig. 1, A–C). The values of pH that produced a 50% reduction in current (IC$_{50}$) were 6.1 ± 0.1 ($I_p$) and 6.2 ± 0.1 ($I_{ss}$) pH units ($n$ = 19 neurons) (Fig. 1, B and C). Also, the ratio of steady-state to peak currents ($I_{ss}/I_p$) decreased with the increase in proton concentration (Fig. 1D), which suggests that desensitization of AMPA receptors was enhanced by protons.

Protons enhance AMPA receptor desensitization

We next examined the effect of acidification on the time course of the onset of desensitization in outside-out patch recordings of AMPA responses (Fig. 2A). The decay of AMPA
The time constants for recovery from desensitization, as determined by fitting each data set with a single exponential, were not altered when pH was changed from 8.0 (τ = 99.8 ± 22.2 ms; n = 5) to 6.0 (τ = 83.4 ± 13.1 ms; n = 5, P > 0.05), which suggests that protons did not change the rate of recovery from desensitization (Fig. 3B).

**Protons inhibit open probability of AMPA receptors**

We next examined whether or not protons were able to reduce the amplitude of non-desensitized AMPA receptor-mediated currents. We tested this possibility by measuring the open probability of the non-desensitized state with nonstationary variance analysis (Fig. 4). Protons reduced the maximum open probability by 35.1 ± 4.6% (pH 8.0, 0.77 ± 0.06; pH 6.0, 0.51 ± 0.06; n = 8 patches, P < 0.01) without changing the conductance of AMPA receptors (pH 8.0, γ = 14.1 ± 2.8 pS; pH 6.0, γ = 13.1 ± 2.4 pS; n = 8 patches, P > 0.05).

**Protons did not change the deactivation of AMPA receptors**

Several modulators of AMPA receptor desensitization, including cyclothiazide, aniracetam, and thiocyanate, also modulate AMPA receptor deactivation (Partin et al. 1996). We therefore used outside-out patches to examine whether or not protons modulate the deactivation kinetics of AMPA receptors. AMPA receptor deactivation was resolved using rapid (1 ms) applications of 10 mM glutamate (Fig. 4A). However, changing pH failed to alter deactivation kinetics (pH 8.0, τ = 3.3 ± 0.1 ms; pH 6.0, τ = 3.1 ± 0.2 ms; n = 8 patches, P > 0.05 by paired t-test) (Fig. 5).

**Voltage-independent modulation by protons**

We also examined the voltage dependence of proton-induced inhibition as well as the pH dependence of the potency of glutamate. Inhibition by protons did not depend on holding potentials ranging from −80 to +40 mV (n = 5) (Fig. 6A), which argues against a simple open channel block. Furthermore, the potency for activation of I_p was not changed by protons (pH 8.0, EC_{50} = 1051 ± 482 μM, n_H = 0.9 ± 0.1; pH 6.0, EC_{50} = 1462 ± 407 μM, n_H = 0.8 ± 0.1; n = 6, P > 0.01) (Fig. 6Bb) whereas the potency for I_p was reduced (pH 8.0, EC_{50} = 66 ± 10 μM, n_H = 1.4 ± 0.1; pH 6.0, EC_{50} = 119 ± 16 μM, n_H = 1.3 ± 0.2; n = 6, P < 0.05) (Fig. 4Bc).
CTZ and organic nitrates reduce proton sensitivity of AMPA receptor desensitization

CTZ reduces AMPA receptor desensitization (Partin et al. 1994; Patneau et al. 1993; Yamada and Tang 1993). We therefore tested if the proton dependence of AMPA receptor–mediated current was altered by this drug. CTZ at a concentration of 100 μM dramatically reduced AMPA receptor desensitization (Fig. 7A). \( I_p \) and, especially, \( I_{ss} \) both were significantly enhanced by CTZ. CTZ shifted the \( I_{ss} \) versus the pH curve to the left by 1.0 pH unit (control, IC\(_{50}\) 5.6 ± 0.1, \( n_H = 1.6 ± 0.1 \); cyclothiazide, IC\(_{50}\) 5.2 ± 0.01, \( n_H = 1.8 ± 0.3 \); \( n = 12, P < 0.0001 \) (Fig. 7B). This result suggests that cyclothiazide reduces proton-mediated enhancement of AMPA receptor desensitization.

GT-21-005, an organic nitrate, substantially reduces AMPA receptor desensitization (Toong et al. 1998). At a concentration of 2 mM, GT-21-005 substantially reduced the desensitization of glutamate-evoked currents (Fig. 7C) and shifted the \( I_{ss} \) versus pH curve to the left by 0.6 pH units (control, IC\(_{50}\) 6.0 ± 0.1, \( n_H = 2.0 ± 0.1 \); GT-21-005, IC\(_{50}\) 5.4 ± 0.1, \( n_H = 2.0 ± 0.3 \); \( n = 7, P < 0.01 \) (Fig. 7D). This result suggests that this organic nitrate also reduces AMPA receptor desensitization by changing the pH sensitivity of desensitization.

Effects of WGA and aniracetam were independent of protons

WGA is a lectin that, with a relatively slow onset (minutes), irreversibly reduces AMPA receptor desensitization (Vyklicky et al. 1991). We therefore pretreated hippocampal slices with WGA (300 μg/ml) for 15 to 30 min and then dissociated CA1 neurons for recording. Pretreating the cells with WGA significantly reduced the desensitization of glutamate-evoked currents (Fig. 8A). However, the pH sensitivity of AMPA receptor desensitization was not changed by WGA (Fig. 8B). The IC\(_{50}\) value was 5.8 ± 0.1 (\( n_H = 2.0 ± 0.3 \)) for control (\( n = 9 \)) and 5.7 ± 0.1 (\( n_H = 1.6 ± 0.1 \)) for cells pretreated with WGA (\( n = 10, P > 0.05 \) (Fig. 8B).

The effect of aniracetam on the pH sensitivity of the currents was more complex (Fig. 8, C and D). Aniracetam (5 mM)
appeared to reduce proton sensitivity between pH 7.0 and pH 6.0 but, at lower pH values, it had no effect (Fig. 8D). A comparison of the IC$_{50}$ values showed no significant difference (control, IC$_{50}$ = 5.9 ± 0.1, n$_H$ = 1.1 ± 0.2; aniracetam, IC$_{50}$ = 5.7 ± 0.1, n$_H$ = 1.8 ± 0.1; n = 5, P > 0.05). We were unable to examine the effects of higher concentrations of this drug because of its limited solubility in physiological solutions.

DISCUSSION

In the present study, we provide evidence that protons enhance AMPA receptor desensitization without altering deactivation kinetics. Although it is likely that the mechanism underlying proton-mediated enhancement of desensitization is complex, our results do not support a simple slowing of recovery from desensitization because the change in extracellular pH from 8.0 to pH 6.0 enhanced receptor desensitization but failed to alter the rate of recovery from desensitization. The result that protons did not change the time constants of deactivation suggests that protons probably do not modulate the binding or unbinding of agonist. However, our results suggest that protons reduce the maximum open probability of non-desensitized receptors. This observation may provide one explanation for proton-induced enhancement of desensitization because, in some models, entry to desensitized states cannot proceed via the open, agonist-bound state of the receptor (Jones and Westbrook 1997). Therefore, a decrease in the probability of channel opening induced by protons might favor receptor desensitization.

The binding site for glutamate consists primarily of a segment of the N-terminus called the S1 segment as well as a second S2 segment located in the extracellular loop (Paas 1998). Both segments, and an additional region of the N-terminus, demonstrate substantial homology with prokaryotic periplasmic bacterial binding proteins (PBPs) and, by analogy, it has been postulated that these segments from two separate binding lobes are capable of physically trapping agonist (the “Venus flytrap” model) (Mano et al. 1996). Closure of the segments would perhaps stabilize the receptor in a high-affinity and non-conducting desensitized conformation. However, recent experiments (Abele et al. 1999) suggest that movements of these segments are much more subtle than are those reported for PBPs, which suggests instead that relatively small rotations of the lobes are associated with receptor desensitization (Abele
et al. 1999; Swanson et al. 1997). In this respect, a single-site mutation within S1 can entirely block desensitization of the GluR3 subunit and various mutations within S2 can also alter desensitization (Stern-Bach et al. 1998). The flip and flop region, which is located toward the C-terminus from S2 but still within the extracellular loop, also controls desensitization, perhaps by altering rotation of the S2 segment. It seems likely that the proton sensor(s) of the AMPA channel is located in or near the S1 or S2 segment and that its occupancy may stabilize desensitized conformation of the AMPA channel.

Our results show that positive modulators of AMPA receptors, such as CTZ and GT-21-005, reduce the proton sensitivity of AMPA receptor desensitization whereas WGA and aniracetam do not. This evidence suggests that there are multiple ways in which drugs can alter desensitization. For example, aniracetam preferentially modulates desensitization of flop splice variants of AMPA receptor subunits (Johansen et al. 1995; Partin et al. 1996), which probably is a consequence of slowing channel closing. In contrast, CTZ preferentially reduces desensitization of flip splice variants (Partin et al. 1994) by stabilizing a non-desensitized agonist-bound closed state (Partin et al. 1996). GT-21-005 is a novel organic nitrate (Yang et al. 1996) and is a member of a large family of S-nitrates that have previously been described and that exhibit properties very different from the prototype nitrate ester nitroglycerin (Thatcher and Weldon 1998). The fact that GT-21-005 also reduces the proton-sensitivity of AMPA receptor desensitization suggests that it may act at the same site or sites as CTZ. In contrast, lectins such as concanavalin A and WGA probably act to reduce desensitization by binding to glycosylation sites on receptor subunits (Everts et al. 1997, 1999). CTZ and aniracetam are unlikely to interact with these glycosylation sites because their effects are in addition to those of lectins (Everts et al. 1997; Vyklicky et al. 1991).

Our study is important for understanding the roles attributed to AMPA receptors in some pathological conditions. Extracellular pH undergoes relatively small changes during synaptic transmission (Chesler and Kaila 1992). In contrast, large decreases in pH occur during intense seizure activity or ischemia and pH levels can decrease by 0.2 to more than 1.0 pH units (Chesler and Kaila 1992; Siesjo 1985; Silver and Erecinska 1992). The activation both of NMDA and of AMPA receptors potentially contributes to neuronal injury during these pathological conditions. Simultaneous acidification of the extracellular space would be expected to limit the degree of excitotoxicity by inhibiting NMDA receptor activity and enhancing AMPA receptor desensitization. Consistent with this suggestion, extracellular acidification, at a level observed during

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** Wheat germ agglutinin (WGA)- and aniracetam-induced inhibition of desensitization were little effected by a change in pH. **A:** glutamate-evoked currents in solutions of different values of pH from a control neuron (top) and from a neuron pretreated with WGA (300 μg/ml) (bottom). Note the reduction of desensitization but lack of change in the pH sensitivity of these currents. **B:** fitting of $I_\text{ss}$ vs. pH curve by Hill equation. The curve was not altered by WGA ($n = 9$ for control neurons, $n = 10$ for neurons pretreated with WGA; $P > 0.05$). **C** and **D:** responses to glutamate in a single neuron before and after treatment with aniracetam (5 mM). No considerable change in the sensitivity of pH was observed.
ischemia, reduces glutamate-mediated neuronal death (Giffard et al. 1990; Kaku et al. 1993).

We thank Drs. D. Bai and M. Jackson for help with outside-out nucleated patch recordings and E. Cerwinska and L. Brandes for technical assistance. This work was supported by the Canadian Institutes of Health Research and the Ontario Neurotrauma Foundation.

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