Contribution of Presynaptic Na\(^+\) Channel Inactivation to Paired-Pulse Synaptic Depression in Cultured Hippocampal Neurons

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He, Yejun, Charles F. Zorumski, and Steven Mennerick. Contribution of presynaptic Na\(^+\) channel inactivation to paired-pulse synaptic depression in cultured hippocampal neurons. J Neurophysiol 87: 925–936, 2002; 10.1152/jn.00225.2001. Paired-pulse depression (PPD) of synaptic transmission is important for neuronal information processing. Historically, depletion of the readily releasable pool of synaptic vesicles has been proposed as the major component of PPD. Recent results suggest, however, that other mechanisms may be involved in PPD, including inactivation of presynaptic voltage-dependent sodium channels (NaChs), which may influence coupling of action potentials to transmitter release. In hippocampal cultures, we have examined the potential role and relative contribution of presynaptic NaCh inactivation in excitatory postsynaptic current (EPSC) PPD. Based on current- and voltage-clamp recordings from somas, our data suggest that NaCh inactivation could potentially participate in PPD. Paired stimulation of somatic action potentials (20- to 100-ms interval) results in subtle changes in action potential shape that are mimicked by low concentrations of tetrodotoxin (TTX) and that appear to be generated by a combination of fast and slow recovery from NaCh inactivation. Delute concentrations of TTX dramatically depress glutamate release. However, we find evidence for only minimal contribution of NaCh inactivation to EPSC PPD under basal conditions. Hyperpolarization of presynaptic elements to speed recovery from inactivation or increasing the driving force on Na\(^+\) ions through active NaChs had minimal effects on PPD while more robustly reversing the effects of pharmacological NaCh blockade. On the other hand, slight depolarization of the presynaptic membrane potential, by elevating extracellular [K\(^-\)], significantly increased PPD and frequency-dependent depression of EPSCs during short trains of action potentials. The results suggest that NaCh inactivation is poised to modulate EPSC amplitude with small tonic depolarizations that likely occur with physiological or pathophysiological activity.

INTRODUCTION

Neurons exhibit short-term and long-term synaptic plasticity that depend on previous firing history. Paired-pulse depression (PPD), a simple form of short-term synaptic plasticity, plays an important role in neuronal information processing (Zucker 1989). However, the mechanisms underlying PPD are poorly understood. Although postsynaptic receptor desensitization can contribute to PPD at some specialized synapses (Ottis et al. 1996; Trussell et al. 1993) or under some experimental conditions (Mennerick and Zorumski 1996; Tong et al. 1995), most synapses exhibit a strong presynaptic component of PPD, likely explained by depletion of an immediately releasable vesicle pool (Zucker 1989, 1999). When the release probability is high, depletion of the readily releasable pool of synaptic vesicles by a conditioning action potential results in synaptic depression to subsequent stimulation.

Alternatively, accumulating evidence suggests that the presynaptic mechanism of PPD may not simply be explained by depletion of readily releasable vesicles. A use-dependent modification of the functional state of the vesicle release machinery or more upstream events, such as modulation of the sodium action potential or Ca\(^{2+}\) influx, may also contribute to PPD through release-independent mechanisms (Betz 1970; Hsu et al. 1996; Waldeck et al. 2000; Wu and Borst 1999).

Although depletion has been proposed to account for EPSC PPD at hippocampal synapses in vitro (Mennerick and Zorumski 1995; Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995), a release-independent mechanism in these cells has been suggested previously, based on the observation that presynaptic depression of transmission decreases PPD less than predicted by the depletion hypothesis (Brody and Yue 2000; Maki et al. 1995). One candidate mechanism to explain the discrepancies between the depletion hypothesis prediction and experimental observations is that changes in presynaptic action potentials may also contribute to PPD (Brody and Yue 2000). In particular, NaCh inactivation (Catterall 2000) was proposed to account for the major component of PPD (Brody and Yue 2000). Such a hypothesis represents a potentially novel synaptic modulatory role for NaChs, which are traditionally thought to participate only in all-or-none control over synaptic transmitter release in vertebrates.

For NaCh inactivation to play a role in PPD, which recovers with a time course of hundreds of milliseconds to seconds, one would expect that inactivation should exhibit a recovery time course more prolonged than the major fast component typically observed (approximately 4 ms) (Martina and Jonas 1997). On the other hand, our recent results suggest that glutamate release from hippocampal neurons is exquisitely sensitive to partial inhibition of the action potential with pharmacological NaCh blockers (Prakriya and Mennerick 2000). Therefore even a small degree of residual NaCh inactivation following conditioning stimulation might dramatically influence transmitter release.

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release to subsequent stimuli either indirectly by altering Ca\(^{2+}\) influx (Prakriya and Mennerick 2000) or by altering action potential propagation (Brody and Yue 2000). NaCh inactivation with a slow time course of recovery has recently been described in the somas and dendrites of hippocampal neurons in slice preparations (Colbert et al. 1997; Jung et al. 1997; Martina and Jonas 1997; Mickus et al. 1999). The existence and relevance of this form of slow inactivation to synaptic transmission at presynaptic terminals is unknown but could conceivably participate in the slow time course of PPD recovery. We investigated the potential contribution of presynaptic NaCh inactivation in EPSC PPD. Our data suggest a limited role for NaCh inactivation in PPD under basal conditions. In contrast, manipulations that slow recovery from presynaptic NaCh inactivation enhanced synaptic depression, suggesting that NaCh inactivation contributes to EPSC depression under depolarizing physiological or pathophysiological conditions.

**METHODS**

**Hippocampal cultures**

Microculture dishes were prepared by coating the bottom of 35-mm plastic culture dishes with 0.15% agarose. On the agarose layer, which serves as a nonpermissive background for cell adhesion, small droplets of collagen (0.5 mg/ml) were sprayed with a microtome. Single-cell suspensions were prepared from *postnatal day 1–3* rat hippocampus using papain and mechanical dispersion (Mennerick et al. 1995) and then plated on the bottom of microculture dishes at an initial density of 75/mm\(^2\). Electrophysiological recordings were carried out 8–15 days following plating. The culture medium consisted of Eagle’s minimal essential media (Invitrogen, Life Technologies, Carlsbad, CA) supplemented with horse serum (5% vol/vol), fetal calf serum (5% vol/vol), d-glucose (17 mM), glutamine (400 \(\mu\)M), penicillin (50 U/ml), and streptomycin (50 \(\mu\)g/ml).

**Electrophysiology**

Whole cell recordings were performed on solitary, excitatory, hippocampal microculture neurons, using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) interfaced to a Pentium III–based computer via a Digidata 1200 acquisition board (Axon Instruments). Recordings were at room temperature. Electrodes had resistances of 1–5–4 M\(\Omega\) for whole cell recordings and 4–7 M\(\Omega\) for nucleated patch recordings (excised outside-out macropatches containing the cell nucleus) (Sather et al. 1992). For whole cell recordings, access resistance (<10 M\(\Omega\)) was compensated 90–100%. Autaptic release of neurotransmitter was stimulated in voltage-clamped solitary neurons with a 2-ms voltage pulse to 0 mV from a holding potential of −70 mV. This stimulation protocol elicits an escaped action potential in the partially clamped axons, which triggers transmitter release (Bekkers and Stevens 1991; Mennerick et al. 1995). Na\(^+\) currents and action potentials were examined from cell bodies. In some cases nucleated patches or young (6–8 days in vitro) cells with very limited neurite arbors were used to study isolated Na\(^+\) currents to provide better spatial clamp. Whole cell or nucleated patch recordings of isolated Na\(^+\) currents were performed following synaptic stimulation, which was used to verify the transmitter phenotype of the neuron. Action potentials were generated by injecting depolarizing current (30–400 pA) in the current-clamp recording mode of the patch amplifier.

Whenever possible, at least three traces in each experimental condition were acquired for analysis. For statistical comparisons, the average of responses in a given condition was used to generate one data point per condition per cell. Raw traces in the figures represent a single response per condition except where noted in the figure legends. For all experiments, the interval between data sweeps was \(\approx\)15 s for Na\(^+\) currents and 25 s for synaptic responses. Control conditions were interleaved with experimental conditions to counterbalance any time-dependent changes. Data sampling frequency was 5–10 kHz. pCLAMP6 software (Axon Instruments) was used for data acquisition and analysis. Data plotting and curve fitting were done with Sigma Plot software (SPSS Science, Chicago, IL). Data are presented in the text and figures as means ± SE.

At the time of experiments, culture medium was replaced with an extracellular recording solution consisting of (in mM) 138 NaCl, 4 KC1, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 10 HEPES (pH 7.25), supplemented with 25 \(\mu\)M 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX) to block any residual N-methyl-D-aspartate (NMDA) receptor activity not blocked by the extracellular Mg\(^{2+}\). One micromolar 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX) was included to record action potentials. Solutions were exchanged via a local multibarrel perfusion pipette with a common delivery port placed within 0.5 mm from the cell under study. The standard pipette solution for autaptic responses and action potential measurements contained (in mM) 140 K-gluconate, 4 NaCl, 0.5 CaCl\(_2\), 5 EGTA, and 10 HEPES, pH 7.25. In experiments in which inhibitory postsynaptic currents (IPSCs) were examined, gluconate was replaced with chloride to set \(E_C\) to approximately 0 mV. To record Na\(^+\) currents, the pipette solution contained cesium methanesulfonate in place of K-gluconate to block voltage-dependent K\(^+\) conductances. To pharmacologically isolate Na\(^+\) currents, bath solution also contained 1 \(\mu\)M NBQX and 50–100 \(\mu\)M Cd\(^{2+}\) to block postsynaptic \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and Ca\(^{2+}\) conductances, respectively. In one set of experiments, we included 5 mM MgATP in the whole cell recording pipette. This manipulation did not have a significant effect on the biexponential rate of recovery from NaCh inactivation (\(P = 0.41\), Student’s \(t\)-test, \(n = 7\)). Other alterations to these standard solutions are given in the text and figure legends.

**Chemicals**

All chemicals were from RBI/Sigma (St. Louis, MO), except for anemone toxin ATX II, which was from Calbiochem (La Jolla, CA). In all cases, the concentration of solvent DMSO (if necessary) in working solution was ≤0.1%.

**RESULTS**

The depletion hypothesis of synaptic depression suggests that when the release probability (\(p_r\)) is high, depletion of a readily releasable pool of synaptic vesicles by the conditioning stimulus causes depression of the synaptic response to subsequent test pulses (Zucker 1989, 1999). Therefore depressing initial \(p_r\) should diminish PPD. However, similar to other recent results (Brody and Yue 2000), we found that 1 \(\mu\)M Cd\(^{2+}\), which depressed conditioning excitatory postsynaptic currents (EPSCs) by 34 ± 2%, produced no significant change in PPD evaluated at a 100-ms paired-pulse interval (−25 ± 2% vs. −24 ± 3%, mean ± SE, \(n = 10\), \(P = 0.4\)). The lack of effect of Cd\(^{2+}\) on PPD is consistent with a component of PPD that is independent of previous transmitter release (release-independent PPD), although there are several possible alternative explanations for this observation, including direct effects of Cd\(^{2+}\) on readily releasable vesicle pool size or effects on underlying facilitation. These caveats notwithstanding, we proceeded with an evaluation of the specific hypothesis that NaCh inactivation accounts for a release-independent component of PPD.
Exquisite sensitivity of glutamate release to partial NaCh blockade

Consistent with previous results, we found that EPSC PPD was prominent at hippocampal synapses in culture at intervals of 20–100 ms (Fig. 1A) with 2 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) in the bath solution. Synaptic depression was 35 ± 4%, 27 ± 4%, and 23 ± 5% at 20-, 50-, and 100-ms intervals, respectively (n = 10). We have recently shown that glutamate release from hippocampal neurons is more sensitive to dilute concentrations of NaCh blockers than GABA release from interneurons grown under the same culture conditions (Prakriya and Mennerick 2000). The sensitivity of glutamate release to partial NaCh blockade led us to consider the possibility that endogenous NaCh currents, peak EPSC amplitudes, and somatic action potential of action potential amplitude (AP PPD) with effects of dilute concentrations of exogenous NaCh blockers.

The synaptic depression observed (Fig. 1A) roughly paralleled changes in action potentials measured in current clamp (Fig. 1, B and C). We found that test somatic action potentials, elicited following conditioning action potentials at intervals of 20–100 ms, exhibited depression of peak amplitude (Fig. 1, B and C). However, the AP PPD seemed minor, exhibiting <5-mV depression at 20 ms and <2-mV depression at 100 ms (Fig. 1C). Action potential half-width was also broadened by conditioning action potentials (Fig. 1C). These changes were similar to the effect of very low tetrodotoxin (TTX) concentrations on EPSCs. Five nanomolar TTX produced <1-mV depression of the peak somatic action potential from excitatory hippocampal neurons (0.8 ± 0.1 mV change, or −1.0 ± 0.2% depression, n = 6, Fig. 1, D1 and D). Despite the small effects on somatic action potential waveform, 5 nM TTX depressed peak EPSC amplitudes by 22 ± 7% (Fig. 1, D2 and E), similar to the depression observed at 100-ms paired-pulse interval (Fig. 1A). Ten nanomolar TTX depressed action potentials by a modest 4.3 ± 0.6 mV (−7 ± 1%, n = 6) and broadened the action potential half-width by 110 ± 20 μs. These changes in action potential waveform are very similar to those elicited by paired action potential stimulation with a 20-ms paired-pulse interval (Fig. 1C), and EPSCs were depressed 55 ± 6% by 10 nM TTX (n = 19). These results suggest that even minor alterations of the action potential, such as would occur with a small degree of NaCh inactivation, may produce significant EPSC depression and participate in release-independent PPD.

FIG. 1. Synaptic paired-pulse depression (PPD), action potential paired-pulse depression (AP PPD), and effect of mild NaCh block on excitatory postsynaptic currents (EPSCs). A: example of synaptic (autaptic) depression obtained from a solitary excitatory hippocampal neuron. The cell was stimulated in voltage-clamp mode with a pair of stimuli to elicit action potentials in the axon. Responses were superimposed and scaled to the conditioning response where necessary to highlight the relative change of the test responses. Note that the synaptic depression is greatest at the shortest paired-pulse interval. Depicted are paired-pulse intervals of 20, 50, and 100 ms. B: in current-clamp mode in another cell, paired action potentials separated by 20 ms were elicited by DC injections. This cell had a resting membrane potential of −66 mV. 6-Nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX; 1 μM) was present to block autaptic depolarizations following stimulation. C: changes in measurements of action potentials averaged across cells at varied paired-pulse intervals. Differences (Δ) were obtained by subtracting measurements of conditioning action potentials from those of test action potentials. Action potential width was measured at 50% of the peak amplitude. The differences of peaks and widths between paired action potentials were statistically significant at all intervals (peak, P < 0.05; width, P < 0.01, paired Student’s t-test). Twenty-millisecond interval, n = 10; 50-ms interval, n = 11; 100-ms interval, n = 12. D1: single sweeps of current-clamped somatic action potentials recorded from a glutamatergic neuron, one sweep in control and the other in 5 nM TTX. The dotted line indicates the action potential peak in control. The action potential height was reduced by 0.5 mV in the presence of TTX in this cell. The peaks are shown at higher resolution in the inset. NBQX (1 μM) and d-asparto-5-phosphonovaric acid (d-APV; 25 μM) were present in both sweeps to block autaptic depolarization. D2: from the same cell depicted in D1, EPSCs were elicited in the presence (thick trace) and absence (thin trace) of 5 nM TTX (no NBQX present). EPSCs exhibited a reliable 13% reduction in this cell. For this and subsequent figures, the currents associated with presynaptic stimulation have been partially blanked for clarity. E: summarized changes in whole cell Na\(^{+}\) currents, peak EPSC amplitudes, and somatic action potential heights by 5 nM TTX (n = 6). Left: averaged percent inhibition of Na\(^{+}\) currents and peak EPSC amplitudes by 5 nM TTX. Right: averaged difference (Δ) between peak action potential heights in control and in 5 nM TTX, obtained by subtracting the height of action potentials in control from the height of action potentials in 5 nM TTX.
The results do not address whether NaCh inactivation underlies the observed changes in action potential waveform and whether the changes in waveform observed at the soma also occur at synaptic terminals.

**Biexponential time course of recovery from NaCh inactivation**

The depression of action potentials at paired-pulse intervals of 50–100 ms is surprising given that NaCh inactivation typically recovers in <10 ms. On the other hand, slow forms of recovery from NaCh inactivation have been described (Colbert et al. 1997; Jung et al. 1997; Mickus et al. 1999) and could participate in PPD. To explore whether changes observed in somatic current-clamp recordings of paired action potentials (Fig. 1, B and C) could result from NaCh inactivation, as opposed to changes in other voltage-gated conductances, recovery of isolated Na⁺ current from inactivating voltage pulses was examined. Figure 2A shows isolated whole cell Na⁺ currents recorded from the soma of a glutamatergic hippocampal neuron. Conditioning stimulation using 2-ms voltage pulses (identical to those used to elicit synaptic glutamate release) caused nearly complete inactivation of voltage-dependent NaChs during the conditioning pulse (Fig. 2A). Recovery from inactivation was estimated by varying the paired-pulse interval in successive trials. Two components were apparent in the recovery process. Fitting pooled data from whole cell recordings revealed a biexponential time course of recovery from NaCh inactivation (Fig. 2B) with time constants of 3.9 and 938 ms and relative amplitudes of 0.92 and 0.08, respectively. Conditioning Na⁺ currents recovered to within 99.5 ± 0.7% in 15 s.

A slow phase of recovery from inactivation has been found in dendritic voltage-dependent NaChs of hippocampal pyramidal neurons in slice preparations (Colbert et al. 1997; Jung et al. 1997; Mickus et al. 1999), although the existence of presynaptic slow inactivation is unknown. Spatial voltage clamp of whole cell Na⁺ currents might be a problem in these synaptically mature neurons and contribute to an artifactually slow recovery from inactivation. To eliminate spatial clamp artifacts, we also studied the time course of recovery from NaCh inactivation in nucleated outside-out patches excised from the soma (Sather et al. 1992). The nucleated patch configuration should provide both good spatial voltage clamp and good preservation of the intracellular environment. Recovery from inactivation of somatic NaChs recorded from nucleated patches also exhibited a slow component (Fig. 2C). The time constants of recovery from a fit to pooled data were 7.7 and 923 ms with relative respective amplitudes of 0.77 and 0.23 (n = 14).

NaCh inactivation in nucleated patches was somewhat slower than that estimated from whole cell recordings. This could be due to different properties of NaChs in the soma and processes, to wash out of a modulating factor from patches, or to some artifact of poor clamp in whole cell recordings. Whatever the reason for the quantitative differences between patches and whole cells, both methods indicate that recovery from NaCh inactivation is biexponential and suggest that hippocampal cells possess a slow recovery from inactivation that, if present in presynaptic elements, could participate in release-independent PPD.

**FIG. 2.** Paired stimulation depressed test Na⁺ currents, and recovery from NaCh inactivation occurred in 2 phases. A: 5 overlaid pairs of leak-subtracted, pharmacologically isolated whole cell Na⁺ currents recorded from a solitary excitatory neuron at various interstimulus intervals. Averages of 3 runs through the sequence of intervals are shown. Paired voltage pulses from −70 to 0 mV for 2 ms were separated by interstimulus intervals varied between 5 ms to 1 s. Holding potentials before and after the stimuli were −70 mV. Each sweep has been scaled to the conditioning Na⁺ current at the 5-ms interval to offset slight rundown or run-down. The diagonal lines represent gaps in the time axis, and the numbers in parentheses represent paired-pulse intervals for the test Na⁺ currents indicated. Fast transients associated with Na⁺ current are residual, incompletely subtracted capacitive currents and denote the onset and offset of the voltage pulse. The scale bar represents the unscaled initial sweep. B: summarized time course of recovery from NaCh inactivation estimated with whole cell patch-clamp configuration. The ratio of Na⁺ currents evoked by the test pulse to Na⁺ currents evoked by the conditioning pulse is plotted against interstimulus intervals. Error bars are not shown when smaller than symbols. The sum of 2 exponentials (smooth curve) was fit to the recovery. Time constants of the 2 components were 3.9 and 938 ms with relative amplitudes of 0.92 and 0.08, respectively (n = 7). C: 3 overlaid pairs of isolated somatic Na⁺ currents recorded from a nucleated patch excised from a solitary excitatory neuron at different interstimulus intervals. The stimulation paradigm was the same as in A, and the conditioning currents have been normalized correspondingly. The scale bar applies to the 1st (unscaled) sweep.

**Blockers of inactivation had complicated actions on action potentials and synaptic signaling**

A potentially straightforward method to test a role for NaCh inactivation in PPD is to block inactivation, then assess the effect of inactivation block on EPSC PPD. On the other hand,
in hippocampal slices the effect of direct inhibition of NaCh inactivation on synaptic transmission is complex (Brand et al. 2000). Anemone toxin II (ATX II), a site-3 NaCh toxin, binds to voltage-dependent NaChs and prolongs the time course of Na\(^+\) current during depolarization by inhibiting inactivation. In contrast to some other modulators of NaCh function, ATX II has little or no effect on activation (Mantegazza et al. 1998). Despite the selective effect of ATX II, work in intact hippocampus suggests that ATX II increases excitability in the soma compartment but decreases excitability in the axon, presumably due to depolarization-induced conduction block (Brand et al. 2000).

Consistent with these prior results, we found that ATX II inhibited NaCh inactivation during voltage pulses to 0 mV (data not shown) but actually increased both AP PPD and synaptic PPD. In current-clamp studies, ATX II had little effect (data not shown) but actually increased both AP PPD and inhibited NaCh inactivation during voltage pulses to 0 mV (Brand et al. 2000).

Soma compartment but decreases excitability in the axon, pre-
contrast to some other modulators of NaCh function, ATX II
NaCh to voltage-dependent NaChs and prolongs the time course of
inactivation through noninactivated Na\(^+\) conductances, which contributed to depression of test action potentials. Similar effects were observed with another blocker of NaCh inactivation, veratridine (1 \(\mu\)M; data not shown). Consistent with the paradoxical actions of these drugs on paired action potentials, we found that EPSC PPD was not relieved by ATX II or by veratridine. PPD actually increased slightly for both drugs at paired-pulse intervals of 20–50 ms (data not shown). We conclude that direct manipulation of NaCh inactivation is not an appropriate strategy for assessing the contribution of NaCh inactivation to basal release-independent EPSC PPD. These results caused us to consider less direct approaches to evaluate the role of NaCh inactivation in release-independent PPD.

**Increasing the driving force on extracellular Na\(^+\) through remaining activated presynaptic NaChs partially relieved PPD**

If NaCh inactivation plays a role in PPD through a release-independent mechanism upstream of transmitter release, then increased Na\(^+\) influx through remaining activatable channels may compensate for inactivation caused by conditioning stimuli. The rationale is that because the Na\(^+\) current underlying the action potential is dependent on the product of electrochemical driving force and membrane conductance (Ohm’s Law), one can compensate for a decrease in conductance (inactivation or pharmacological channel block) with an increase in driving force (elevated [Na\(^+\)]\(_o\)). Indeed, we recently found that increased Na\(^+\) driving force could partially alleviate the synaptic depression induced by pharmacological blockade of NaChs (Prakriya and Mennerick 2000). In the present experiments, we compared PPD in 120 mM [Na\(^+\)]\(_o\) (supplemented with 40 mM choline chloride to maintain osmolarity and charge) and 160 mM [Na\(^+\)]\(_o\). On average, raising [Na\(^+\)]\(_o\) by 40 mM increased peak conditioning EPSC amplitudes by 28 ± 5% (\(n = 10\), primarily due to postsynaptic effects of the altered cation concentrations (Prakriya and Mennerick 2000). On the same set of cells, PPD was consistently, although minimally, less prominent in 160 mM [Na\(^+\)]\(_o\), than in 120 mM [Na\(^+\)]\(_o\), at the 20-ms paired-pulse interval tested (\(P < 0.01\), Fig. 3, A and B). This was not because of a direct effect of elevated [Na\(^+\)]\(_o\), on inactivation recovery, because whole cell Na\(^+\) current inactivation was similar in 120 mM and 160 mM [Na\(^+\)]\(_o\) at a 20-ms paired-pulse interval (9.6 ± 1.9% test inactivation in 120 mM [Na\(^+\)]\(_o\), 9.3 ± 2.1% inactivation in 160 mM [Na\(^+\)]\(_o\), n = 10, P = 0.5, paired t-test). We found no significant effect of elevated [Na\(^+\)]\(_o\) on PPD evaluated at a 100-ms paired-pulse interval (−16 ± 4% vs. −13 ± 3% PPD in 120 vs. 160 mM [Na\(^+\)]\(_o\), respectively, n = 13, P = 0.15). This suggests that slow inactivation, the amplitude of which recovers little between 20 and 100 ms, is unlikely to participate in PPD.

Raising [Na\(^+\)]\(_o\), by 40 mM was able to partially relieve PPD, but the effect was quite small. We wondered whether this was because increasing the driving force on extracellular Na\(^+\) was weak in compensating for NaCh inactivation, and/or because inactivation of presynaptic NaChs did not contribute much to PPD. To help distinguish these two possibilities, we studied the effect of raising [Na\(^+\)]\(_o\) on riluzole (2-amino-6-trifluorome-thoxybenzothiazole) block of single EPSCs. Riluzole is a state-dependent blocker of NaChs, promoting NaCh inactivation (Hebert et al. 1994; Prakriya and Mennerick 2000; Taylor and Meldrum 1995). Riluzole (3 \(\mu\)M) block of whole cell Na\(^+\) current was similar in 120 and 160 mM [Na\(^+\)]\(_o\) (−5 ± 1% and −8 ± 1% change, respectively, n = 7). On the other hand, riluzole-induced EPSC depression was twice as large in 120 mM [Na\(^+\)]\(_o\) compared with 160 mM [Na\(^+\)]\(_o\) (Fig. 3C). Thus the small effect of raised [Na\(^+\)]\(_o\) on PPD may reflect a small contribution of NaCh inactivation to baseline PPD rather than to ineffectiveness of elevated [Na\(^+\)]\(_o\), as an experimental manipulation.

The rationale for the preceding experiments is that increasing the driving force through noninactivated Na\(^+\) channels compensates for the Na\(^+\) channels that are putatively inactivated by conditioning stimulation. However, it is possible that raising [Na\(^+\)]\(_o\), has effects on other processes relevant to PPD. Ca\(^{2+}\) current inactivation is another candidate mechanism for
release-independent PPD. Perhaps raising \([\text{Na}^+]_o\) compensates for inactivated \(\text{Ca}^{2+}\) channels by increasing action potential height and recruiting \(\text{Ca}^{2+}\) channels in the synaptic terminal that were not activated by the smaller action potential in low \([\text{Na}^+]_o\). To test this possibility, we pharmacologically simulated \(\text{Ca}^{2+}\) channel inactivation using \(\text{Cd}^{2+}\), a nonselective \(\text{Ca}^{2+}\) channel blocker, and examined single-stimulus EPSCs. Raising \([\text{Na}^+]_o\) did not relieve inhibition of isolated EPSCs by 1 \(\mu\text{M Cd}^{2+}\) (−46 ± 4% change in 120 mM \([\text{Na}^+]_o\), and −51 ± 4% change in 160 mM \([\text{Na}^+]_o\), \(n = 10\)). This suggests that \(\text{Ca}^{2+}\) channel inactivation likely does not participate in the small, \(\text{Na}^-\)-sensitive component of PPD and that increasing \([\text{Na}^+]_o\) is at least partially selective.

We also changed the \(\text{Na}^+\) driving force by reducing intracellular \([\text{Na}^+]_i\) from 4 to 0.5 mM (Brody and Yue 2000). However, on loading cells with 0.5 mM \([\text{Na}^+]_o\), for 10–20 min, we observed no relief in PPD (20-ms paired-pulse interval) from cells recorded with the standard pipette solution from the same plating (−51 ± 11% PPD with 0.5 mM \([\text{Na}^+]_o\), \(n = 6\); −45 ± 11% PPD with 4 mM \([\text{Na}^+]_o\), \(n = 6\), \(P = 0.35\)). This result is again consistent with the idea that \(\text{Na}^+\) channel inactivation plays little role in PPD, although we cannot exclude the possibility that axons and presynaptic terminals were not adequately perfused with the whole cell pipette solution. Further evidence presented below also supports a small contribution of \(\text{Na}^+\) channel inactivation to a release-independent component of PPD under basal conditions.

**Hyperpolarization between stimuli significantly enhances recovery from \(\text{Na}^+\) channel inactivation, but does not relieve frequency-dependent depression**

Recovery from \(\text{Na}^+\) channel inactivation is voltage dependent (Cuterall 2000). Thus the resting potential of presynaptic elements might be manipulated to test the role of \(\text{Na}^+\) channel inactivation in PPD. Confirming that hyperpolarization speeds recovery from inactivation, we found that PPD of \(\text{Na}^+\) current was decreased from 11.3 ± 2.3% at −70 mV to 7.0 ± 1.8% at −110 mV using a 20-ms paired-pulse interval (\(n = 6\) excitatory neurons, \(P < 0.01\)). Inactivation was similarly reduced at a hyperpolarized holding potential of −90 mV (\(n = 6\) neurons, \(P < 0.01\), data not shown). We then altered the rate of recovery from \(\text{Na}^+\) channel inactivation by changing somatic holding potentials between stimuli and studied the consequence on EPSC PPD. As a positive control, we used the voltage- and state-dependent \(\text{Na}^+\) blocker riluzole (Hebert et al. 1994; Prakriya and Mennerick 2000; Taylor and Meldrum 1995). Because the voltage control over the axon is not precise, we used the depression of EPSCs by riluzole as an indication of the effective influence of the somatic clamp over presynaptic membrane potential. As previously observed (Prakriya and Mennerick 2000), riluzole depression of EPSCs was largely relieved by hyperpolarizing prepulses. Ten micromolar riluzole altered EPSCs by −33 ± 11% at −70 mV, and only −13 ± 8% at −110 mV (\(n = 3\)).

Despite the effect of holding potential on riluzole block, we found no significant difference in PPD at the two holding potentials. With a 20-ms paired-pulse interval studied in seven neurons, PPD was −13 ± 9% with a holding potential of −70 mV between pulses and −18 ± 10% with a holding potential of −110 mV imposed between pulses (\(P = 0.13\)). Even in the cells with greatest depression (>40%, \(n = 2\)), there was no evidence of a change in depression with the more negative holding potential (data not shown). We also examined longer (100 ms) paired-pulse intervals. In either saline containing 2 mM \(\text{Ca}^{2+}\) and 1 mM \(\text{Mg}^{2+}\) (\(P = 0.33\), \(n = 5\)) or saline containing 1 mM \(\text{Ca}^{2+}\) and 2 mM \(\text{Mg}^{2+}\) to reduce putative depletion (\(P = 0.64\), \(n = 10\)), there was no difference in PPD with −70 versus −110 mV holding potentials between pulses. Although EPSC inhibition by riluzole, which shifts steady-state \(\text{Na}^+\) channel inactivation to more negative potentials (Hebert et al. 1994; Prakriya and Mennerick 2000; Taylor and Meldrum 1995), suggested that we were able to influence membrane potential of presynaptic elements with the somatic voltage clamp, PPD was not affected by direct hyperpolarization.

It is possible that the altered membrane potential imposed between pulses did not effectively reach presynaptic terminals during the relatively brief paired-pulse intervals in these experiments. As another strategy to hyperpolarize unclamped presynaptic terminals, we removed extracellular \(\text{K}^+\). Decreasing \([\text{K}^+]_o\) from 4 to 0 mM hyperpolarized the resting potential of neuronal cell bodies by −10 mV (Prakriya and Mennerick 2000), which should enhance recovery from \(\text{Na}^+\) inactivation.

We have previously shown that removal of extracellular \([\text{K}^+]_o\) effectively decreases the degree of riluzole block (Prakriya and Mennerick 2000). This treatment also effectively reduced AP PPD measured at a 20-ms paired-pulse interval (Fig. 4A). Baseline (4 mM \([\text{K}^+]_o\)) AP PPD was −6 ± 1% but was 0 ± 2% in 0 mM \([\text{K}^+]_o\) (\(n = 5\)). Removal of \([\text{K}^+]_o\) had no effect on the conditioning EPSC amplitude (Fig. 4B) (also see Prakriya and Mennerick 2000), and PPD was not altered in 0 mM \([\text{K}^+]_o\) (Fig. 4, B and C). Therefore neither strong hyperpolarization imposed at cell bodies between stimuli nor removal of external \(\text{K}^+\) relieved PPD.

Although some studies suggest that action potential invasion is highly reliable (Allen and Stevens 1994; Cox et al. 2000; Mackenzie and Murphy 1998), \(\text{Na}^+\) channel inactivation could participate in PPD by promoting branch failure, such that entire axonal branches are not invaded by test action potentials. If this mechanism is important in PPD, then reducing action potential threshold should alleviate branch failure and decrease PPD. Threshold can effectively be reduced by altering extracellular divalent cation concentration (Hille 1992; Prakriya and Mennerick 2000). In an extracellular saline containing 2.0 mM \(\text{Ca}^{2+}\) and 2.5 mM \(\text{Mg}^{2+}\), conditioning EPSCs were the same size as conditioning EPSCs in an extracellular saline containing 1.1 mM \(\text{Ca}^{2+}\) and no added \(\text{Mg}^{2+}\) (3 ± 3% difference in conditioning EPSCs, \(n = 9\)). In the low-divalent saline, action potential threshold is decreased by approximately 6 mV (Prakriya and Mennerick 2000), yet we detected no effect on PPD measured at a 20-ms paired-pulse interval (−22 ± 9% PPD in high-divalent saline and −19 ± 9% PPD in low-divalent saline, \(n = 9\), \(P = 0.3\)).

Our results failed to detect evidence for a strong role of \(\text{Na}^+\) channel inactivation in PPD under physiological conditions. However, in the nervous system, neurons usually fire in trains of impulses rather than with single or paired action potentials. We considered the possibility that inactivation, although small with single conditioning pulses, may accumulate during trains of action potentials and contribute to synaptic depression. Inactivation of somatic and dendritic \(\text{Na}^+\)s of hippocampal CA1 pyramidal neurons accumulates during a stimulus train (Colbert et al. 1997; Jung et al. 1997; Mickus et al. 1999),
which might influence neuronal responsiveness to subsequent stimulation. We delivered a short train of depolarizing pulses (from −70 to 0 mV for 2 ms each) at 50 Hz and examined the relationship between NaCh inactivation and depression of EPSCs during the train. Consistent with earlier reports in slice preparations (Colbert et al. 1997; Jung et al. 1997; Mickus et al. 1999), inactivation of somatic NaChs from hippocampal neurons in response to trains of depolarization was cumulative (Fig. 4D). Whole cell Na⁺ current progressively decreased with six repetitive stimuli delivered at 50 Hz. The sixth response of the train was depressed by 25 ± 3% relative to the first conditioning current. The recovery from this cumulative inactivation was very slow; Na⁺ current was still depressed 18 ± 2% 100 ms after the sixth pulse in the train. This depression was much more pronounced than the depression following a single conditioning stimulus (Fig. 2A), where the Na⁺ current was depressed 100 ms after a conditioning pulse by only approximately 5%. Hyperpolarization to −90 mV between pulses reduced train-induced inactivation of Na⁺ current (33 ± 3% inactivation by train at −70 mV vs. 26 ± 3% at −90 mV, n = 6, P < 0.01).

To test for a role of accumulated NaCh inactivation in synaptic depression, we compared frequency-dependent synaptic depression, using a baseline extracellular saline of 1 mM Ca²⁺ and 2 mM Mg²⁺ to reduce any contribution of depletion. We hyperpolarized unclamped terminals as in Fig. 4B, by omitting extracellular K⁺. As with paired stimuli, extracellular solution without K⁺ did not affect conditioning EPSC amplitude (2 ± 2% increase over that in 4 mM [K⁺]₀, n = 27), nor did it influence the frequency-dependent synaptic depression during the short train of stimuli (Fig. 4E, n = 19). A similar result was also obtained with a train stimulation protocol in which six stimuli at 50 Hz was followed by a single stimulus 50 or 100 ms later (data not shown). Thus a manipulation that speeds recovery from NaCh inactivation had no effect on PPD or depression during short trains of action potentials.

Depolarization between stimuli significantly slows recovery from NaCh inactivation and substantially enhances PPD and frequency-dependent depression

Hyperpolarization and increased Na⁺ driving force had little effect on EPSC PPD. However, it is possible that under conditions of intense physiological or pathophysiological activity, [K⁺]₀ accumulation would promote tonic depolarization that might slow NaCh inactivation, thus promoting additional EPSC PPD. In the hippocampus, rises in [K⁺]₀ of 6–9 mM above baseline occur with modest stimulation rates of 10 Hz for several seconds (Krnjevic et al. 1980; Somjen and Giacchino 1985). To test the influence of such rises, we used increased [K⁺]₀ to depolarize unclamped processes (Prakriya and Mennerick 2000). In current-clamp recordings, increasing [K⁺]₀ from 4 to 10 mM depolarized the resting potential of neuronal cell bodies from −66.9 ± 0.9 mV to −51.7 ± 0.8 mV (n = 33, Fig. 5A). Additionally, 10 mM [K⁺]₀ decreased the peak of conditioning action potentials by 5.8 ± 1.7 mV and increased action potential half-width by 0.32 ± 0.03 ms (n = 33). AP PPD increased dramatically in the presence of 10 mM [K⁺]₀ (Fig. 5B). At a 20-ms paired-pulse interval, AP PPD was −8 ± 3% in 4 mM [K⁺]₀ and −27 ± 6% in 10 mM [K⁺]₀. The half-amplitude width of test action potentials was broad-
the depletion hypothesis alone (but see Prakriya and Mennerick 2000). In contrast to the depletion prediction, PPD was enhanced at all three paired-pulse intervals tested (Fig. 6, A and C), again suggesting the existence of a release-independent component of PPD under depolarizing conditions. Similar to PPD in 4 mM [K⁺]₀, the degree of PPD in 10 mM [K⁺]₀ also depended on interstimulus interval (Fig. 6C). At the shortest interval tested (i.e., 20 ms), PPD increased from −28 ± 4% in 4 mM [K⁺]₀ to −75 ± 6% in 10 mM [K⁺]₀ (n = 6). PPD roughly doubled at the other paired-pulse intervals examined.

We also examined whether GABAergic IPSC PPD was similarly affected by increased [K⁺]₀. Because IPSCs are not as sensitive to NaCh block as EPSCs (Prakriya and Mennerick 2000), a smaller effect of elevated [K⁺]₀ on IPSCs would be consistent with a contribution of Na⁺ current inactivation to the increased EPSC PPD. Conditioning IPSCs were slightly potentiated by increased [K⁺]₀ up to concentrations of 12 mM (11 ± 7% increase, n = 7). Additionally, PPD of IPSCs, examined at an interval of 100 ms, was unaffected by ≤12 mM [K⁺]₀ (−41 ± 8% vs. −32 ± 16% PPD, n = 7, P = 0.45; Fig. 6, B and C). These data provide indirect evidence for the involvement of NaCh inactivation in the additional EPSC PPD observed during depolarization. In addition, the data suggest a major difference in the susceptibility of glutamate and GABA neurotransmission to tonic depolarization.

In 10 mM [K⁺]₀, there was significant depression of conditioning EPSCs (approximately 30%). While classical, depletion-dependent mechanisms predict a decrease in PPD with depressed conditioning EPSCs, we previously found that partial pharmacological block of NaChs prompted PPD by selectively blocking low pᵢ synapses (Prakriya and Mennerick 2000). Thus part of the increased PPD in 10 mM K⁺ may arise from an increase in steady-state NaCh inactivation rather than from slowing of recovery from inactivation after a conditioning action potential. To determine whether slowing of inactivation alone may contribute to the increase in PPD under depolarizing conditions, we examined the effect of small increases in [K⁺]₀ that had no effect on conditioning EPSCs and thus did not promote sufficient steady-state NaCh inactivation to depress basal transmission.

EPSC depression was augmented by as little as a 2-mM shift in [K⁺]₀, which had no significant effect on conditioning EPSCs. Figure 6D shows the effect of 6-mM total [K⁺]₀ on a 50-Hz train of EPSCs, similar to the protocol used in Fig. 4E. Compared with 4 mM [K⁺]₀, 6 mM [K⁺]₀ depolarized the resting potential of neuronal cell bodies from −66.6 ± 2.4 mV to −62.2 ± 2.3 mV (n = 12). This had no effect on the conditioning EPSC amplitude (2 ± 2% change, n = 34), excluding the contribution of a release-dependent mechanism to PPD. However, 6 mM [K⁺]₀ enhanced frequency-dependent synaptic depression during a train (Fig. 6D), including depression of the second EPSC of the train, equivalent to PPD. A test stimulus delivered 50 ms following the end of the train was not affected by 6 mM [K⁺]₀ (Fig. 6D, n = 34). Assuming involvement of NaCh inactivation in the augmented depression, this latter result suggests that the slow component of NaCh inactivation (approximately 1-s time constant in 4 mM [K⁺]₀) does not play a strong role in presynaptic depression, at least under these mildly depolarizing conditions. Because single action potentials may transiently raise local [K⁺]₀ by as much as 1 mM in intact tissue (Adelman and Fitzhugh 1975), these data...
suggest that release-independent frequency-dependent EPSC depression may become quite prominent during periods of intense physiological or pathophysiological activity.

To test more directly for a role of NaCh inactivation in the increased EPSC PPD observed under depolarizing conditions, we examined the effect of increased \([\text{Na}^+]_o\) on the EPSC PPD observed in elevated \([\text{K}^+]_o\). We again used a 40-mM change in \([\text{Na}^+]_o\) as an experimental manipulation designed to compensate for NaCh inactivation. As in the standard saline, increasing \([\text{K}^+]_o\) from 4 to 10 mM in the presence of 120 mM \([\text{Na}^+]_o\) increased PPD from \(-47 \pm 6\%\) to \(-79 \pm 5\%\) (Fig. 7A), with little effect on the conditioning EPSC in the eight cells tested.

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

**FIG. 7.** Increased \([\text{Na}^+]_o\) reduced the additional PPD induced by elevated \([\text{K}^+]_o\). A: effect of 10 mM \([\text{K}^+]_o\) (right traces) or 160 mM \([\text{Na}^+]_o\) (bottom traces). Note that the increase in PPD induced by elevated \([\text{K}^+]_o\) is less dramatic in 160 mM \([\text{Na}^+]_o\) than in 120 mM \([\text{Na}^+]_o\). Traces represent the averaged waveform of 3 repetitions in each condition. B: summary of the results from experiments like that depicted in A. As in Fig. 3, there was a small but statistically significant decrease in depression with elevated \([\text{Na}^+]_o\) in 4 mM \([\text{K}^+]_o\). A repeated measures ANOVA suggested that the effect of elevated \([\text{Na}^+]_o\) was greater in 10 mM \([\text{K}^+]_o\). * \(P < 0.05\), ** \(P < 0.01\).

\((-10 \pm 10\%\) change). As observed previously (Fig. 3, A and B), the increase from 120 to 160 mM \([\text{Na}^+]_o\) had a minor effect on PPD in 4 mM \([\text{K}^+]_o\) \((-43 \pm 6\%\), \(P = 0.03\)\). However, under the depolarizing condition, 160 mM \([\text{Na}^+]_o\) more effectively relieved PPD (Fig. 7, A and B; \(-79 \pm 5\%\) vs. \(-66 \pm 5\%\). A repeated measures ANOVA revealed a statistically significant interaction between \([\text{Na}^+]_o\) and \([\text{K}^+]_o\) on PPD (\(P < 0.01\), \(n = 8\)). These results strongly suggest that alteration of the \([\text{Na}^+]_o\) action potential, likely through increased NaCh inactivation, may become quite prominent during periods of intense physiological or pathophysiological activity.

**FIG. 6.** Effect of elevated \([\text{K}^+]_o\) on PPD. A: effect of a 6-mM increase in \([\text{K}^+]_o\) on EPSC PPD at 100-ms paired-pulse interval. Conditioning EPSCs have been scaled to highlight the increased depression. The inset shows unscaled conditioning EPSCs, which were little affected by increased \([\text{K}^+]_o\) in this cell. B: IPSCs also recorded at a 100-ms paired-pulse interval and with an increase of 8 mM \([\text{K}^+]_o\), showed little change in PPD and a moderate increase in conditioning IPSCs (inset). The traces in A and B represent averaged waveforms of \(\geq 2\) sweeps per condition. C: summary of the effect of 10 mM \([\text{K}^+]_o\), high \([\text{K}^+]_o\) (gray bars) on EPSC PPD at 20 ms (\(n = 6\)), 50 ms (\(n = 10\)), and 100 ms (\(n = 15\)). ** \(P < 0.01\). Also shown is a summary of the effect of 12 mM \([\text{K}^+]_o\), high \([\text{K}^+]_o\) on IPSCs at the 100-ms paired-pulse interval (\(n = 7\)). D: an increase of as little as 2 mM \([\text{K}^+]_o\) (6 mM total) significantly enhanced depression at each time point during a train of 6 pulses (\(n = 34\), \(P < 0.01\) paired \(t\)-tests, indicated by asterisks). However, a test pulse delivered 50 ms after the final pulse of the train showed that the depression induced by 6 mM \([\text{K}^+]_o\) had already recovered to 4-mM levels. This may suggest that the additional depression induced in 6 mM \([\text{K}^+]_o\) is due to slowing of fast inactivation rather than to promotion of slow inactivation. To limit release-dependent depression of transmission, all experiments in this panel were performed in extracellular solution containing 1 mM \([\text{Ca}^{2+}]_o\) and 2 mM \([\text{Mg}^{2+}]_o\).
tivation, is responsible for the increased PPD observed in the presence of 10 mM [K\textsuperscript{+}]\textsubscript{o}. Like the relief from riluzole block (Fig. 3C), the larger relief of PPD by elevated [Na\textsuperscript{+}]\textsubscript{o} under depolarizing conditions suggests that the minor effect of elevated [Na\textsuperscript{+}]\textsubscript{o} under baseline conditions (i.e., 4 mM [K\textsuperscript{+}]\textsubscript{o}) is not due to ineffectiveness of the increased extracellular [Na\textsuperscript{+}]\textsubscript{o} but rather to a small contribution of NaCh inactivation to basal PPD.

As another test that NaCh inactivation participates prominently in the increased PPD under depolarizing conditions, we employed the use-dependent NaCh blocker, riluzole. Riluzole has little effect on NaChs at rest, but its apparent affinity is much greater for inactivated NaChs (Hebert et al. 1994; Prakriya and Mennerick 2000; Taylor and Meldrum 1995). In concentration-response experiments, we found that the threshold for riluzole effects on individual EPSCs occurred at approximately 0.5 \mu M (data not shown). In 4 mM [K\textsuperscript{+}]\textsubscript{o}, 0.5 \mu M riluzole depressed conditioning EPSCs by 8 \pm 2% and did not significantly increase PPD at a 20-ms interval (−41 \pm 4% baseline, −43 \pm 4% in 0.5 \mu M riluzole, P = 0.09; n = 17, Fig. 8, A and B). This suggests that neither a riluzole-induced increase in steady-state inactivation nor slowed recovery from inactivation affects PPD under basal conditions. In modestly elevated [K\textsuperscript{+}]\textsubscript{o} (6−7 mM total extracellular [K\textsuperscript{+}]\textsubscript{o}), PPD was significantly increased (−41 \pm 4% to −47 \pm 4%, P < 0.001, n = 17). Importantly, this increased PPD was augmented even further by 0.5 \mu M riluzole (−47 \pm 4% vs. −58 \pm 5%, P < 0.001; Fig. 8, A and B). However, conditioning EPSCs were not affected by either the elevated [K\textsuperscript{+}]\textsubscript{o} (−2 \pm 2% change), or the riluzole (−12 \pm 3%, P = 0.16 compared with riluzole’s effects on conditioning EPSCs in 4 mM [K\textsuperscript{+}]\textsubscript{o}). These data strongly suggest that NaCh inactivation becomes more prominent in elevated [K\textsuperscript{+}]\textsubscript{o}, and contributes to EPSC PPD. Specifically, because the increases in PPD with elevated [K\textsuperscript{+}]\textsubscript{o} and riluzole were independent of changes in conditioning EPSCs, these data suggest that slowing of recovery from inactivation (apart from increased steady-state inactivation) plays a prominent role in the increased synaptic depression.

**DISCUSSION**

PPD is a robust phenomenon at many synapses in the CNS and PNS (Zucker 1989, 1999). Although vesicle depletion has been proposed to explain the presynaptic component of depression at many synapses (Betz 1970; Mennerick and Zorumski 1995; Zucker 1989, 1999), other presynaptic mechanisms have been suggested to explain discrepancies between the depletion model predictions and experimental observations. To explain an observed lack of proportionality between pharmacologically induced presynaptic depression and frequency-dependent depression, a release-independent mechanism was recently proposed, which could include NaCh inactivation and associated depression of action-potential signaling (Brody and Yue 2000).

Because glutamate release is highly sensitive to partial NaCh blockade (Prakriya and Mennerick 2000), and because somatic action potentials are clearly depressed by NaCh inactivation at synaptically relevant frequencies, we examined the potential role of NaCh inactivation in EPSC PPD in hippocampal neurons in culture.

We fail to find evidence for a strong role of NaCh inactivation in PPD under basal conditions. The evidence for this conclusion is threefold. First, increasing Na\textsuperscript{+} driving force only mildly relieved PPD while more dramatically diminishing EPSC depression induced by riluzole and PPD induced by elevated [K\textsuperscript{+}]\textsubscript{o}. Second, hyperpolarization with either direct voltage pulses or omitting [K\textsuperscript{+}]\textsubscript{o} did not diminish PPD or depression during trains of impulses. Finally, the inactivation-dependent NaCh blocker riluzole, at concentrations that very slightly inhibited conditioning EPSCs, did not significantly affect basal PPD.

On the other hand, several results implicate a role of NaCh inactivation in the increased PPD observed in elevated [K\textsuperscript{+}]\textsubscript{o}. As mentioned above, increased Na\textsuperscript{+} driving force more effectively relieved PPD in 10 mM [K\textsuperscript{+}]\textsubscript{o} than PPD in basal 4 mM [K\textsuperscript{+}]\textsubscript{o}. In addition, riluzole at a concentration that has no effect on basal PPD, significantly increased PPD in moderately elevated [K\textsuperscript{+}]\textsubscript{o}. We therefore suggest that NaCh availability is poised to influence the efficacy of synaptic transmission, depending on the depolarization state of the axon.

Unfortunately, pharmacological tools that interfere directly with NaCh inactivation did not prove useful in dissecting the role of NaCh inactivation in PPD. This is because these agents dramatically interfered with conditioning action potentials, prolonging them by approximately 40%. Paradoxically, these agents slightly increase EPSC PPD and AP PPD. The increased AP PPD is likely due to a combination of increased depolar-
depression induced by Ca\(^{2+}\) such as those examining Cd\(^{2+}\) (Stevens 1996; Stevens and Tsujimoto 1995). Yet some results, apsises (Mennerick and Zorumski 1995; Rosenmund and Yue 2000)? First, the actual quantitative contribution of release-dependent depression to total PPD remains uncertain. Earlier work suggests a strong component of release-dependent synaptic depression that increases action potential depression promotes EPSC PPD.

Because blockers of NaCh inactivation proved inadequate for evaluating the role of NaCh inactivation in basal PPD, we were forced to use more indirect tools to test a role of NaCh inactivation in EPSC depression. We previously found that increased extracellular sodium can partially compensate for pharmacological NaCh blockade (Prakriya and Mennerick 2000). In the present study, this manipulation was used to compensate for putative NaCh inactivation produced by conditioning action potentials. While indirect, altering [Na\(^{+}\)]\(_o\) driving force exhibits selectivity; increased [Na\(^{+}\)]\(_o\), partially rescued synaptic transmission from pharmacological NaCh block and PPD in high [K\(^{+}\)]\(_o\), but it did not relieve synaptic depression induced by Ca\(^{2+}\) channel block.

Given that we failed to find evidence for a substantial contribution of NaCh inactivation to basal PPD, it seems likely that presynaptic terminal action potentials behave differently than the somatic action potentials measured directly in our current-clamp experiments. Indeed, a substantial difference between the shape of somatic and presynaptic action potentials was recently observed in hippocampal slices (Geiger and Jonas 2000). Our results might be explained if terminal NaChs recover from inactivation faster than somatic NaChs. Inactivation is regulated by phosphorylation (Catterall 2000; Franseschetti et al. 2000), which may differ between the cell body and presynaptic elements. It is also possible that other short-term modulatory mechanisms at presynaptic terminals, such as synaptic facilitation (Magleby 1987; Zucker 1989, 1999) could mask the contribution of NaCh inactivation to PPD. Finally, our results could be explained if terminal NaChs exhibit higher pharmacological sensitivity to blockers than somatic NaChs. This may be unlikely as presynaptic channels would need to exhibit high sensitivity both to riluzole and to TTX (Prakriya and Mennerick 2000), representing two different classes of NaCh blockers. However, this hypothesis would account for the apparent strong sensitivity of glutamate release to low concentrations of TTX and riluzole, compared with weak effects of these drugs on somatic action potentials. If a difference in pharmacological sensitivity exists, then greater terminal AP PPD than that observed in Fig. 1 may be needed to substantially depress glutamate release. NaChs with low TTX sensitivity have been described in CNS neurons (White et al. 1993), but whether this relative insensitivity extends to other classes of NaCh blockers is unknown.

If NaCh inactivation does not strongly contribute to PPD under normal conditions, what is the source of the apparent release-independent PPD observed (see RESULTS) (Brody and Yue 2000)? First, the actual quantitative contribution of release-independent depression to total PPD remains uncertain. Earlier work suggests a strong component of release-dependent PPD, likely representing vesicle depletion, at these same synapses (Mennerick and Zorumski 1995; Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995). Yet some results, such as those examining Cd\(^{2+}\) effect on PPD (see RESULTS), appear inconsistent with the depletion hypothesis as the sole explanation of PPD. Augmented K\(^{+}\) currents could be the source of release-independent PPD, but we would have expected an increase in PPD with [K\(^{+}\)]\(_o\) removal (Fig. 4) if this were the major contributor. Rather, it may be more likely that downstream events not directly related to depletion may participate in PPD. At the calyx of Held synapse, it was recently suggested that intense stimulation results in decreased p, in addition to vesicle depletion (Wu and Borst 1999). A similar mechanism could also be active in hippocampal synapses, as could other forms of functional adaptation of vesicle release machinery (Hsu et al. 1996; Waldeck et al. 2000). Ca\(^{2+}\) channel inactivation may potentially play a role in release-independent depression. However, we observed no effect of raised [Na\(^{+}\)]\(_o\) on Cd\(^{2+}\) inhibition of EPSCs (see RESULTS). Therefore direct inactivation of Ca\(^{2+}\) channels is unlikely to underlie the component of release-independent synaptic depression that is sensitive to elevated [Na\(^{+}\)]\(_o\) in normal solutions and in elevated [K\(^{+}\)]\(_o\).

At presynaptic terminals where the question of action potential coupling to Ca\(^{2+}\) influx and transmitter release can be studied more directly, action potential waveform changes have variable effects on Ca\(^{2+}\) influx and transmitter release (Borst and Sakmann 1999; Charlton et al. 1982; Sabatini and Regehr 1997). It is likely that the details of action potential coupling to transmitter release will vary among synapses of different morphology and cell type. In fact, from our results, we conclude that even among cells from the same animals maintained under identical conditions, NaCh inactivation under depolarizing conditions is differentially involved in glutamate versus GABA release. This has interesting implications for synaptic transmission during intense activity. The present work shows a clear difference in susceptibility of EPSC and IPSC PPD to elevated [K\(^{+}\)]\(_o\); IPSC PPD was relatively unaffected by depolarization, while EPSC PPD was nearly doubled at the same paired-pulse interval. Additionally, conditioning EPSCs were slightly depressed by elevated [K\(^{+}\)]\(_o\), while conditioning IPSCs were slightly potentiated. During single action potentials, local extracellular [K\(^{+}\)]\(_o\) may be increased by as much as 1 mM (Adelman and Fitzhugh 1975), and much larger increases in [K\(^{+}\)]\(_o\) may be achieved during trains of intense activity (Krnjevic et al. 1980; Malenka et al. 1981; Somjen and Giacchino 1985). In addition, background synaptic activity may significantly depolarize the resting potential of neurons (Destexhe and Pare 1999), although it is unknown whether this effect is relevant to presynaptic terminals. The relative insensitivity of IPSCs to increases in [K\(^{+}\)]\(_o\) ≤12 mM parallels the relative insensitivity of GABA release to partial pharmacological NaCh block (Prakriya and Mennerick 2000). The differences in susceptibility of IPSC and EPSC frequency-dependent depression in the face of depolarization has interesting implications for synaptic modulation. The relative persistence of GABAAergic transmission under conditions of tonic depolarization may provide a negative feedback mechanism that limits the possibility of runaway excitation.

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REFERENCES


