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L-Proline Activates Glutamate and Glycine Receptors in Cultured Rat Dorsal Horn Neurons

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SUMMARY

The pharmacological actions of L-proline on excitatory and inhibitory amino acid receptors have been characterized under voltage-clamp conditions, using cultured dissociated neurons from the dorsal horn of the rat spinal cord. At a holding potential of −62 mV, millimolar concentrations of L-proline elicited an inward current that was partially antagonized by D-(−)-2-amino-5-phosphonopentanoic acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and strychnine and was virtually abolished (97% block) by a combination of all three antagonists. Currents evoked by D-proline were abolished by strychnine alone. APV-, CNQX-, and strychnine-sensitive components of L-proline-evoked currents were isolated using various combinations of the three antagonists. These currents were identical to currents elicited by *N*-methyl-D-aspartate (NMDA), kainate, and glycine, respectively, with respect to antagonist specificity, reversal potential, and ionic permeability. The APV- and strychnine-sensitive currents also showed a time dependence similar to that of the currents elicited by NMDA and glycine. EC_{50} values could not be calculated, because the response did not saturate within the tested range of L-proline concentrations (0.3–50 mM). Estimates of relative potency were obtained, however, by comparison with responses elicited by selective agonists. The APV-sensitive, CNQX-sensitive, and strychnine-sensitive currents evoked by 10 mM L-proline were comparable in size to currents elicited by 15 μM NMDA, 5 μM kainate, and 30 μM glycine, respectively. L-Proline was found to elicit an increase in intracellular $[Ca^{2+}]$ that was dependent upon Ca^{2+} entry into the cell. These Ca^{2+} responses were enhanced by strychnine and partially antagonized by APV, CNQX, or Mg^{2+}. Our results using dorsal horn neurons grown in culture indicate that L-proline is a weak agonist at strychnine-sensitive glycine receptors and at both NMDA and non-NMDA glutamate receptors. These observations should help in interpreting the confusing array of L-proline actions that have been described using more intact nervous system preparations. Furthermore, the ability of L-proline to stimulate Ca^{2+} entry after activation of excitatory amino acid receptors implicates L-proline as a potential endogenous excitotoxin.

L-Proline has both excitatory and inhibitory effects when applied to different areas of the mammalian central nervous system. Such contradictory actions suggest either that L-proline produces complex actions through indirect effects mediated by neuronal circuitry or that it can act on both excitatory and inhibitory receptors directly. Because the pharmacological characteristics of L-proline have not been precisely characterized, this issue is unresolved. The usefulness of a more detailed analysis of the neuropharmacology of L-proline is suggested by studies implicating L-proline in neurotransmission. These include the demonstration of Na^+-dependent, high affinity uptake (1–3) and Ca^{2+}-dependent, K^+-evoked release (1, 4, 5) of L-proline in brain slices and synaptosomes. The suggestion that L-proline may function as a neurotransmitter has been largely ignored, however, due to a failure to associate L-proline with specific or unique nerve pathways, a poor understanding of its receptor selectivity, and its apparent low potency as an agonist.

Inhibitory effects of L-proline have been observed in several areas of the mammalian nervous system. Curtis and Johnston (6) observed that iontophoretically applied L-proline inhibits the spontaneous firing of cat spinal interneurons and that this effect is antagonized by the selective glycine receptor antagonist strychnine. However, excitation, not inhibition, was observed by other investigators using a similar experimental preparation (7), implying that the inhibitory action of L-proline may have been indirect. Nevertheless, strychnine partially antagonizes L-proline-evoked hyperpolarizations of frog ventral roots, although only at lower doses of L-proline (8). Finally, strychnine-insensitive inhibition by L-proline of spontaneous firing has been observed in cat cerebellum (9). Taken together, these results imply that some of the inhibitory actions of L-proline may be mediated by a direct agonist action at glycine receptors.

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; APV, D-(−)-2-amino-5-phosphonopentanoic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum essential medium.

This suggestion is supported by the ability of L-proline to displace strychnine bound to rat spinal cord synaptic membranes (10).

The excitatory action of L-proline is mediated at least in part by activation of NMDA receptors. NMDA receptor antagonists partially block L-proline-elicited depolarizations of rat ventral roots (11) and hippocampal pyramidal cells (12) and displace L-proline binding to rat brain synaptosomal membranes (13). Furthermore, glutamate receptor antagonists block the neurotoxic effects of L-proline injections into rat hippocampus (14). On the other hand, observations in rat hippocampus (15, 16), cat cerebral cortex and spinal cord (7), and chick retina (17) suggest that L-proline may function also as an antagonist of the effects of glutamate. It has, therefore, remained unclear whether L-proline is an agonist or partial agonist at these receptors. In addition, L-proline has a depolarizing action, which is resistant to NMDA receptor antagonists, that has not been fully characterized (11, 12).

The potency of the action of L-proline at excitatory and inhibitory amino acid receptors is not known. It has been suggested that the high concentrations of L-proline required to evoke depolarization in brain slices may be due to the presence of high affinity uptake systems for L-proline in the brain (3). By analogy with glutamate, a large excess of agonist applied to brain slices would be required to ensure that sufficient agonist gets past the uptake systems to activate the receptors. Previous assessments of L-proline potency have been further complicated by the possible multiple sites of action of L-proline. The potential effects of uptake can be overcome by recording from cells in tissue culture, where effective control of applied agonist concentration is most feasible. Therefore, using embryonic rat spinal cord neurons dissociated and grown in culture, we have defined the receptor subtypes mediating the actions of L-proline in neurons and directly assessed L-proline potency at these receptors. To investigate a possible role of Ca^{2+} entry in L-proline-mediated neurotoxicity, we measured $[Ca^{2+}]_i$ in unclamped neurons, by using indo-1. Preliminary results have been described in abstract form (18, 19).

Materials and Methods

Preparation of dorsal horn neurons. Pregnant Sprague-Dawley rats were decapitated at 14–16 days of gestation. The dorsal one third of the spinal cord was isolated from several embryos and was incubated for 20 min at 37° in MEM modified for suspension culture (GIBCO), containing 0.25% trypsin (GIBCO). After gentle trituration with a fire-polished Pasteur pipette, the cells were resuspended and plated (approximately 250,000 cells/dish) onto glass coverslips bearing a confluent layer of rat cortical astrocytes. The culture medium consisted of MEM (GIBCO) supplemented with 5% heat-inactivated horse serum (GIBCO), 1% MEM vitamins (GIBCO), and 8 mg/ml glucose. Cultures were maintained in a 5% CO_2 moist-air incubator and were fed weekly by replacing one half of the medium volume with fresh medium. Cells were used for experiments after 10–25 days in culture.

Electrophysiology. Coverslips were secured in a 0.5-ml Lucite chamber, which was continuously perfused (approximately 5 ml/min) at room temperature with a recording solution containing (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, and 10 HEPES, with 0.1% glucose and 0.5 μM tetrodotoxin (Sigma). An alternative recording solution, in which NaCl was replaced with sodium gluconate, was used for some experiments, as indicated in Results. To attenuate K^+ conductances, 5 mM $BaCl_2$ and 2 mM 4-aminopyridine were added to the recording solutions when current-voltage relationships were to be determined. These solutions were adjusted to a pH of 7.3 with NaOH and to 330 mOsM with sucrose. Patch electrodes (4–8 MΩ) were filled with a solution containing (in mM) 130 cesium gluconate, 10 KCl, 5 HEPES, 5 EGTA, 2 $MgCl_2$, and 0.5 $CaCl_2$, adjusted to a pH of 7.1 with KOH and to 315 mOsM with sucrose. Whole-cell patch recordings were conducted using an Axoclamp 2A amplifier (Axon Instruments), in discontinuous voltage-clamp mode. Membrane potentials were adjusted for a junction potential of −12 mV that occurred between the cesium gluconate-based electrode solution and the NaCl-based recording solution. Drug-evoked currents were measured at a holding potential of −62 mV.

Drugs and their application. Solutions containing L-proline (Sigma), D-proline (Sigma), kainate (Sigma), NMDA (Tocris Neuramin), glycine (Sigma), CNQX (Tocris Neuramin), APV (Cambridge Research and Tocris Neuramin), and strychnine (Sigma) were prepared using the standard recording solutions, and pH was adjusted as needed. At the highest doses of L-proline (20, 30, and 50 mM), no sucrose was added to the recording solutions that contained L-proline, in order to maintain a relatively uniform osmolarity with the drug-free recording solutions. At these L-proline concentrations, the osmolarities were 320, 330, and 350 mOsM, respectively. Unless otherwise indicated in Results, all drug-containing solutions also contained 5 μM glycine, in order to saturate the NMDA receptor glycine site. These solutions were delivered to a selected neuron by gravity feed through a Y tube that was positioned to deliver a broad and fast stream of solution (20). This allowed precise control of the drug concentrations and rapid exchange of the drug-containing and drug-free solutions. Drug applications lasted 4–5 sec. The L-proline and D-proline used in these experiments were independently analyzed with an Applied Biosystems model 420 amino acid analyzer. For every 56,000 parts L-proline, there was <1 part glutamate and 4 parts glycine or serine. For every 50,000 parts D-proline, there was <1 part glutamate, 4 parts glycine, and 25 parts serine. At the typical concentrations of proline used in these experiments (10 mM), these levels of contamination are insufficient to activate glutamate (21) or glycine (22) receptors.

Data acquisition and analysis. Membrane current and voltage were continuously monitored with a Gould chart recorder. For purposes of analysis, data were sampled (125 Hz) using pCLAMP software (Axon Instruments) and a 386-based computer. Current-voltage relationships were determined with repeated computer-driven 50–100-msec voltage steps during continuous (20–30 sec) drug applications, after the drug-evoked currents had achieved a steady state. Leak currents were measured by repeating the same series of voltage steps immediately before or after exposure to drug. The drug-activated currents were calculated as the difference between these two measures.

The amplitudes of agonist-activated currents were quantified by measuring the response at a fixed time point 3 sec after drug onset. Data obtained in this way did not differ qualitatively from measurements of the peak current response. This method was chosen, however, because the peak current, unlike the current measured at 3 sec, was highly sensitive to variability in the rate of drug onset (50–150 msec). Dose-response curves were constructed by normalizing the measured currents to the highest drug dose used (50 mM) and fitting the data with a form of the Michaelis-Menten equation, as described previously (23). Individual data points that reflected occasional fast electrical artifacts generated by our drug application circuitry were deleted from the displayed current traces.

Measurement of $[Ca^{2+}]_i$. Cultured dorsal horn neurons were loaded with the calcium indicator dye indo-1 (Molecular Probes), by a 30-min exposure to a 10 μM concentration of the acetoxymethyl ester form of the dye in the standard recording solution with 0.025% pluronic acid. To allow for complete deesterification of the dye, experimental measurements were not initiated for at least 1 hr after the loading period. Measurements of $[Ca^{2+}]_i$ were performed using the same recording chamber, recording solution, and drug application system as described above for electrophysiological experiments. Epifluorescent illumination with 350-nm light excited the dye, and emissions at 405 and 485 nm were continuously monitored by dual photomultiplier tubes that col-

lected light through a 20-μm-diameter circular aperture centered over a single neuronal cell body. The intensities of emitted light were digitized and stored at a rate of 40 Hz. We calculated $[Ca^{2+}]_i$ by using background-subtracted emitted intensities and the equation:

$$[Ca^{2+}]_i = K_d \times F_{485_{min}}/F_{485_{max}} \times (ratio_{405/485_{max}} - ratio_{405/485})/(ratio_{405/485} - ratio_{405/485_{min}})$$

in which K_d is the apparent dissociation constant of indo-1 for Ca^{2+}, measured in this system using a series of calibrated Ca^{2+}-buffered solutions containing 40 μM indo-1 free acid. In a few cases, at the end of an experiment, the neurons were permeabilized with the Ca^{2+} ionophore ionomycin (Sigma), and $F_{485_{min}}$ and $ratio_{405/485_{min}}$ were measured by exposing the cell to a solution containing 0 added Ca^{2+} and 10 mM EGTA. $F_{485_{max}}$ and $ratio_{405/485_{max}}$ were then measured by exposing the neurons to a saturating $[Ca^{2+}]$. Finally, addition of $MnCl_2$ quenched the fluorescence to background levels, indicating that very little dye was present as the non-Ca^{2+}-sensitive acetoxymethyl ester. Noise generated by the photomultiplier tubes has been reduced in the displayed traces of $[Ca^{2+}]_i$ by plotting the rolling average of each point ± two points.

Examination of antagonist specificity. L-Proline was coapplied with selective antagonists to different receptors to identify the receptor types activated by L-proline. In order to assess potency of L-proline at each receptor type activated under such conditions, the specificity and potency of the antagonists used must be well defined. We, therefore, evaluated the ability of APV, CNQX, and strychine to inhibit currents elicited by NMDA (50 μM), kainate (30 μM), and glycine (100 μM). Strychnine (5 μM) had no effect on currents elicited by kainate or NMDA ($n = 2$) but completely abolished glycine-evoked currents ($n = 4$). APV (30 μM) was also very specific, with no effect on glycine- and kainate-evoked currents ($n = 2$), whereas NMDA-evoked inward currents were completely abolished ($n = 3$) by APV in the absence of added glycine. However, CNQX was neither as potent nor as specific as the other antagonists. Coapplication of CNQX (10 μM) blocked 92.2 ± 2.1% (mean ± standard error; $n = 13$) of the kainate-evoked (30 μM) currents but also blocked 21.0 ± 3.4% ($n = 6$) of NMDA-evoked (50 μM) currents in the presence of 5 μM glycine. Noncompetitive antagonism of NMDA-evoked responses by CNQX has been observed previously (24) and is primarily due to competitive binding of CNQX to the strychnine-insensitive glycine binding site on the NMDA receptor (25). We were able to counteract this competition partially, by elevating glycine to 100 μM and adding strychnine (5 μM). This was possible because of the complete block of 100 μM glycine-evoked currents by strychnine. Under these conditions, CNQX (10 μM) antagonized only 7.9 ± 1.4% of NMDA-evoked currents, compared with 50 μM NMDA with 5 μM glycine ($n = 5$). Thus, by manipulating glycine concentrations in conjunction with the antagonists, we were able to isolate and preserve (with <10% error) currents mediated exclusively by each one of three classes of receptor.

Results

L-Proline- and D-proline-evoked currents. In an effort to clarify the mechanisms of action of L-proline, L-proline-evoked currents were recorded and dissected pharmacologically. In all cells tested ($n = 108$), millimolar concentrations of L-proline elicited inward currents at a holding potential of −62 mV (Fig. 1A, control). The amplitude of currents evoked by 10 mM L-proline from dorsal horn neurons in culture 10–25 days ranged from 100 to 600 pA. Although there was little variation between neurons of the same age, current amplitude tended to be greater in neurons cultured for longer periods. L-Proline-evoked currents were accompanied by a large increase in current noise and showed a variable amount of desensitization from cell to cell (approximately 15–30% after 4 sec).

The current response to L-proline is sensitive to antagonists

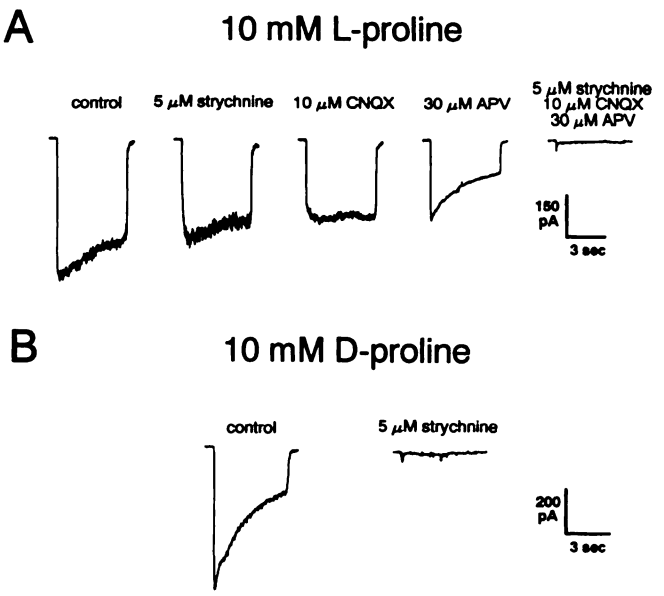

Fig. 1. Effects of antagonists on currents elicited by L- and D-proline. A, *Left trace*, inward current elicited by 10 mM L-proline with 5 μM added glycine, in a representative neuron, at a holding potential of −62 mV. Simultaneous application of 5 μM strychnine, 10 μM CNQX, or 30 μM APV partially antagonized the L-proline-evoked current (*middle traces*). Simultaneous application of all three antagonists virtually abolished the L-proline-evoked current (*right trace*). B, Application of 10 mM D-proline to a different neuron elicited an inward current that was abolished by simultaneous application of 5 μM strychnine. Strychnine also abolished the response to D-proline with 5 μM added glycine (data not shown).

against three different classes of receptor. In the example of Fig. 1A, the currents were partially antagonized by simultaneous application of L-proline with APV (30 μM), CNQX (10 μM), or strychnine (5 μM). The current remaining in the presence of each antagonist had a different decay time course and noise level during drug application, compared with the control L-proline current. Coapplication of all three antagonists virtually abolished (97.3 ± 0.3% block; $n = 10$) the L-proline-evoked current (Fig. 1A). These data suggest that L-proline simultaneously activates three classes of receptor on dorsal horn neurons, the NMDA and non-NMDA glutamate receptors and the strychnine-sensitive glycine receptor.

The stereospecificity of proline action was determined by testing the effects of D-proline on dorsal horn neurons. Millimolar concentrations of D-proline elicited desensitizing inward currents in each cell tested ($n = 11$; Fig. 1B). In contrast to L-proline-evoked currents, the response to D-proline was insensitive to APV and CNQX, whereas it was completely abolished by 5 μM strychnine alone with ($n = 3$) or without ($n = 3$) 5 μM added glycine (Fig. 1B). The amplitude of currents evoked by 10 mM D-proline without added glycine ranged from 50 to 250 pA. There was little increase in current noise associated with the D-proline-evoked current, and the current exhibited a greater degree of desensitization (approximately 45–75% after 4 sec) than did that evoked by L-proline. Thus, although both D- and L-proline can activate the strychnine-sensitive glycine receptor, only L-proline can additionally activate the NMDA and non-NMDA subtypes of glutamate receptors.

Isolation of three components of the L-proline-evoked current. We dissected the response to L-proline into APV-, CNQX-, and strychnine-sensitive components (Fig. 2A) by using various combinations of two of the antagonists and

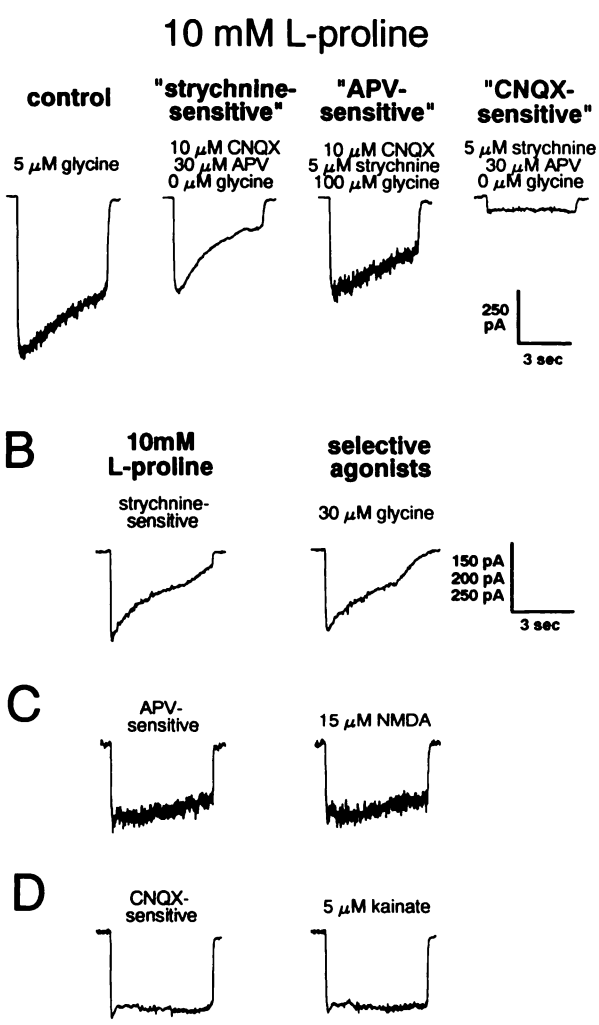

Fig. 2. Isolation of three components of L-proline-evoked currents and comparison with selective agonists. A, *Left trace*, control response to 10 mM L-proline with 5 μM glycine, at a holding potential of −62 mV. *Right traces*, In the same neuron, a "strychnine-sensitive" component of this current was isolated by simultaneous application of 10 μM CNQX and 30 μM APV with no added glycine. An "APV-sensitive" component was isolated by simultaneous application of 10 μM CNQX, 5 μM strychnine, and 100 μM glycine. A "CNQX-sensitive" component was isolated using 5 μM strychnine and 30 μM APV and no added glycine. B–D, *Left*, strychnine-, APV-, and CNQX-sensitive 10 mM L-proline-evoked currents, as in A. *Right*, currents evoked by 30 μM glycine, 15 μM NMDA, and 5 μM kainate. Each *row* illustrates data from a different neuron.

manipulating the concentration of glycine. An exclusively strychnine-sensitive L-proline-evoked current was isolated by coapplication of L-proline (10 mM), APV (30 μM), and CNQX (10 μM), with no added glycine. Compared with control responses to L-proline, the increase in current noise associated with the strychnine-sensitive response was markedly reduced. Furthermore, the current desensitized over the duration of the drug application (Fig. 2A), consistent with other observations of glycine-evoked currents in voltage-clamped neurons (26). Indeed, when the strychnine-sensitive L-proline response was compared with the response to 30 μM glycine on the same neuron, the currents were remarkably similar (Fig. 2B). This adds further support to the conclusion that L-proline activates the strychnine-sensitive glycine receptor and that the strychnine-sensitive component of the L-proline response is cleanly isolated by using 30 μM APV and 10 μM CNQX.

The APV-sensitive component of the L-proline-evoked current was isolated by simultaneous application of L-proline (10 mM), CNQX (10 μM), and strychnine (5 μM). Glycine (100 μM) was included to lessen the antagonistic effect of CNQX on NMDA receptor-mediated currents (see Materials and Methods). Under these conditions, currents evoked by L-proline desensitized and exhibited an increase in agonist-induced noise that was greater than that elicited by L-proline alone (Fig. 2A). Increases in current noise are characteristic of NMDA-evoked currents in these cells. In Fig. 2C, the APV-sensitive component of the L-proline response is compared with the response to 15 μM NMDA in the same cell and is found to be similar. These observations suggest that the responses to L-proline in the presence of 10 μM CNQX, 5 μM strychnine, and 100 μM glycine are predominantly due to direct NMDA receptor activation. Finally, to determine whether L-proline can function as an agonist of the strychnine-insensitive glycine site of the NMDA receptor, we compared the potency of L-proline versus NMDA in activating APV-sensitive currents, with and without added glycine. Without added glycine, the APV-sensitive current responses to both L-proline and NMDA were attenuated approximately equally ($n = 2$; data not shown), suggesting that 10 mM L-proline can serve as an agonist of the NMDA receptor but not of the glycine site of the NMDA receptor.

The remaining component of the response to L-proline is CNQX sensitive. It was isolated by coapplication of L-proline (10 mM), APV (30 μM), and strychnine (5 μM), with no added glycine. In contrast to the strychnine-sensitive, APV-sensitive, and control L-proline-evoked currents, the CNQX-sensitive current did not decay over time during sustained drug applications (Fig. 2A). Like the strychnine-sensitive current, there was little to no increase in agonist-induced noise, consistent with the interpretation that the noise increase, normally due to activation of NMDA receptors, is blocked under these conditions. This CNQX-sensitive response was compared with the response to 5 μM kainate and found to be similar (Fig. 2D). Thus, the CNQX-sensitive component of the L-proline response is also well isolated.

Our failure to observe desensitization may have resulted from an inability of our perfusion system to resolve fast desensitization or from the negligible current desensitization seen with low concentrations of low affinity agonists of non-NMDA receptors (21). To resolve this issue, we used L-cysteate, a low potency glutamate receptor agonist that is known to elicit CNQX-sensitive desensitizing currents (21). Concentrations of L-cysteate exceeding 5 mM evoked clearly desensitizing currents in the presence of 30 μM APV ($n = 5$). However, lower concentrations (~1 mM), which were chosen to evoke responses comparable in magnitude to 10 mM L-proline-evoked currents, exhibited barely detectable desensitization ($n = 5$).

We compared the relative size of the APV-, CNQX-, and strychnine-sensitive components of the currents evoked by L-proline in seven neurons. Using 10 mM L-proline with a holding potential of −62 mV, the APV-sensitive currents were 189 ± 40 pA, the CNQX-sensitive currents were 88 ± 17 pA, and the strychnine-sensitive currents were 117 ± 17 pA. In order to make a meaningful comparison of these values, however, each should be normalized by the reversal potential appropriate for the receptor/channel involved. These reversal potentials were determined as described below.

In some cases, the sum of the isolated components of the L-

proline response was greater than the response to L-proline alone. This is likely to be due to the imperfect voltage control inherently part of voltage-clamping of a cell with complex branching morphology. Receptors on both the soma and neurites are activated by our drug application system. NMDA and non-NMDA receptors are expressed at high levels on the neurites of dorsal horn neurons in culture (27), raising the possibility that, even with perfect voltage control of somal currents, currents generated at the neurites might induce a partially unclamped change in membrane potential. The greater the voltage error, the smaller the driving force on the currents and the smaller the currents. The current responses to L-proline in which neuritic voltage control is poor would be more depressed by incomplete neuritic voltage control than the responses to the smaller isolated components of the L-proline response, accounting for the larger amplitude of the summed isolated components.

Current-voltage relationships. The identities of the receptors activated by L-proline were further characterized by comparison of the predicted and experimental permeability characteristics of each component. The APV- and CNQX-sensitive components are expected to have linear current-voltage relationships, with reversal potentials near 0 mV, like the NMDA (in the absence of Mg^{2+}) and non-NMDA responses activated by selective agonists. To obtain these data for the L-proline response, we applied brief (50–100 msec) voltage steps from the holding potential of −62 mV to a wide range of voltages, before, during, and after sustained drug applications (see Materials and Methods). Current-voltage curves for the APV- and CNQX-sensitive L-proline-evoked responses are illustrated in Fig. 3. For comparison, this relationship was also determined for a kainate-evoked response in the same cell. For each curve in Fig. 3, the current amplitude was linearly related to the holding membrane potential. The reversal potential of the APV-sensitive current (3.5 ± 2.1 mV; $n = 4$) and the CNQX-sensitive current (4.0 ± 2.2 mV; $n = 5$) did not differ substantially from that of the kainate-evoked currents (2.1 ± 2.2 mV; $n = 7$). Therefore, like kainate-evoked currents, both APV- and CNQX-sensitive L-proline-evoked currents appear to be mediated by a cation conductance that is nonselective between Cs^+ and Na^+.

It was expected that the strychnine-sensitive component of the response to L-proline would be due to activation of the Cl^--permeable glycine receptor. Thus, the current-voltage relationships for glycine and strychnine-sensitive L-proline responses should be similar, and the reversal potentials for both responses should vary with changes in extracellular [Cl^-]. The strychnine-sensitive currents evoked by glycine (Fig. 4A) and by L-proline (Fig. 4B) exhibited marked outward rectification. Strychnine-sensitive currents evoked by L-proline reversed at −51.0 ± 2.3 mV ($n = 5$), whereas glycine-evoked currents reversed at −51.5 ± 2.5 mV ($n = 4$). Lowering the extracellular [Cl^-] in the recording solution by exchanging sodium gluconate for NaCl caused a positive shift in the reversal potential, of approximately 25–30 mV, for each agonist (Fig. 4). These results indicate the successful isolation of a strychnine-sensitive L-

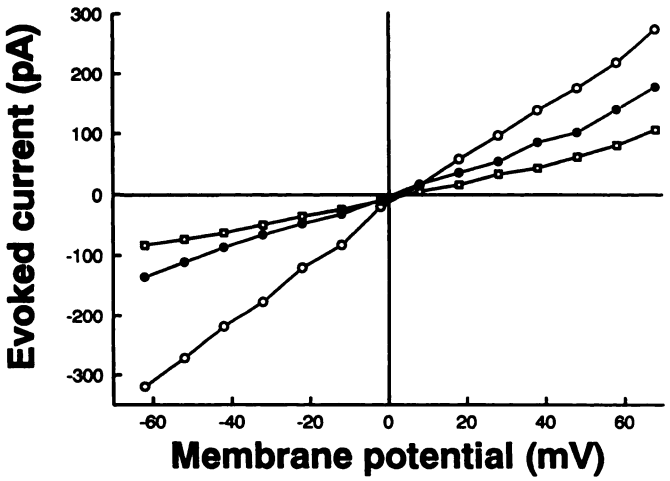

Fig. 3. Current-voltage relationship for APV- and CNQX-sensitive L-proline-evoked currents and kainate-evoked currents in the same neuron. ○, 15 μM kainate; ●, APV-sensitive L-proline-evoked current, with 10 mM L-proline, 10 μM CNQX, 5 μM strychnine, and 100 μM glycine; □, CNQX-sensitive current, with 10 mM L-proline, 5 μM strychnine, 30 μM APV, and no added glycine. Data were obtained as described in Materials and Methods.

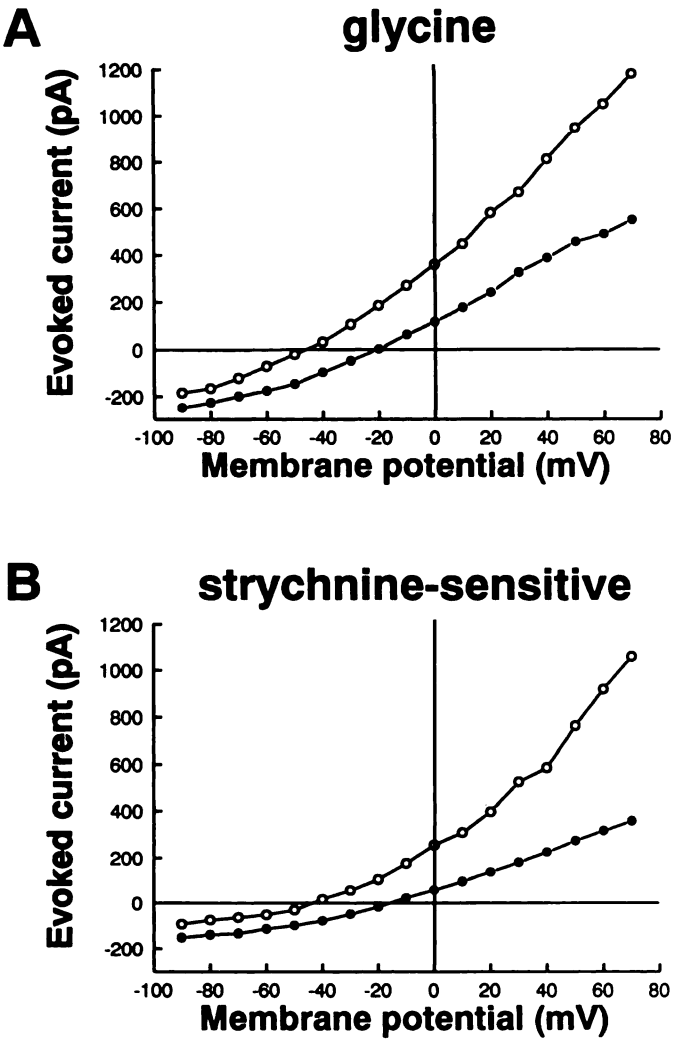

Fig. 4. Current-voltage relationship for strychnine-sensitive L-proline-evoked currents and glycine-evoked currents after manipulation of extracellular [Cl^-]. A, Currents evoked by 30 μM glycine in bath solution containing 150 mM NaCl (○) or 150 mM sodium gluconate (●). B, Currents evoked in the same cell by 10 mM L-proline, 10 μM CNQX, and 30 μM APV with no added glycine. ○, NaCl bath solution; ●, sodium gluconate bath solution. Data were obtained as described in Materials and Methods.

proline-evoked current that, like glycine-evoked currents, is mediated by an anionic conductance.

Agonist potency of L-proline. Another unresolved issue concerning L-proline action on neurons is its potency. It has been suggested that high concentrations of L-proline are required to activate neurons in brain slice preparations because high affinity proline uptake causes a decrease in the concentration of agonist reaching the receptors (3). This can be readily tested in tissue culture, where large volumes of drug can be continuously applied to a single cell growing on a monolayer of astrocytes. Under these conditions, agonist uptake cannot influence agonist concentration. Using the same pharmacological method for isolating currents, we attempted to determine the potency of L-proline at each of the three receptors it activates and to make comparisons with the potency of the selective agonists for those receptors.

We applied various doses of L-proline, 0.3, 1, 3, 5, 10, 20, 30, and 50 mM. Higher doses of L-proline were not used, because they would have significantly altered the osmolarity of the drug solution. Within this range of concentrations, the dose-response relations were virtually identical for the APV- ($n = 4$) and CNQX-sensitive ($n = 5$) L-proline-evoked currents (Fig. 5, A and B). In each case, the first detectable response was typically observed with 1 mM L-proline, and the current amplitude could be approximated by a sigmoidal function of L-proline concentration. In contrast, significant strychnine-sensitive L-proline-evoked currents ($n = 4$) were detected with 0.3 mM L-proline (Fig. 5C). The strychnine-sensitive current amplitudes could also be approximated by a sigmoidal function that exhibited a smaller slope in the 5–50 mM L-proline range than that of the APV- or CNQX-sensitive currents.

As an alternative means of expressing the potency of L-proline action on each of the three receptors, we determined the concentration of the selective agonist for each receptor that yielded a response comparable to that elicited by 10 mM L-proline (Fig. 2, B–D). The mean amplitude of APV-sensitive currents was 106 ± 4% of the mean amplitude of currents evoked by 15 μM NMDA ($n = 6$). The CNQX-sensitive current was 93 ± 13% ($n = 6$) of 5 μM kainate-evoked currents. The strychnine-sensitive current was 102 ± 2% ($n = 5$) of currents elicited by 30 μM glycine.

Tests for antagonist action of L- and D-proline. Both L- and D-proline were tested for antagonist activity at the NMDA, non-NMDA, and glycine receptors. Concentrations of L-proline that were near threshold for receptor activation (100 μM) did not antagonize currents evoked by 30 μM kainate, 30 μM glycine, or 20 μM NMDA. We also tested for additivity of currents evoked by higher concentrations of L-proline (3 or 10 mM) with currents evoked by selective agonists. If L-proline-evoked currents add to currents activated by nonsaturating doses of selective receptor agonists, then antagonist action of L-proline is unlikely. We found that isolated APV-, CNQX-, and strychnine-sensitive L-proline-evoked currents were additive with currents evoked by NMDA, kainate, and glycine, respectively. In contrast, 10 mM D-proline (with 5 μM strychnine added to block glycine receptor-mediated currents) did attenuate currents elicited by 30 μM kainate, by 28.1 ± 3.3% ($n = 4$), and also reduced currents elicited by 50 μM NMDA, by 23.0 ± 3.7% ($n = 5$). Coapplication of 100 μM D-proline, which by itself elicited little or no response, with 30 μM glycine did not attenuate the glycine-evoked currents ($n = 2$).

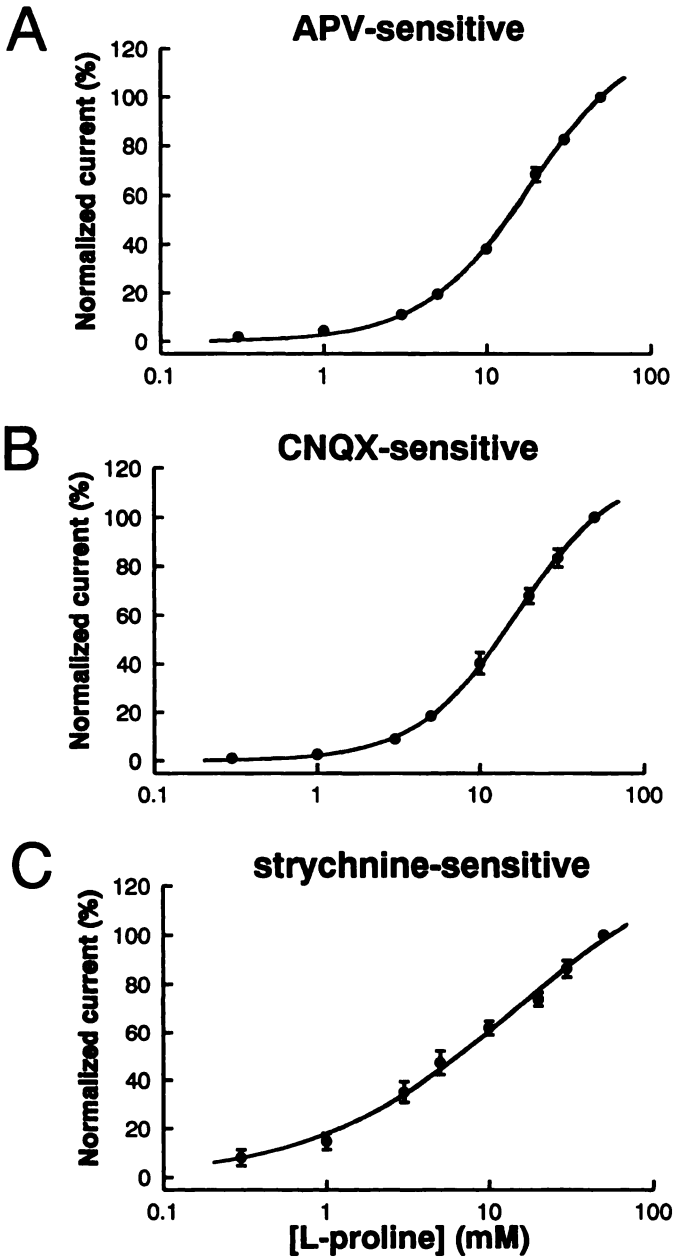

Fig. 5. Dose-response relationships for APV-, CNQX-, and strychnine-sensitive L-proline-evoked currents. In each case, responses to 0.3, 1, 3, 5, 10, 20, 30, and 50 mM L-proline were normalized relative to the response to 50 mM L-proline. A, L-Proline with 10 μM CNQX, 5 μM strychnine, and 100 μM glycine ($n = 4$). B, L-Proline with 5 μM strychnine, 30 μM APV, and no added glycine ($n = 5$). C, L-Proline with 10 μM CNQX, 30 μM APV, and no added glycine ($n = 4$). *Vertical bars*, 1 SE. All curves were fitted using a form of the Michaelis-Menton equation, as described previously (23).

L-Proline-evoked increases in $[Ca^{2+}]_i$. Interaction between glutamate receptor activation and membrane potential is a critical factor in determining the elevation of $[Ca^{2+}]_i$ evoked by glutamate receptor agonists (28). Therefore, to characterize the effect of L-proline on $[Ca^{2+}]_i$ we have studied neurons in which the membrane potential was free to fluctuate under the influence of applied drugs. L-Proline evoked increases in $[Ca^{2+}]_i$ in all cultured dorsal horn neurons tested ($n = 35$). A concentration of L-proline greater than 1 mM was ordinarily required to elevate $[Ca^{2+}]_i$ significantly, and further increases in the

concentration of L-proline caused progressively increasing $[Ca^{2+}]_i$ responses (Fig. 6A). In 24 neurons tested, 10 mM L-proline evoked a mean change in $[Ca^{2+}]_i$ of 252 ± 60 nM above resting levels of approximately 50 nM. As illustrated in Fig. 6C, coapplication of 30 μM APV with 10 mM L-proline reduced the peak change in $[Ca^{2+}]_i$ above base line to 14 ± 4% of control responses to 10 mM L-proline (n = 13). Similarly, 1–5 mM Mg^{2+} reduced the response to 12 ± 5% of control (n = 11). Coapplication of CNQX (10 μM) reduced the response to 58 ± 6% (n = 9), whereas APV and CNQX applied together reduced the response to 5 ± 7% of control (n = 8; Fig. 6B). Strychnine, on the other hand, increased the peak $[Ca^{2+}]_i$ response to 247 ± 32% of control (n = 6; Fig. 6B).

To investigate the mechanisms by which L-proline elevated $[Ca^{2+}]_i$ in these neurons, we omitted Ca^{2+} from the extracellular recording solution. As shown in Fig. 6D, the reduction of $[Ca^{2+}]$ in the extracellular solution to nominally Ca^{2+}-free conditions completely blocked any elevation of $[Ca^{2+}]_i$ by 10 mM L-proline (n = 3). This observation indicates that Ca^{2+} entry is required for L-proline-evoked increases in $[Ca^{2+}]_i$, but it does not exclude a possible contribution of Ca^{2+} release from intracellular stores after Ca^{2+} entry.

Discussion

Our results demonstrate that L-proline activates both the non-NMDA and the NMDA-type glutamate receptors. L-Proline action on NMDA receptors agrees with previous observations in rat ventral root (11) and hippocampal slice (12). In those studies, the depolarizing action of L-proline was partially blocked by NMDA receptor antagonists. The incompleteness of the block may be explained by our finding that L-proline also activates CNQX-sensitive, non-NMDA glutamate receptors. A weak antagonist effect of CNQX on responses to L-proline in hippocampus was observed by Nadler *et al.* (12), although it was not clear whether this effect was due to antagonist action at non-NMDA receptors or weak antagonism of NMDA receptors by CNQX.

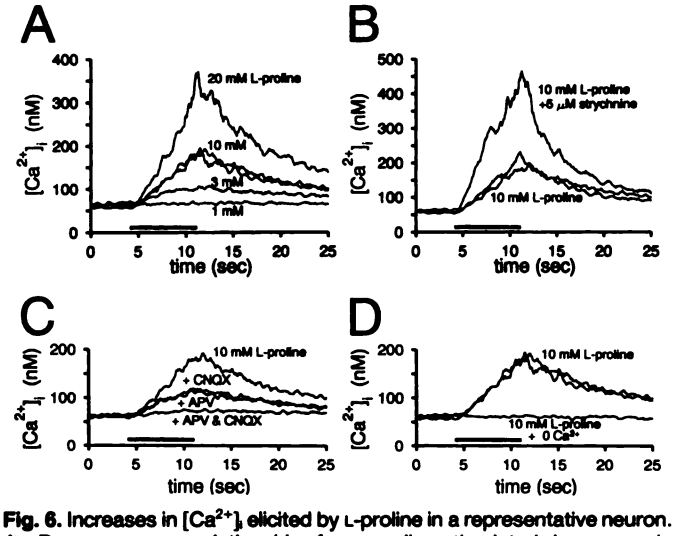

Fig. 6. Increases in $[Ca^{2+}]_i$ elicited by L-proline in a representative neuron. A, Dose-response relationship for L-proline-stimulated increases in $[Ca^{2+}]_i$. At L-proline concentrations exceeding 1 mM, $[Ca^{2+}]_i$ increased during the duration of exposure to L-proline (*horizontal bar*), reaching a peak level that recovered to base-line levels within 2 min after withdrawal of drug. The response to 10 mM L-proline recovered after experimental manipulations, as demonstrated by a second application in this and the other examples. B, Coapplication of strychnine (5 μM) enhanced the increase in $[Ca^{2+}]_i$ evoked by 10 mM L-proline. C, Coapplication of APV (30 μM) or CNQX (10 μM) reduced the 10 mM L-proline-stimulated increase in $[Ca^{2+}]_i$. Furthermore, simultaneous application of both antagonists virtually abolished this increase. D, These increases in $[Ca^{2+}]_i$ are mediated by Ca^{2+} entry, because they did not occur after the removal of extracellular Ca^{2+}.

It has been suggested that L-proline can act as an antagonist of glutamate receptors, based on its antagonism of glutamate-based spreading depression in chick retina (17), electrical stimulation-evoked population excitatory postsynaptic potentials in rat hippocampus (16), and glutamate-evoked increases in firing rate in hippocampus (15), cerebral cortex, and spinal cord (7). Because the present study fully characterized the agonist actions of proline at ionotropic receptors, it was possible to perform experiments that assessed its potency as an antagonist as well. Our results are similar to those of Ault *et al.* (11), in that they provide no support for antagonist action of L-proline at glutamate receptors. The apparent antagonist action observed in previous studies possibly reflects activation of inhibitory interneurons, depolarization block, or the ability of L-proline to inhibit glutamate release at concentrations comparable to those at which it is an effective glutamate receptor agonist. Millimolar concentrations of L-proline have been shown to inhibit glutamate release evoked by electrical stimulation in rat cortical slices (29) and by elevated K^+ concentrations in avian brain slices (30). We observed a moderate antagonistic potency of D-proline at glutamate receptors. However, it seems unlikely that the L-proline used in previous studies was sufficiently contaminated with D-proline for this action to account for the results.

Although Curtis and Johnston (6) described a strychnine-sensitive inhibition of the firing of cat spinal interneurons by L-proline, in a more detailed study using cat spinal interneurons L-proline alone did not inhibit the firing of those cells (7). Rather, for most neurons L-proline was excitatory, whereas in others it antagonized the excitatory action of glutamate but had no effect when applied alone. Nistri and Morelli (8) observed apparently Cl^--dependent and partially strychnine-blockable effects of L-proline on frog ventral roots. However, they also observed that Cl^--dependent depolarizing potentials evoked by L-proline were strychnine- resistant, and concentrations of L-proline exceeding 2 mM evoked strychnine-insensitive hyperpolarizations, making a mechanistic interpretation of their results difficult. Under the conditions of our experiments, it has been possible to isolate a Cl^--dependent, strychnine-sensitive response to L-proline, with inactivation properties and outward rectification typical of glycine receptors. The observation that D-proline activates exclusively glycine receptors predicts that its impact on membrane potential will depend solely on the Cl^- gradient in the cell under study. Thus, the depolarizing effect of D-proline applied to rat ventral roots (11) may be due an outward Cl^- gradient.

The glutamate receptor agonist action of L-proline is surprising, given the absence of any net charge on the molecule. This feature stands in contrast to previously described, higher potency, agonists of glutamate receptors, which are negatively charged, possessing two acidic groups (21, 31). Even at the highest dose of L-proline we tested, the CNQX-sensitive currents elicited by L-proline exhibited no detectable desensitization. All previously described agonists that activate both NMDA- and non-NMDA-type glutamate receptors evoke a

desensitizing CNQX-sensitive current (21). However, our results using L-cysteate indicate that our experiments do not resolve whether the action of L-proline at CNQX-sensitive glutamate receptors is kainate-like (nondesensitizing) or quisqualate-like (rapidly desensitizing).

Our results demonstrate that glycine receptor activation by proline exhibits little, if any, stereospecificity. The approximately 10-fold more potent strychnine-sensitive glycine receptor agonist alanine also shows little stereospecificity in its action at glycine receptors (22). Both proline and alanine can be contrasted with serine, because only the L-isomer of serine is a potent agonist of the glycine receptor. Despite the weak potency of proline, this information may be of use in determining the features of the agonist binding site of the glycine receptor.

We measured large increases in $[Ca^{2+}]_i$ evoked by L-proline in dorsal horn neurons. This elevation of $[Ca^{2+}]_i$ requires extracellular Ca^{2+}, suggesting that it is mediated principally by entry of Ca^{2+} into the neuron. It is likely that Ca^{2+} enters neurons in response to L-proline via two main routes. First, voltage-gated Ca^{2+} channels are activated by glutamate receptor-mediated depolarization of the membrane and, second, Ca^{2+} may enter directly through NMDA (32) and non-NMDA (33) glutamate-gated channels. Predictably, the $[Ca^{2+}]_i$ increases are fully blocked by APV and CNQX applied together. Although we did not detect L-proline-evoked elevations of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, we did not do an exhaustive search for indications of L-proline-sensitive second messenger-mediated responses. Thus, our data do not bear on the possibility that L-proline might also activate metabotropic (guanine nucleotide-binding protein-linked) receptors at significantly lower concentrations.

Glycine receptor activation also influences the $[Ca^{2+}]_i$ response to L-proline. It strongly depresses the ability of glutamate receptors to cause Ca^{2+} entry, because coapplication of strychnine to block glycine receptors yields a >2-fold increase in the size of the $[Ca^{2+}]_i$ response. Under our experimental conditions, in the absence of added extracellular Mg^{2+}, the effect of glycine receptor activation on Ca^{2+} transients probably reflects a reduction in the depolarization evoked by glutamate receptor activation, thereby reducing Ca^{2+} entry via voltage-gated Ca^{2+} channels. Under physiological conditions, however, glycine receptor-mediated attenuation of membrane depolarization may also decrease the voltage-dependent relief of Mg^{2+}-blocked NMDA receptors (34, 35).

Our observation that L-proline can evoke large increases in $[Ca^{2+}]_i$ in neurons suggests that Ca^{2+} entry into neurons may be a critical mechanism in L-proline-induced neuronal cell death. Nadler *et al.* (14) observed similarities between L-proline- and NMDA-mediated neurotoxicity in rat hippocampus but noted that the neurotoxic potency of L-proline versus NMDA was surpisingly high, given the weak potency of L-proline as an excitant in these neurons. This may be due to the ability of L-proline to activate NMDA and non-NMDA receptors simultaneously. Indeed, the mixed agonist action of L-proline may explain why the neurotoxic effects of intrahippocampal L-proline injections were fully antagonized by the nonspecific glutamate receptor antagonist kynurenate but only partially antagonized by a specific NMDA receptor antagonist (14). Non-NMDA receptor agonists can by themselves be excitotoxins, probably also by stimulating Ca^{2+} entry (36). In spinal cord neurons, the simultaneous activation of glycine receptors and glutamate receptors depresses the magnitude of $[Ca^{2+}]_i$ elevations evoked by L-proline. This may be protective against potential neurotoxic actions of L-proline. However, the neurotoxic potency of L-proline may be expected to be greater in other brain regions, where receptor distributions differ markedly from those in spinal cord. For example, area CA1 in the hippocampus has a lesser relative density of glycine receptors versus glutamate receptors and an overall manyfold higher absolute density of NMDA receptors (37).

Previously, it has been suggested that L-proline-mediated excitotoxicity contributes to the neurological abnormalities associated with hyperprolinemia (14). Type I and type II hyperprolinemia are inherited disorders of proline metabolism (38). Each has been occasionally associated with neurological abnormalities, such as seizures or mental retardation. In affected patients, plasma concentrations of L-proline can be up to 20 times greater than normal levels, and cerebrospinal fluid concentrations (normally 4.2 μM) are also considerably elevated (38, 39). L-Proline concentrations in brain may be even higher earlier in development (40). Our results suggest that these concentrations approach the threshold for activation of glutamate receptors. Prolonged exposure to a comparably low concentration of quinolinate, a weak NMDA receptor agonist, has neurotoxic effects on cultured neurons over a period of weeks (41). Furthermore, prolonged elevation of brain quinolinate concentrations has been considered as a potential contributor to the pathogenesis of Huntington's chorea (42). By analogy, we propose that the neurological abnormalities associated with hyperprolinemia may be secondary to neurotoxic effects of long term exposure to elevated brain concentrations of L-proline in hyperprolinemic patients. Such neurotoxic effects of L-proline would be expected to be most dramatic in brain regions with higher densities of NMDA and non-NMDA glutamate receptors.

Although there is a great deal of evidence consistent with the possibility that L-proline has a physiological role in central nervous system neurotransmission (1–5, 9), there has been no accurate assessment of its neuroactive potency. In previous studies using bath application to isolated spinal cord (11) or hippocampal slices (12), millimolar concentrations of L-proline were necessary to achieve appreciable effects, although significant effects with concentrations as low as 100 μM were obtained in the frog ventral root (8). Our ability to isolate individual components of the L-proline-evoked currents and to control drug concentrations precisely has allowed us to make direct measurement of L-proline potency. Nonsaturation of the responses at the highest tested dose of L-proline (50 mM) prohibited determination of EC_{50} values for activation of any receptor subtype. However, with embryonic rat dorsal horn neurons grown in tissue culture, the estimated L-proline EC_{50} values for activation of glycine receptors, NMDA receptors, and non-NMDA glutamate receptors are 10–20 mM, 20–50 mM, and 50–100 mM, respectively, based on comparisons with equipotent concentrations of selective agonists of known potency. These values are approximately 1000-fold greater than those of amino acid transmitters such as L-glutamate and glycine. However, it is not known whether neurons from other regions of the central nervous system may express glycine, NMDA, or non-NMDA receptors with higher affinity for L-proline than embryonic dorsal horn neurons. Furthermore, there are no reliable esti-

mates of the transmitter concentration in the synaptic cleft of central synapses. Therefore, although our data suggest it is unlikely, we cannot rule out the possibility of a significant role for L-proline as a neurotransmitter at synapses with glutamate or glycine receptors.

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