Characterization of L-Homocysteate–Induced Currents in Purkinje Cells From Wild-Type and NMDA Receptor Knockout Mice

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Yuzaki, Michisuke and John A. Connor. Characterization of L-homocysteate–induced currents in Purkinje cells from wild-type and NMDA receptor knockout mice. J. Neurophysiol. 82: 2820–2826, 1999. L-Homocysteic acid (HCA), an endogenous excitatory amino acid in the mammalian CNS, potently activates N-methyl-D-aspartate (NMDA) receptors in hippocampal neurons. However, the responses to HCA in Purkinje cells, which lack functional NMDA receptors, have been largely unexplored: HCA may activate conventional non-NMDA receptors by its mixed agonistic action on both NMDA and non-NMDA receptors, or it may activate a novel non-NMDA receptor that has high affinity for HCA. To test these possibilities, we compared the responses to HCA in cultured Purkinje cells with those in hippocampal neurons by using the whole cell patch-clamp technique. To clearly isolate HCA responses mediated by non-NMDA receptors, we complemented pharmacological methods by using neurons from mutant mice (NR1−/−) that lack functional NMDA receptors. A moderate dose of HCA (100 μM) induced substantial responses in Purkinje cells. These responses were blocked by non-NMDA receptor antagonists but were insensitive to NMDA receptor antagonists. HCA also activated responses mediated by non-NMDA receptors in both wild-type and NR1−/− hippocampal neurons. HCA responses in Purkinje cells had a pharmacological profile (EC50 and Hill coefficient) very similar to that of non-NMDA receptor components of HCA responses in hippocampal neurons. Moreover, the amplitude of the non-NMDA receptor component of HCA responses was directly correlated with that of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)– and kainate-induced responses in both types of neurons. Finally, in both types of neurons, HCA currents mediated by non-NMDA receptors were potently blocked by the AMPA receptor antagonist GYKI52466. These findings indicate that HCA-activated AMPA receptors in Purkinje cells are similar to those in hippocampal neurons and that there is no distinct HCA-prefering receptor in Purkinje cells. We also found that in hippocampal neurons, the EC50 of HCA for non-NMDA receptors and for NMDA receptors were more similar than originally reported; this finding indicates that HCA is similar to other mixed agonists, such as glutamate. HCA responses may appear to be selective at NMDA receptors in cells that express these receptors, such as hippocampal neurons; in cells that express few functional NMDA receptors, such as Purkinje cells, HCA may appear to be selective at non-NMDA receptors.

INTRODUCTION

L-homocysteic acid (HCA), a sulfur-containing analogue of L-glutamate, fulfills several criteria for an endogenous neurotransmitter. It is released from rat brain slices either by depolarization in a Ca2+-dependent manner (Do et al. 1986) or by electrical stimulation (Klančnik et al. 1992). It is also taken up by a specific low-affinity uptake system (Cox et al. 1977; Ito et al. 1991). Because HCA responses are potently blocked by N-methyl-D-aspartate (NMDA) receptor antagonists such as D,L-2-amino-5-phosphonovalerate (APV) but are little affected by non-NMDA receptor antagonists such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX), HCA has been considered to act predominantly via NMDA receptors in neurons from various regions, including neocortex (Knopfel et al. 1987), striatum (Do et al. 1986), hippocampus (Ito et al. 1991; Patneau and Mayer 1990; Provini et al. 1991), olfactory cortex (Collins and Brown 1986), deep cerebellar nuclei (Audinat et al. 1990), and spinal cord (Mayer and Westbrook 1984).

In the cerebellum, HCA is thought to be involved in climbing fiber–Purkinje cell synapses, because the elimination of climbing fibers led to decreased release of HCA in cerebellar slices from adult rats (Vollenweider et al. 1990). Yet it has not been clear what receptors are activated by HCA in Purkinje cells. If HCA acts predominantly on NMDA receptors, as it is thought to act in other neurons, it should not activate significant currents in Purkinje cells, which have few functional NMDA receptors (Audinat et al. 1990; Kimura et al. 1985; Yuzaki et al. 1996b). However, the fact that HCA induces substantial currents that are insensitive to APV but sensitive to CNQX in Purkinje cells (Audinat et al. 1990; Lee et al. 1988) indicates that HCA activates non-NMDA receptors. Thus a unique subtype of glutamate receptor may exist in Purkinje cells (Vollenweider et al. 1990). Alternatively, because at high concentrations HCA substantially activates non-NMDA receptors in hippocampal neurons (Patneau and Mayer 1990), the apparent activation of non-NMDA receptors in Purkinje cells may reflect the mixed action of HCA on both receptor types.

A comparison of non-NMDA receptor activation by HCA in the two types of neurons would differentiate between these possibilities. If the HCA responses in hippocampal neurons had no non-NMDA receptor component, or if the pharmacological properties of this component, such as dose-response relationship and antagonist profiles, were different from those in Purkinje cells, then these findings would indicate that Purkinje cells contain a specific receptor for HCA. However, there have been several technical obstacles to the pharmacological characterization of endogenous excitatory amino acid (EAA) candidates such as HCA. First, the action of endogenous EAA often has a mixed action involving both NMDA and non-NMDA receptors. Second, glutamate receptor antagonists are not always selective. For example, when mixed agonists are applied in the presence of NMDA receptor antagonists to...
identify the components mediated by non-NMDA receptors, NMDA receptor current may break through at higher concentrations of agonists.

Moreover, receptor classification based on pharmacological agonists and antagonists is not infallible. For example, we previously demonstrated that NMDA receptor antagonists, such as APV, show antagonistic effects on aspartate responses in Purkinje cells from mice with disrupted NMDAR1 genes (NR1\(^{-/-}\) mice) (Yuzaki et al. 1996a). However, because Purkinje cells express little NR2 protein (Monyer et al. 1994), NR1\(^{-/-}\) Purkinje cells contain no proteins related to conventional NMDA receptors. Thus those “NMDA receptor-specific” antagonists may bind to proteins other than NMDA receptors in Purkinje cells. Such potential pitfalls limit the conclusiveness of studies that rely only on current pharmacological antagonists. To circumvent these problems, we used neurons from NR1\(^{-/-}\) mice to complement conventional pharmacological antagonists in characterizing the HCA responses in Purkinje cells.

**METHODS**

**Culture preparation**

Primary cultures were prepared from neonatal mice within 3–4 h of birth as previously described (Forrest et al. 1994; Yuzaki and Mikoshiba 1992). Cultures were analyzed after 8–15 d in vitro. For unambiguous identification of Purkinje cells in immature cultures, we routinely performed calbindinD28k staining as previously reported (Yuzaki et al. 1996b). NR1 geotype was determined by polymerase chain reaction studies of DNA samples prepared from mouse tail clips (Yuzaki et al. 1996b).

**Recording system**

Membrane currents were measured by using standard whole cell patch-clamp methods with a List EPC-7 amplifier (Medical Systems, Greenvale, NY) as previously reported (Yuzaki et al. 1996a,b). Saline solution in the electrodes was composed of (in mM) 130 CsMeSO\(_3\), 10 CsCl, 2 MgCl\(_2\), 0.1 CaCl\(_2\), 5.5 EGTA, 10 HEPES, and 2 Na\(_2\)-ATP (pH was adjusted to 7.3 with CsOH). Dextran-conjugated rhodamine (0.625 mg/ml; Molecular Probes, Eugene, OR) was added. Electrodes filled with the recording saline had resistance of ~6–7 M\(\Omega\). Series resistance was 11–13 M\(\Omega\) and was partially (50–60%) compensated. The bath solution contained (in mM) 150 NaCl, 4.5 KCl, 2 CaCl\(_2\), 10 HEPES, and 20 glucose (pH 7.3). Mg\(^{2+}\) was excluded from the external solution throughout the experiments unless otherwise stated. Tetrodotoxin (TTX, 1 \(\mu\)M) and picROTOxin (100 \(\mu\)M) were added to the solution to block spontaneous electrical activity and glycine or \(\gamma\)-aminobutyric acid (GABA) channels. All drugs were dissolved in the recording solution. The chamber was continuously perfused (1–2 ml/min) at room temperature. Drugs were applied by the “Y-tube” method, which had a time constant of 8–9 ms for solution exchange at the tip of the electrode and 15–20 ms in the area surrounding the neuron (Yuzaki et al. 1996b). Currents were filtered at 1 kHz and digitized at 3 kHz. Membrane potential was corrected for the liquid junction potential.

**Drugs**

NMDA, kainate, HCA, glycine, TTX, and picROTOxin were obtained from Sigma (St. Louis, MO); 7-Ch-kyrenenate (7-Ch-Kyn), AMPA, and 1-(4-aminophenyl)-4-methyl-7,8-methyleneoxy-5H-2,3-benzodizepine HCl (GYK152466) were obtained from Research Biochemicals (Natick, MA); and 3-(DL)-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP) and NBQX were obtained from Tocris Cookson (Ballwin, MO).

**RESULTS**

**HCA responses in neurons from wild-type and NR1-knockout mice**

First, responses to HCA (100 \(\mu\)M) were pharmacologically characterized in wild-type neurons. In voltage-clamped (–60 mV) wild-type Purkinje cells, HCA induced large currents (1.5 ± 0.2 nA, mean ± SE, \(n = 18\)) that were insensitive to an NMDA receptor-antagonist mixture containing CPP (50 \(\mu\)M), 7-Ch-Kyn (0.5 \(\mu\)M), and Mg\(^{2+}\) (1 mM; Fig. 1A, top trace). These currents were not blocked by Mg\(^{2+}\) at all holding potentials (Fig. 1B), but they were completely blocked by the non-NMDA receptor antagonist NBQX (5 \(\mu\)M; Fig. 1A). Thus HCA activated non-NMDA receptors in wild-type Purkinje cells.

Earlier pharmacological studies of hippocampal neurons showed that HCA activated non-NMDA receptors with an \(EC_{50}\) of 477 and a Hill coefficient of 1.4 (Patneau and Mayer 1990), indicating that 100 \(\mu\)M HCA would activate only 10% of maximal currents. If these results were applicable to Purkinje cells, maximal currents would be expected to reach 15 nA, whereas observed maximal steady-state non-NMDA receptor currents were ~3 nA (e.g., Fig. 1B). These results suggest that the pharmacology of HCA responses in Purkinje cells may differ from the reported responses in hippocampal neurons.

In contrast, HCA-induced currents in wild-type hippocampal neurons were substantially reduced by the NMDA receptor-antagonist mixture (14 ± 3% of currents in controls; \(n = 26\)) but were relatively insensitive to NBQX (89 ± 4% of currents in controls, \(n = 26\); Fig. 1A, bottom trace). The HCA-induced current was reduced by Mg\(^{2+}\) in a voltage-dependent manner (Fig. 1B), as with classical NMDA receptors. These results are consistent with earlier reports that HCA, at low doses, acts as a selective NMDA receptor agonist in hippocampal neurons (Patneau and Mayer 1990). However, it should be noted that the fact that residual HCA-induced currents in the presence of NMDA receptor antagonists were blocked by the addition of NBQX (Fig. 1C) suggests that non-NMDA receptors were also activated by 100 \(\mu\)M HCA.

To confirm these results, we used neurons from NR1\(^{-/-}\) mice. As with wild-type mice, HCA induced large currents (1.8 ± 0.2 nA, \(n = 15\)) that were insensitive to the NMDA receptor-antagonist mixture in Purkinje cells (Fig. 1C, top trace). These currents were not blocked by Mg\(^{2+}\) at all holding potentials (Fig. 1D), but they were completely blocked by the non-NMDA receptor antagonist NBQX (5 \(\mu\)M; Fig. 1C). Similarly, HCA activated substantial currents in NR1\(^{-/-}\) hippocampal neurons that showed no response to 100 \(\mu\)M NMDA (Fig. 1C, bottom trace). The currents were not blocked by Mg\(^{2+}\) at all holding potentials (Fig. 1B) but were completely blocked by NBQX (Fig. 1A). Thus 100 \(\mu\)M HCA appears to activate non-NMDA receptors in both hippocampal neurons and Purkinje cells from NR1\(^{-/-}\) mice. These results were comparable with those obtained in wild-type neurons in the presence of NMDA receptor antagonists, indicating the validity of pharmacological isolation of non-NMDA receptor components in wild-type neurons. Moreover, the suggestion that a subtype of the classic NMDA receptor is involved in HCA responses in Purkinje cells (Vollenweider et al. 1990) is un-
likely to be valid, because HCA induced similar responses in neurons lacking functional NMDA receptors.

Non-NMDA receptor activation in Purkinje cells may result from the mixed action of HCA on non-NMDA receptors. In wild-type hippocampal neurons, the potent activation of NMDA receptors, which are abundantly expressed, can mask the minor activation of non-NMDA receptors by 100 μM HCA. In contrast, HCA may induce large currents in Purkinje cells that express abundant non-NMDA receptors but have no functional NMDA receptors. It is also possible that Purkinje cells express distinct non-NMDA receptors that have higher affinity for HCA than do those in hippocampal neurons. To test these two possibilities, we next compared the pharmacological properties of HCA responses in Purkinje cells with those in hippocampal neurons.

**Dose-response relationship of non-NMDA receptor component of HCA response**

First, we analyzed the concentration-response curves of HCA-activated responses mediated by non-NMDA receptors (Fig. 2). In wild-type neurons, HCA was applied in the presence of the NMDA receptor antagonist cocktail to block NMDA receptor–mediated responses. Measurement of the peak current response at non-NMDA receptors was complicated by the extremely rapid onset of desensitization, which can have a time constant of <10 ms (Brorson et al. 1995; Patneau and Mayer 1990) (greater than the speed of our drug application) in whole cell recordings. Moreover, in large cells such as Purkinje cells, the large electrotonic distance of den-
drites can potentially distort the rapid phase of desensitization. Furthermore, the EC\textsubscript{50} values obtained by analyzing steady-state currents, but not the rapid inactivating phase, have been shown to reflect the ligand affinity obtained from binding assays (Patneau and Mayer 1990) and biological assays (Brorson et al. 1995). Therefore we analyzed the steady-state current to compare the receptors activated by HCA in the two types of neurons.

The amplitudes of steady-state currents were well fit by the logistic equation for wild-type and NR1\textsuperscript{2/2} hippocampal neurons: wild-type neurons had an EC\textsubscript{50} of 106 ± 12 μM (mean ± SE, n = 6) and a Hill coefficient of 1.8 ± 0.2, whereas NR1\textsuperscript{2/2} hippocampal neurons had an EC\textsubscript{50} of 98 ± 11 μM (n = 7) and a Hill coefficient of 1.6 ± 0.1 (Fig. 2A). The logistic equation also showed a similar dose-response relationship for Purkinje cells from wild-type and NR1\textsuperscript{2/2} mice: wild-type cells had an EC\textsubscript{50} of 96 ± 9 μM (n = 7) and a Hill coefficient of 1.6 ± 0.1, whereas cells from NR1\textsuperscript{2/2} mice had an EC\textsubscript{50} of 88 ± 10 μM (n = 9) and a Hill coefficient of 1.4 ± 0.1 (Fig. 2B). Conversely, HCA activated NMDA receptors with an EC\textsubscript{50} of 14 ± 4 μM (n = 5) and a Hill coefficient of 1.4 ± 0.3, consistent with the previously reported EC\textsubscript{50} of 12.9 μM (Patneau and Mayer 1990). Thus non-NMDA receptors in hippocampal and Purkinje neurons (both NR1\textsuperscript{2/2} and wild-type) have very similar EC\textsubscript{50}s for HCA. These results support the hypothesis that HCA is a mixed agonist that activates conventional non-NMDA receptors in both Purkinje cells and hippocampal neurons.

It is unlikely that the EC\textsubscript{50} value we obtained for HCA at AMPA receptors, which was lower than previously reported values (Patneau and Mayer 1990), was caused by the voltage drop across the series resistance. If we had underestimated EC\textsubscript{50} because of the voltage-clamp error, we would have obtained much lower values of EC\textsubscript{50} for HCA at NMDA receptors, because the larger HCA-induced NMDA receptor currents would have caused larger voltage errors. However, the EC\textsubscript{50} of HCA at NMDA receptors in our study was very similar to the reported value.

Although the differences were not statistically significant, the EC\textsubscript{50}s for HCA at AMPA receptors tended to be larger in wild-type neurons than in NR1\textsuperscript{2/2} neurons. Similarly, they were larger in hippocampal neurons than in Purkinje cells. This finding suggests that EC\textsubscript{50}s may increase in the presence of functional NMDA receptors. The possibility that, at higher concentrations of HCA, NMDA receptors may be activated even in the presence of blockers and thereby increase the apparent EC\textsubscript{50}s is ruled out by the use of NR1\textsuperscript{2/2} neurons.

**Relationship between HCA current and AMPA or kainate current**

If HCA is a mixed agonist that activates similar non-NMDA receptors in both Purkinje cells and hippocampal neurons, cells that show large HCA responses mediated by non-NMDA receptors should have abundant non-NMDA receptors. We compared non-NMDA receptor currents induced by AMPA (20 μM), kainate (10 μM), and HCA (100 μM). The amplitude of HCA currents in all types of neurons was strongly correlated
with that of AMPA currents (Fig. 3A) and kainate currents (Fig. 3B). In contrast, NMDA receptor currents induced by 100 μM NMDA were not correlated with HCA currents mediated by non-NMDA receptors (Fig. 3C). These results are consistent with the hypothesis that 100 μM HCA potently activates conventional non-NMDA receptors in both types of neurons.

Interestingly, non-NMDA currents in NR1<sup>−/−</sup> hippocampal neurons were larger than those in wild-type counterparts (<i>P</i> < 0.01, Fig. 3D). This difference may be caused by the up-regulation of non-NMDA receptors in the absence of functional NMDA receptors. To test this possibility, we treated cultures of wild-type hippocampal neurons with the NMDA receptor antagonist cocktail from day 0 to day 14 in vitro and compared AMPA-induced currents with those in untreated sister cultures. Unlike NR1<sup>−/−</sup> neurons, neurons treated with NMDA receptor antagonists displayed smaller responses to 20 μM AMPA (892 ± 107 pA, <i>n</i> = 25) than did control neurons (1,356 ± 185 pA, <i>n</i> = 17). Moreover, if the loss of functional NMDA receptors were involved in the up-regulation of functional non-NMDA receptors, the amplitude of non-NMDA receptor currents would be inversely correlated with that of NMDA receptor currents. However, there was no correlation between the amplitudes of NMDA and non-NMDA currents (Fig. 3C). Thus the larger AMPA current in NR1<sup>−/−</sup> hippocampal neurons may reflect other factors. The presence of NR1 and some NR2 mRNAs as early as embryonic day 13 suggests that regulation of non-NMDA receptors may be determined by the functions of NMDA receptors at earlier stages in neurodevelopment. It is also possible that the increase in non-NMDA currents may require complete loss of NMDA receptor function, a condition that may not be achieved by pharmacological blockade of NMDA receptors.

**Sensitivity of HCA currents to GYKI52466**

To further confirm the hypothesis that HCA activates conventional non-NMDA receptors, and to test which non-NMDA receptors are activated by HCA, we analyzed the dose-inhibition relationship of GYKI52466, a potent and selective antagonist for AMPA receptors, in HCA responses in NR1<sup>−/−</sup> hippocampal and Purkinje cells. In hippocampal cells, the IC<sub>50</sub> was 561 μM (<i>n</i> = 7), and the Hill coefficient was 1.0 ± 0.1; in Purkinje cells, these values were 6 ± 2 μM (<i>n</i> = 6) and 1.1 ± 0.2 (Fig. 1E). GYKI52466 is reported to inhibit AMPA receptors in wild-type hippocampal and Purkinje cells with IC<sub>50</sub>s between 7.5 and 10 μM, whereas it inhibits kainate receptors in Purkinje cells with an IC<sub>50</sub> of 105 μM (Herrling et al. 1989; Provini et al. 1991; Renard et al. 1995). Thus our findings indicate that HCA activated the AMPA subtype of non-NMDA receptors in both hippocampal and Purkinje cells.

**DISCUSSION**

Although it has been suggested that HCA is involved in climbing-fiber–Purkinje cell synapses, the receptors activated by HCA in Purkinje cells have not been well characterized. In this study, by taking advantage of NR1<sup>−/−</sup> neurons, we have unambiguously demonstrated that HCA activates AMPA receptors in Purkinje cells by its mixed action on both NMDA and non-NMDA receptors. First, non-NMDA receptors activated by HCA in Purkinje cells had pharmacological profiles (EC<sub>50</sub> and Hill coefficient) very similar to those of non-NMDA receptors activated by HCA in hippocampal neurons (Fig. 2). Second, the amplitude of the non-NMDA receptor component of HCA responses was directly correlated with that of AMPA-
and kainate-induced responses in both types of neurons (Fig. 3). Finally, in both types of neurons, HCA currents mediated by non-NMDA receptors were potentely blocked by the AMPA receptor antagonist GYKI52466, with similar IC_{50}s (Fig. 1E).

Thus unlike aspartate, which activates receptors that are distinct from both NMDA and non-NMDA receptors, HCA has no distinct preferential receptor in Purkinje cells. In addition, HCA does not activate the aspartate receptors (Yuzaki et al. 1996a).

We have demonstrated that 100 μM HCA potently activates non-NMDA receptors in Purkinje cells, whereas it appears to selectively activate NMDA receptors in wild-type hippocampal neurons. This apparent discrepancy can be explained by the mixed action of HCA on both NMDA and non-NMDA receptors. The ratio of the EC_{50} for equilibrium responses at non-NMDA receptors to the EC_{50} for equilibrium responses at NMDA receptors reflects the degree of selectivity of the agonist at NMDA receptors. L-glutamate, for example, which has mixed action on NMDA and non-NMDA receptors, has a ratio of 8.2 (Patneau and Mayer 1990). Because the EC_{50} of HCA was 12.9 μM for NMDA receptors and 477 μM for AMPA receptors, yielding a ratio of 36.9, HCA was reported to be a selective agonist at NMDA receptors (Patneau and Mayer 1990). However, we obtained a ratio of 7.5 in wild-type hippocampal neurons, in which the EC_{50} of HCA was 14 μM for NMDA receptors and 106 μM for AMPA receptors. Therefore we propose that HCA is similar to other mixed agonists, such as glutamate, and that it has only modest selectivity at NMDA receptors. Because the maximum amplitude of equilibrium responses to non-NMDA receptor agonists is usually <10% of the maximal response that can be evoked by saturating doses of NMDA agonists (Patneau and Mayer 1990), HCA responses may appear to be selective at NMDA receptors in cells that express these receptors, such as hippocampal neurons. In cells that express few functional NMDA receptors, such as Purkinje cells and NR1^+/− hippocampal neurons, HCA may appear to be selective at non-NMDA receptors.

Given that HCA is a mixed agonist that has selectivity very similar to that of glutamate at NMDA and non-NMDA receptors, what could be the functional role of this endogenous amino acid? Several important differences exist between glutamate and HCA. HCA is much less effective than glutamate in activating metabotropic glutamate receptors (Poirier and Roberts 1993). HCA is taken up, at least partially, by a specific uptake system that does not involve known glutamate transporters (Ito et al. 1991). In addition, unlike glutamate, which is released from both nerve terminals and surrounding glia, HCA is released only from glia (Grandes et al. 1991). Recently, glutamate released from glia has been shown to modulate synaptic neurotransmission (Araque et al. 1999). Thus HCA released from surrounding glia may also modify basic neurotransmission, making further characterization of its release and actions on neurons important in understanding integrative functions.

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