N-Ethylmaleimide Blocks Depolarization-Induced Suppression of Inhibition and Enhances GABA Release in the Rat Hippocampal Slice *In Vitro*

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Regulation of synaptic, $GABA_A$ receptor-mediated inhibition is a process of critical importance to normal brain function. Recently, we have described a phenomenon in hippocampus of a transient, yet marked, decrease in spontaneous, $GABA_A$ receptor-mediated IPSCs after depolarization activated Ca^{2+} influx into a pyramidal cell. This process, depolarization-induced suppression of inhibition (DSI), is absent in hippocampal cells that previously had been exposed to pertussis toxin *in vivo*, implicating a G-protein in the DSI process. To circumvent the problem that a single cell cannot be studied before and after G-protein block using the pertussis toxin pretreatment method, we have used the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM), which blocks pertussis toxin-sensitive G-proteins, to determine whether acute inhibition of G-proteins can eliminate DSI of spontaneous IPSCs (sIPSCs). In whole-cell recordings from CA1 pyramidal cells that were first determined to express DSI, we have found that NEM does block DSI of sIPSCs. We also report that DSI of monosynaptic, evoked IPSCs is blocked by NEM, suggesting that a similar mechanism underlies both forms of DSI. It was of interest that DSI was abolished at a time when NEM had increased, not decreased, GABA transmission. Indeed, NEM greatly increased quantal GABA release by a Ca^{2+}-independent mechanism, an observation with potentially important implications for understanding synaptic GABA release.

Key words: NEM; hippocampus; GABA; mIPSCs; transmitter release; IPSC

DSI is a phenomenon whereby activation of certain principal cells in the CNS sufficient to cause voltage-dependent Ca^{2+} influx is followed by a reduction in the GABAergic synaptic input onto the cells for a period of 1–2 min. DSI seems to involve a retrograde signal that is generated in the principal cell and inhibits the output from the presynaptic interneuron (Alger and Pitler, 1995). DSI has been found in hippocampal pyramidal cells (Pitler and Alger, 1992, 1994) and cerebellar Purkinje cells (Llano et al., 1991; Vincent et al., 1992; Vincent and Marty, 1993).

DSI is not observed in slices from hippocampi of pertussis toxin-treated animals (Pitler and Alger, 1994), implicating a pertussis toxin-sensitive G-protein in the DSI signal pathway. The results of the pertussis toxin test were clear, but the test is time consuming and laborious. Most important, it suffers from the drawback that the same cell cannot be studied before and after G-protein block. This limits the types of experiments that can be performed and the conclusions that can be drawn. Intracellular manipulation of G-protein function suffers from some of the same limitations. Hence, a more convenient method for readily manipulating G-protein function would be very useful.

Thus far, DSI has been studied as the transient reduction in spontaneously occurring, action potential-dependent IPSCs (sIPSCs). We have observed what seems to be DSI of evoked, monosynaptic IPSCs. To determine whether suppression of evoked IPSCs involves the same mechanism as does DSI of sIPSCs, the G-protein sensitivity of the DSI of the evoked IPSCs should be tested. However, unlike DSI of sIPSCs, the occurrence of DSI of evoked IPSCs is variable, occurring only 50–75% of the time. A convincing test of the G-protein role in DSI of evoked IPSCs requires knowing that a given cell is capable of DSI before blocking the G-proteins. Pretreating hippocampi with pertussis toxin would be a very inefficient procedure.

NEM is a sulfhydryl alkylating agent that can block pertussis toxin-sensitive G-protein actions in peripheral (Shapiro et al., 1994) and invertebrate (Fryer, 1992) neurons. In pharmacological experiments, NEM can decouple G-protein receptors from their substrates in central neurons (e.g., Kitamura and Nomura, 1987; Shinoda et al., 1990); there are very few data on the physiological actions of NEM in CNS, however. The present experiments have two purposes: (1) generally, to determine whether NEM is a useful tool for the study of G-protein-mediated actions in the hippocampal slice; and (2) specifically, to test the hypothesis that DSI of sIPSCs and of evoked monosynaptic IPSCs will be similarly affected by NEM. We used whole-cell recordings in the rat hippocampal slice to examine these issues.

We find that NEM blocks pertussis toxin-sensitive $GABA_B$ actions and that NEM blocks DSI of sIPSCs and of evoked IPSCs, implying an underlying similarity in the two processes. Unexpectedly, we also find that NEM greatly increases the release of GABA from presynaptic nerve terminals.

MATERIALS AND METHODS

Preparation of slices. After adult male Sprague Dawley rats (125–250 gm) were deeply anesthetized with halothane and decapitated, the brain was removed and the hippocampi dissected free. Hippocampi were placed on

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an agar block in a slicing chamber containing partially frozen saline and sectioned transversely at 400 μm intervals with a Vibratome (Technical Products International). The slices recovered in a holding chamber at the interface of a physiological saline and humidified 95% O_2/5% CO_2 atmosphere at room temperature. After a minimum 1 hr incubation, a single slice was transferred to a submerged, perfusion-type chamber (Nicoll and Alger, 1981), where it was perfused at 0.3–1.0 ml/min at 29–31°C.

Solutions. Patch electrodes with resistances of 3–6 MΩ were filled with 145–160 mM CsCl, 2 mM BAPTA, 0.2 mM $CaCl_2$, 1 mM MgATP, 1 mM $MgCl_2$, 5 mM 2-(triethylamino)-*N*-(2,6-dimethylphenyl)acetamide (QX-314), 10 mM HEPES, and 0.3 mM Tris-GTP, pH 7.35. QX-314 blocks postsynaptic $GABA_B$ responses (Nathan et al., 1990; Andrade, 1991) and certain K^+ channels (Andrade, 1991; Oda et al., 1992), as well as Na^+ action potentials (Hille, 1992). In a few cells, as noted, KCl or KCH_3SO_3 replaced CsCl. Extracellular saline contained 124 mM NaCl, 26 mM $NaHCO_3$, 3.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, and 10 mM glucose. CNQX (10 or 20 μM) and APV (50 μM) were present in all experiments to block ionotropic glutamate responses. TTX (0.5 μM) was present for experiments on miniature IPSCs (mIPSCs). Carbachol (10–25 μM) enhances sIPSCs (Pitler and Alger, 1992a) and DSI of sIPSCs (Pitler and Alger, 1994) and was present for all experiments on sIPSCs, but not for experiments on mIPSCs or evoked IPSCs.

CNQX, baclofen, and 4,5,6,7,-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP) were purchased from Research Biochemicals (Natick, MA), BAPTA from Molecular Probes (Eugene, OR), and TTX from Calbiochem (La Jolla, CA). QX-314 was a generous gift from Astra (Sodertalje, Sweden) or was purchased from Alamone Labs (Jerusalem, Israel). All other drugs and chemicals were obtained from Sigma (St. Louis, MO). Drugs were either iontophoretically applied or bath-applied.

Whole-cell recordings and data analysis. Data reported in this paper were obtained from 45 cells. CA1 pyramidal cell recordings were obtained using the "blind," whole-cell, patch-clamp recording technique (Blanton et al., 1989). Cells were voltage-clamped near their resting potential immediately after break-in. Acceptable cells had resting potentials more than or equal to −55 mV and input resistances >40 MΩ. Series resistance was <12 MΩ at the beginning of an experiment and was compensated by ~80%, except for experiments on mIPSCs. Cells were discarded if series resistance increased to >30 MΩ during an experiment. Liquid junction potentials were small and were not corrected for.

Iontophoresis of baclofen or THIP was carried out in some experiments. Baclofen was solubilized at 40 mM in 100 mM NaCl and acidified to pH 3 using 1 M HCl. THIP was solubilized at 50 mM in distilled water at pH 3.5. Both baclofen and THIP were present at full strength in the iontophoretic pipettes. Drugs were ejected from glass pipettes positioned close to the recording pipette. Iontophoretic currents of 100–500 nA lasting either 1 or 2 sec were used.

DSI of sIPSCs was quantified in some experiments by integrating (using the Fetchan subroutine of pClamp 6.0, Axon Instruments) the current traces over 2 sec time bins for 10 sec before and for ≥60 sec after the depolarizing current pulse. This gives a measure of total charge crossing the membrane and is a robust index of the amount of synaptic activity present. Spontaneous IPSCs were filtered at 1 kHz and digitized at 5 kHz. The baseline for integration of each trace was set manually, and rigorous visual inspection of all traces was performed to ensure that the analysis was not corrupted by the presence of events with aberrant waveforms or by periods of unstable holding current fluctuation. The quantitative results using this method faithfully agreed with the conclusions based on visual inspection of the data. For experiments on evoked monosynaptic IPSCs, afferent stimuli were delivered, in different experiments, to either stratum (st.) radiatum, st. pyramidale, or st. oriens, continuously at 0.5 or 1 Hz. A 1 sec depolarizing step to near 0 mV to induce DSI was delivered every 90 or 120 sec. Evoked IPSCs were filtered at 2 or 3 kHz and stored on VHS videotape. Responses were digitized at 10 or 20 kHz and analyzed with pClamp. IPSCs were averaged at each time interval over three to six complete DSI trials. To quantify DSI of evoked IPSCs, we compared the mean of 6–12 IPSCs in the control (pre-DSI) period with the mean of the same number of IPSCs after the DSI step. Because DSI is often not maximal immediately after the step, but can take from 1 to 3 sec to develop (Pitler and Alger, 1994), we usually omitted the first two or three IPSCs during the DSI period.

Effects of NEM on control IPSC amplitudes and DSI were assessed by one-way ANOVA with repeated measures followed by Tukey's *post hoc* test for multiple comparisons, or by Kruskal–Wallis ANOVA on Ranks with Dunn's *post hoc* test in Sigma Stat 3.0 (Jandel Scientific, San Rafael, CA). A parametric test could not always be used because NEM significantly reduces IPSC amplitude variance (see Results). Kolmogorov–Smirnov (K–S) tests of cumulative frequency distributions were used to assess the significance of NEM effects on mIPSCs. For K–S tests, a significance level of $p < 0.005$ was chosen. Averaged cumulative amplitude distributions were obtained by normalizing individual cumulative amplitude distributions to the median amplitude of the corresponding control distributions. Data from individual cells were combined and averaged by calculating the normalized amplitude at fixed cumulative frequency intervals. Statistical significance of differences between coefficients of variation (CVs) was assessed according to Zar (1984); the significance level chosen was $p < 0.05$. *t* tests were otherwise used to determine significance of effects ($p \leq 0.05$), and all data are reported as mean ± SEM.

In initial experiments, we prepared NEM as a stock solution at 250 mM in distilled water. At this concentration, NEM crystals did not seem to be readily soluble, and it was necessary to crush the crystals and vigorously sonicate the suspension before use. In more recent experiments, NEM has been solubilized in DMSO at a concentration of 500 mM. At the experimental doses of 250 or 300 μM NEM, DMSO (0.05–0.06%) had no effect on cellular properties by itself. Trial and error established that NEM, applied at 250 or 300 μM for 10–15 min at our relatively slow perfusion rate, had marked effects on IPSCs. Lower concentrations or much shorter applications often had much less effect. NEM concentrations higher than 300 μM, or applications prolonged beyond 20 min, profoundly depressed inhibitory synaptic transmission. The effects of NEM that we have recorded seem to be irreversible with up to 1 hr of washing.

RESULTS

We used responses to the pertussis toxin-sensitive, G-protein-dependent action of the $GABA_B$ receptor agonist baclofen to determine whether NEM blocks G-protein effects. In neocortical slices, Ong and Kerr (1995) found that baclofen-induced depression of epileptiform discharges can be prevented by NEM, but they did not examine the baclofen response at the cellular level. In whole-cell recordings from hippocampal CA1 pyramidal cells, we found that 250 μM NEM applied for 10–12 min blocked the outward current induced by iontophoretic application of baclofen (Fig. 1). QX-314 was not present, and KCH_3SO_3 was the predominant salt in the electrode solution for these experiments. In control solution, multiple applications of baclofen to each cell yielded a mean peak of $I_{baclofen}$ of 32.7 ± 2.3 pA, whereas in the same cells after NEM treatment it was only 10.3 ± 3.5 pA ($n = 3$). The difference was significant at $p < 0.02$ (paired *t* test). NEM had no marked effects on holding current or input conductance of the cells with these perfusion times. Baclofen can also decrease IPSCs by acting on presynaptic $GABA_B$ receptors (Davies et al., 1990). In two voltage-clamped cells, we noted that NEM reduced the presynaptic inhibition of IPSCs induced by iontophoretic baclofen from 55 and 69% to 20 and 12%, respectively (compare *inset traces* in Fig. 1).

In whole-cell recordings from CA1 pyramidal cells filled with Cl^--based salt solution, sIPSCs are visible in the presence of CNQX and APV. The sIPSC frequency is enhanced by carbachol, which also blocks possible confounding effects of K^+ currents. As reported previously (Pitler and Alger, 1994), a 1 sec voltage step from the holding potential to 0 mV was followed after a brief delay by a dramatic reduction in the occurrence of sIPSCs for ~1 min, after which the sIPSCs recovered (Fig. 2*A*, *left column*). This is the period of DSI. After establishing that DSI did occur in a given cell, we bath-applied NEM. In every case (9 of 9 cells), NEM dramatically reduced DSI of sIPSCs (e.g., Fig. 2*A, right column*). NEM had no major effects on the holding current or input conductance of the cells, although in some cases with KCH_3SO_3-containing electrodes an outward current of ~50 pA developed. Figure 2*A*, *right column*, illustrates, however, that

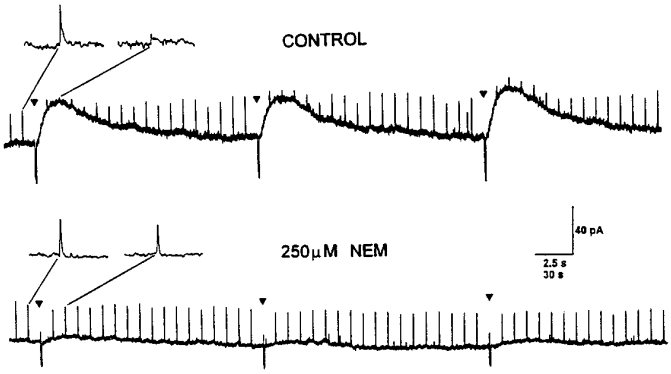

Figure 1. NEM blocks iontophoretic baclofen responses. Whole-cell voltage-clamp recordings from a CA1 pyramidal cell in the presence of APV and CNQX (see Materials and Methods). *Sharp upward deflections* represent monosynaptic GABA IPSCs elicited at 10 sec intervals. The slow outward currents were elicited by iontophoretic ejection of baclofen at 3 min intervals shown by *arrowheads* (350 nA for 1 sec; iontophoretic pipette contained 40 mM baclofen). Responses in the *top row* were obtained in control solution. In control solution, baclofen also reduced the evoked IPSCs, probably via activation of presynaptic $GABA_B$ receptors. *Lower traces* were obtained immediately after a 10 min application of 250 μM NEM. Note that NEM virtually abolished the postsynaptic baclofen response with very little effect on the baseline $GABA_A$ IPSC, but the IPSCs were no longer reduced by baclofen. The results suggest that NEM can block the G-protein-coupled responses mediated by both presynaptic and postsynaptic $GABA_B$ receptors. KCH_3SO_3-containing electrodes without QX-314 included were used in these experiments. $V_H = -60$ mV.

NEM increased sIPSC activity during the control period. The integral of the sIPSC activity over time represents the total charge transfer across the membrane, a convenient measure for quantifying the total amount of IPSC activity. Figure 2*B* represents group data from three typical cells subjected to three DSI trials in control and then three after applying NEM. NEM increased the charge transfer by an average of 26.7 ± 0.1%. Comparison of activity in 10 sec blocks before and after the DSI-inducing pulse in the present experiments reveals that, whereas DSI represented a 70.2 ± 2.1% reduction in charge transfer before NEM application, the same DSI-inducing voltage pulse produced only a 14.3 ± 1.4% reduction after NEM (significant at $p < 0.001$). The effect of NEM on DSI was gradual, and it took ~8 min of NEM application before the maximal block of DSI was seen (e.g., Fig. 3). The effects of NEM on DSI were irreversible with up to 1 hr of washing. The results confirm the prediction that NEM would block DSI of spontaneous IPSCs.

We next addressed the issue of whether NEM would affect DSI of evoked monosynaptic IPSCs. Monosynaptic IPSCs (evoked in the absence of carbachol with moderate to high stimulus intensities at a frequency of 1 or 0.5 Hz) underwent a brief period of decline when the stimuli began but then stabilized. Shortly after a DSI-inducing voltage step was delivered to a pyramidal cell, monosynaptic IPSC amplitudes were depressed for a period of ~1 min before recovering fully (e.g., Fig. 4*A*), a time course that closely mimics that of DSI of sIPSCs. If suppression of monosynaptic IPSCs does in fact represent DSI, then it should be blocked by NEM. We tested this prediction, and in 9 of 10 cells we found that DSI was clearly reduced by NEM (e.g., Fig. 4*B*). Of five cells having IPSCs of comparable size and all subjected to identical experimental protocols, an ANOVA revealed that NEM entirely abolished DSI in four cells (i.e., in NEM there was no statistically significant difference in any cell between IPSCs during the DSI period and in the immediately preceding control period) and reduced it in the fifth. For the group, the mean decrease in the IPSC amplitude during DSI was 32.8 ± 6.1% in control but only 7.8 ± 3.6% in NEM, a significant reduction ($p < 0.01$; $n = 5$).

It was of interest that, as also shown in Figure 4*B*, the monosynaptic IPSC amplitudes, like the sIPSC activity, were consistently increased in NEM during the time at which NEM had blocked DSI. ANOVA indicated that this increase was significant in each cell ($n = 5$). For the group, the mean increase caused by NEM was 43.4 ± 10.0%. As with sIPSCs, the NEM effect on evoked IPSCs was of gradual onset and, after the period of enhanced responsiveness induced by NEM, evoked IPSC amplitudes gradually became depressed.

Because bath-applied NEM could affect both presynaptic and postsynaptic cells, we wished to determine where NEM actually exerted its effects on GABAergic transmission. The increase in evoked and spontaneous IPSCs could, in principle, be explained either by increased release of GABA or increased postsynaptic $GABA_A$ receptor responsiveness.

We began by investigating the responses to iontophoretic application of the selective $GABA_A$ agonist, THIP, and comparing THIP responses and monosynaptic IPSCs in the same cells before and during NEM application. At 2 min intervals, we elicited an IPSC and then, 15 sec later, a THIP response. After a control period of at least 6 min of stable responses, we applied 300 μM NEM for 11 min. As can be seen in Figure 5, the THIP responses were virtually unaltered at the same time the monosynaptic IPSCs were substantially increased. Typical responses are shown for one cell in Figure 5*A* and the group data from all five cells in Figure 5*B,C*. The right-most traces in Figure 5*A* demonstrate that both THIP responses and IPSCs were abolished by bicuculline, as expected. After 10 min in NEM, the mean IPSC amplitude had significantly increased to 125.8 ± 9.7% of control ($p < 0.05$), whereas the THIP response was unaltered (95.3 ± 3.8% of control; $n = 5$). Hence, despite its increase in synaptically evoked $GABA_A$ responses, NEM did not affect $GABA_A$ receptors as determined by iontophoresis. These observations suggested that NEM increased IPSCs by a presynaptic mechanism.

Two other pieces of evidence provide further support for this conclusion. In the presence of TTX, the largest spontaneous IPSCs are abolished, indicating they are dependent on action potential firing in the interneurons (Alger and Nicoll, 1980; Collingridge et al., 1984). Remaining in TTX, however, is a population of small, spontaneous IPSCs that represent quantal or mIPSCs (Collingridge et al., 1984; Edwards et al., 1990). We found that NEM substantially increased mIPSC frequency. The increase was of gradual onset (e.g., Fig. 6, *top trace*) but became very substantial in magnitude. Addition of 10 μM bicuculline to the bath (Fig. 6, *arrow, top trace*) blocked all spontaneous activity, indicating that the events were $GABA_A$ mIPSCs. The mIPSC frequency increased from 4.4 ± 0.8 Hz in control solution to 33.9 ± 5.1 Hz in NEM ($n = 6$). The difference is significant at $p \leq 0.004$. The NEM-induced increase in mIPSC frequency is most simply explained as a presynaptic action. There was no change in the mIPSC amplitude distributions in five of six cells as determined by K–S tests on individual cells. The cumulative frequency distribution of mIPSC amplitudes for the group data are shown in Figure 6*C*. In the group data, before NEM the mean mIPSC amplitude was 13.2 ± 2.5 pA, whereas in NEM it was 15.3 ± 3.3 pA ($n = 6$; numbers of mIPSCs per cell: 113–700 in control, 197–824 in NEM).

In principle, NEM might increase mIPSC frequency by increasing voltage-dependent Ca^{2+} influx into presynaptic nerve termi-

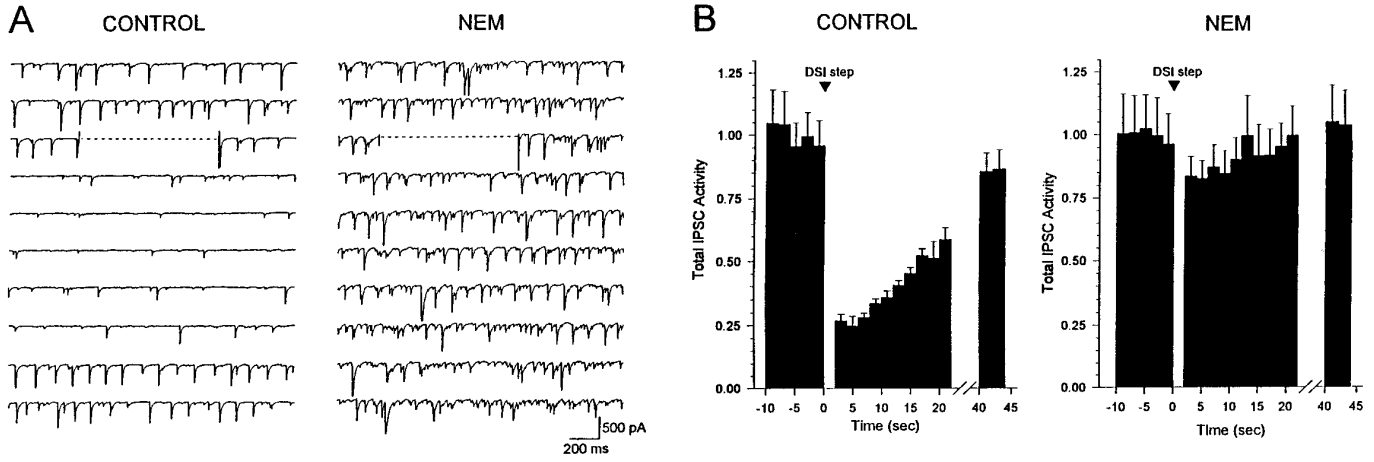

Figure 2. NEM blocks DSI of spontaneous $GABA_A$ergic IPSCs. *A*, Traces of spontaneous monosynaptic (50 μM APV, 10 μM CNQX, and 10 μM carbachol were present) IPSCs recorded under whole-cell voltage clamp in a CA1 pyramidal cell. At the intervals marked by *dotted lines*, the cell was depolarized to 0 mV ($V_H = -60$ mV) for 1 sec. In control conditions (*left*), the depolarizing pulse was followed by a period of markedly suppressed sIPSC activity. In the *right column*, traces from the same cell 3 min after a 7 min perfusion with 250 μM NEM demonstrate that NEM blocked IPSC suppression. Traces in each column were consecutive except for the *lowest two*, which were recorded 1 min after the pulse and show recovery from DSI. *B*, Histograms represent data from three experiments such as that shown in *A*; each cell was recorded first in control saline and then again after NEM treatment. Total IPSC activity was quantified by integrating the baseline activity versus time, yielding a measure of net inward charge crossing the membrane. Data were grouped in 2 sec bins. Total activity in each bin was then normalized to the amount of activity in the pre-DSI period for each cell and then averaged across cells. At time 0 (*downward arrowhead*) a 1 sec, 60 mV depolarizing step ($V_H = -60$ mV) was given to induce DSI [represented by the period of suppression of activity in the graph of control data (*left*)]. Data from three trials were obtained from each cell in each condition.

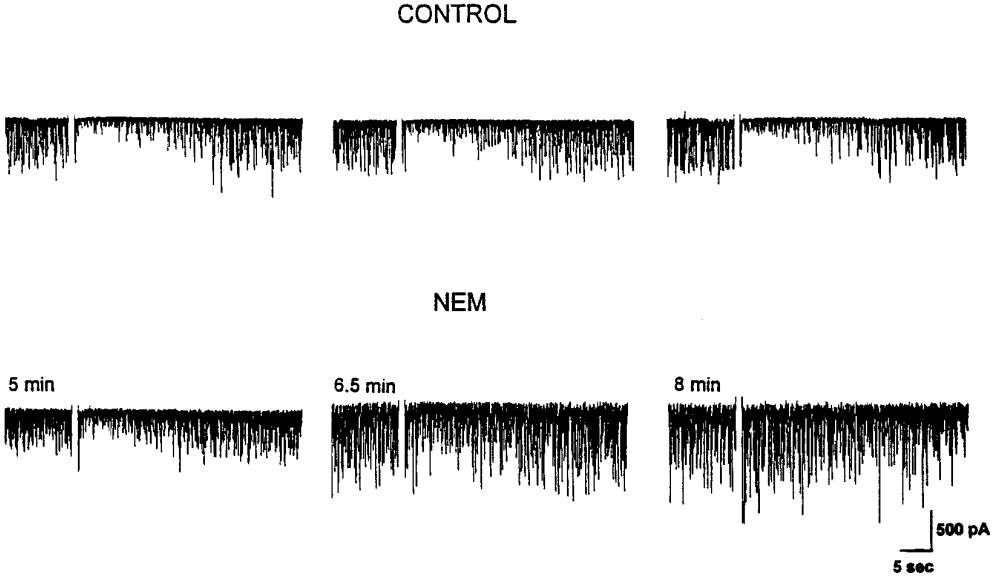

Figure 3. The onset of the NEM effect was gradual and often associated with an increase in sIPSC activity. The *top three traces* were from consecutive DSI trials in control conditions (10 μM CNQX, 50 μM APV, and 10 μM carbachol). The *bottom three traces* were recorded at the indicated times after beginning perfusion with 250 μM NEM. The *small gaps* indicate when 1 sec depolarizing voltage steps to 0 mV were given.

nals, as does perfusion with an elevated K^+-containing solution (Doze et al., 1995), despite the presence of TTX. However, NEM still increased TTX-resistant mIPSC frequency, even in the absence of added external Ca^{2+} (nominally 0 mM $[Ca^{2+}]_o$) and 4 mM $[Mg^{2+}]_o$ ($n = 6$; numbers of mIPSCs per cell: 150–334 in control, 370–1563 in NEM). There was no change in the mean mIPSC amplitudes in NEM (21.1 ± 5.53 pA to 21.5 ± 2.75 pA, control and NEM, respectively). Even in a bath solution with 8 mM Mg^{2+}, 0 Ca^{2+}, and 100 μM EGTA, NEM still markedly increased mIPSC frequency (Fig. 7) ($n = 4$; numbers of mIPSCs per cell: 120–660 in control, 799–2182 in NEM). Combining data from all three conditions (TTX alone; TTX in combination with 0 Ca^{2+}, 4 Mg^{2+}; or TTX plus 0 Ca^{2+}, 8 Mg^{2+}, 100 μM EGTA; a one-way ANOVA indicated mIPSC frequencies in the control groups did not differ) indicated that NEM caused an increase from 4.8 ± 0.67 Hz to 24.1 ± 4.6 Hz ($n = 16$), a difference that was significant at $p < 0.001$ (paired t test). When all data were taken together, there was no difference in mean mIPSC amplitudes between control and NEM (20.0 ± 2.85 to 24.1 ± 3.2, respectively; $n = 16$). There was no difference in the pre-NEM mIPSCs between the TTX only and TTX plus 0 Ca/4 mM Mg groups. In the EGTA-containing solution, there was an apparent increase in mean mIPSC size (28.5 ± 4.76 pA to 41.4 ± 4.75 pA, control and NEM, respectively; $n = 4$). The significance of the larger mIPSCs in the EGTA-containing solutions is not clear. It is caused mainly by an increase in the skew of the mIPSC distribution toward larger events, which greatly affects the mean sizes. The modal mIPSC values were in the range of 15–20 to 20–25 pA and changed much less in NEM. To guard against the possibility that sampling bias or other source of variability caused a spurious impression of larger

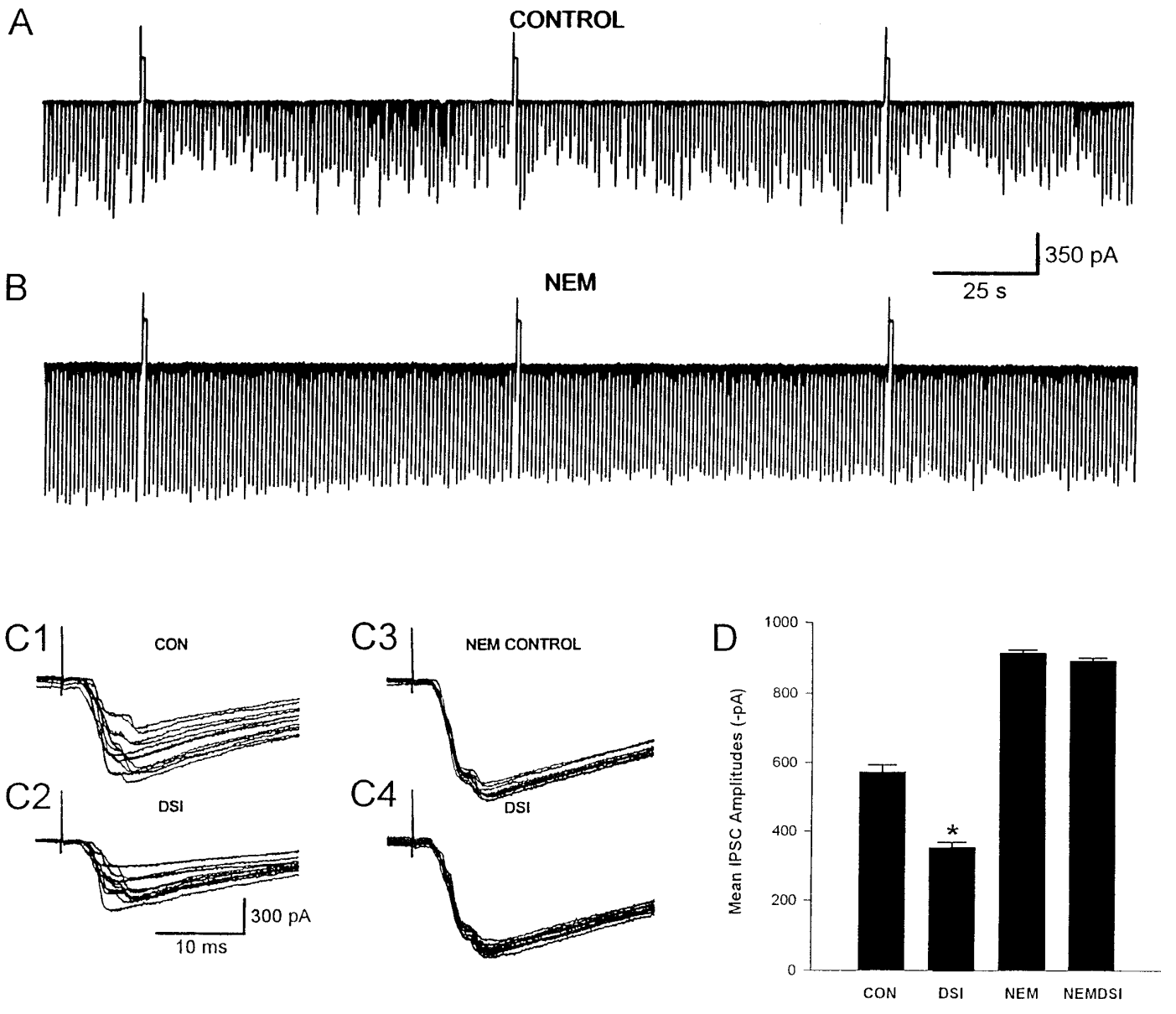

Figure 4. NEM blocks DSI of evoked IPSCs. Monosynaptic IPSCs (20 μM CNQX, 50 μM APV) were evoked at 0.5 Hz by electrical stimulation in st. pyramidale and recorded under whole-cell voltage clamp. DSI-inducing voltage steps to 0 mV were delivered every 90 sec. *A*, Three consecutive DSI trials in control conditions. *B*, Three consecutive trials recorded 3 min after a 10 min perfusion of 250 μM NEM. *C1*, Twelve consecutive evoked IPSCs from just before the DSI pulse in control conditions. The IPSCs, all evoked with the same intensity stimulation, were highly variable in amplitude. *C2*, Twelve consecutive IPSCs beginning at the fourth response after a DSI pulse in control conditions. *C3*, Twelve consecutive IPSCs evoked after NEM treatment just before the same voltage step that induced DSI in control conditions. Note the large size and relative lack of variability in responses. *C4*, Twelve consecutive IPSCs evoked immediately after the voltage step after NEM application. *D*, Histogram of IPSC amplitudes from five consecutive trials in each of the indicated conditions ($n = 60$ in each condition). Twelve responses were taken before the depolarizing voltage step (*CON*), during the DSI period (*DSI*), then again during NEM before the step (*NEM*), and finally after the step (*NEMDSI*). One-way ANOVA on ranks followed by Student–Newman–Keuls tests indicates a significant reduction in mean IPSCs during DSI in control conditions (*asterisk*), but not with NEM present. IPSCs in NEM were significantly larger than in control ($p < 0.05$). All data taken from the same CA1 cell. Similar results have been obtained from eight of the nine other cells tested.

mIPSC amplitudes, for 10 of 16 cells we analyzed the mIPSC data in two ways: (1) by measuring all mIPSCs occurring in a fixed time interval in control and then NEM solution, and (2) by measuring a fixed number of events in control solution and then NEM (for a given cell this number ranged from 150 in both conditions to 700 in both). There was very little difference when the data were replotted. Because NEM blocks $GABA_B$ responses and because $GABA_B$ receptor activation reduces mIPSC frequency, we considered the possibility that NEM might increase mIPSCs by blocking a tonic $GABA_B$ receptor activation. However, in three cells we applied the $GABA_B$ antagonist CGP 35348 and found it does not increase mIPSC frequency. In two of the cells, we subsequently added NEM to the CGP 35348-containing solution and observed the same marked increases reported above. Thus, the major effect of NEM on mIPSCs was an increase in their frequency by a mechanism not involving presynaptic $GABA_B$ receptors.

Further evidence that NEM effects on IPSC are primarily presynaptic comes from an analysis of the evoked IPSC amplitude

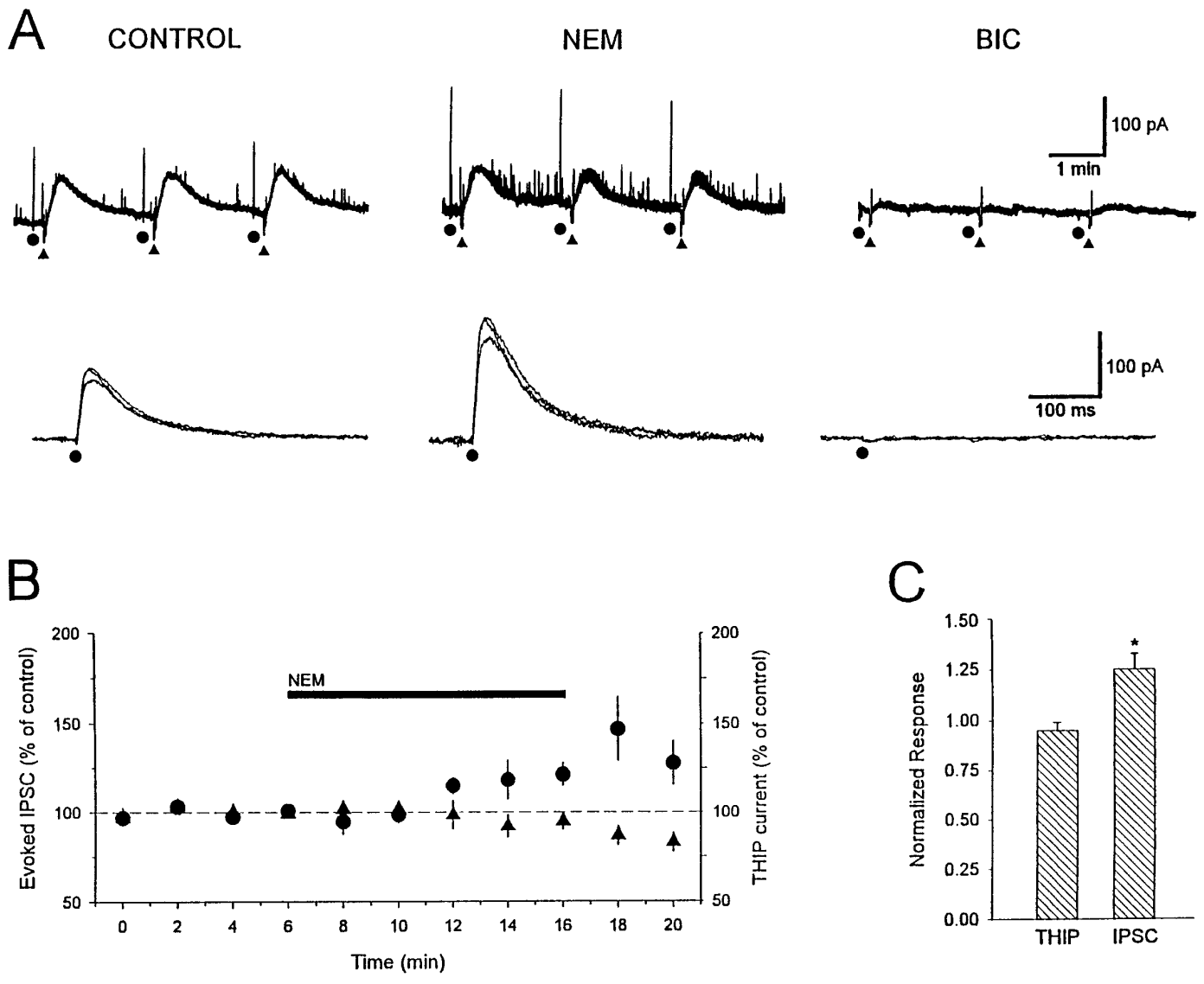

Figure 5. NEM increases the amplitudes of monosynaptically evoked IPSCs but not iontophoretic THIP responses. Iontophoresis of the $GABA_A$ agonist THIP (50 mM) was accomplished by an ejecting current of +170 nA lasting 2 sec; a constant retaining current of −10 nA was on at all times. Cells were voltage-clamped at potentials between −50 and −60 mV. *A*, Groups of current traces recorded sequentially from a single CA1 pyramidal cell ($V_H = -50$ mV). Each evoked IPSC (*filled circles*) is followed by iontophoresis of the $GABA_A$ agonist THIP (*filled triangles*) in control, during the end of a 10 min application of 300 μM NEM, and finally during application of 50 μM bicuculline methiodide (*BIC*), which blocked both kinds of response. Note that only the IPSCs and not the THIP currents are enhanced in the presence of NEM. *Below* each trace are the corresponding superimposed records of the IPSCs on an expanded time scale. *B*, Time course of action of NEM (application indicated by *solid bar*) on evoked IPSCs (*filled circles*) and THIP currents (*filled triangles*) recorded from five pyramidal cells. *C*, Bar graph summarizing the effects of NEM on the THIP currents and the IPSCs recorded during the 10th minute of application ($n = 5$). Compared with the THIP current, the IPSC is significantly increased in the presence of NEM (paired Student's *t* test; $p < 0.05$). All cells were recorded with patch pipettes containing KCH_3SO_3.

variability. As we and others have observed previously (Miles and Wong, 1984; Alger et al., 1996; Vincent and Marty, 1996), repeatedly evoked IPSCs can fluctuate markedly despite constant stimulus intensity (e.g., Fig. 4*A*). The magnitude of the fluctuations in normal saline indicates that they represent large multiquantal events, generated evidently by action potentials in presynaptic cells, because they do not appear in TTX. The CV of the responses provides a measure of this variability that is independent of absolute response amplitude. NEM, in addition to increasing IPSC amplitudes, also decreased the CVs of the responses, from a mean of −0.22 ± 0.03% in control to −0.14 ± 0.02% (compare Fig. 4, *A* and *B*). The difference in CV of evoked IPSC amplitudes in control versus CVs in NEM was statistically significant in four of five cells tested. Although interpretation of changes in CV can be complex (Faber and Korn, 1991), this decline in CV is consistent with the conclusion that NEM increases IPSCs via a presynaptic mechanism (Miles and Wong, 1984; Discussion in Alger et al., 1996; Vincent and Marty, 1996).

DISCUSSION

These results support several conclusions: (1) NEM seems to be a useful tool for the electrophysiological investigation of G-protein-related phenomena in the vertebrate CNS, as it is in peripheral and invertebrate neurons, and (2) NEM blocks DSI. Because DSI of sIPSCs is blocked by *in vivo* pertussis toxin pretreatment of hippocampi, it was predicted that NEM would

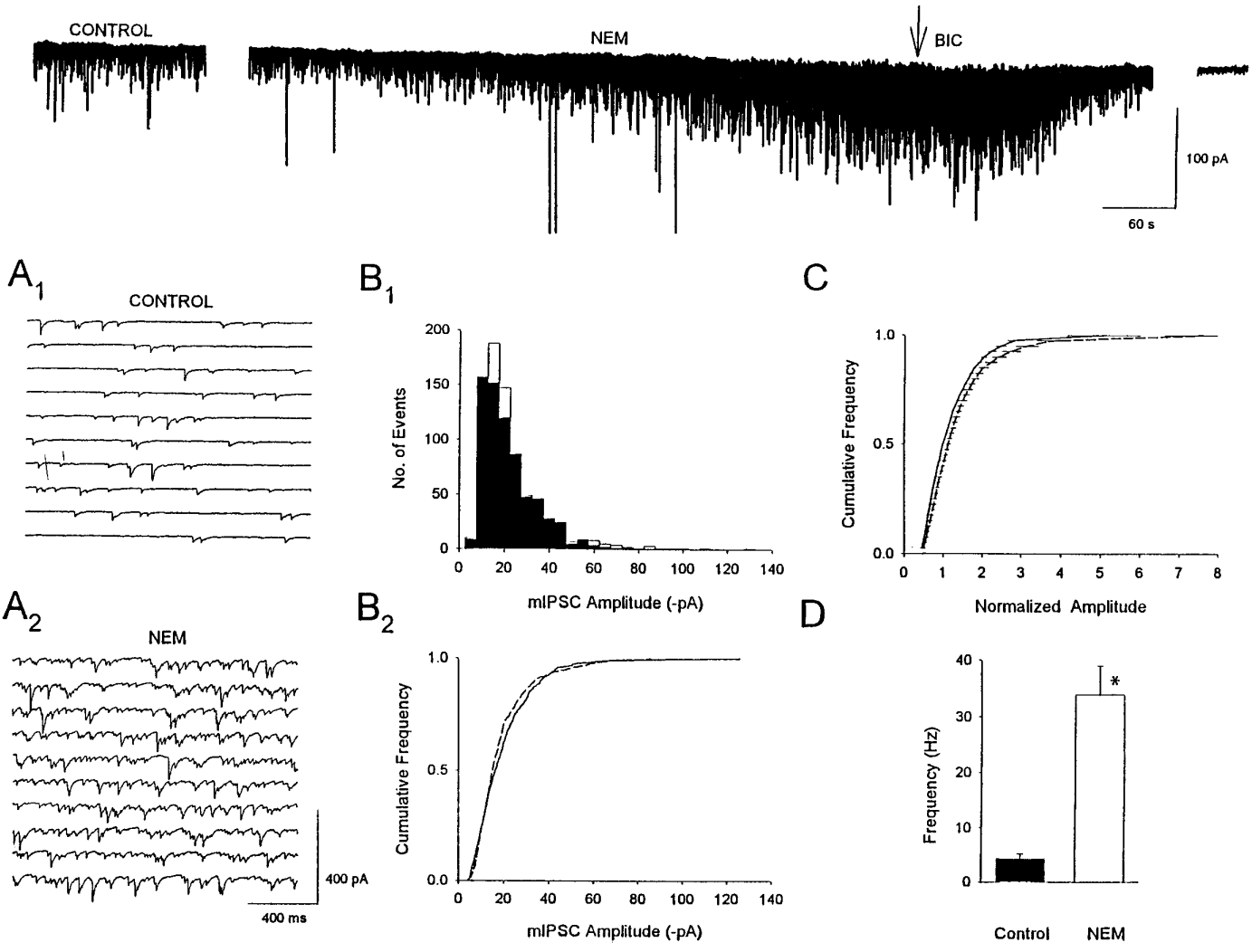

Figure 6. NEM increases mIPSC frequency. *Top traces* from an experiment on spontaneous mIPSCs in the presence of APV, CNQX, and TTX. After a period of baseline recording in control saline (*left*), 250 μM NEM was added to the perfusate. NEM had been present for 4 min by the beginning of the portion of the trace marked NEM. When 10 μM bicuculline methiodide was added to the bath at the point indicated by the *arrow*, the activity began to decline. Eight minutes later, when the right-most portion of trace was recorded, all activity was blocked. *A1*, Ten consecutive traces of activity in control saline (plus TTX) from *top traces* shown at faster sweep speed. *A2*, Ten consecutive traces 12 min after 250 μM NEM had been added to the bath. *B1*, mIPSC amplitude histograms before (*filled bars*) and after (*open bars*) NEM was added; 700 events were measured in each condition. In control, 700 mIPSCs were detected in 154 sec; in NEM, 700 mIPSCs were detected in 21.3 sec. *B2*, Cumulative frequency histogram of the data in *B1* for control (*solid line*) and NEM (*dashed line*) data. All data in *A* and *B* were recorded from the same cell. A K–S test confirmed that there was no significant difference between the curves. *C*, Cumulative frequency plots for group data from six cells (control, *solid line*; NEM, *dashed line*). *D*, mIPSC frequencies in control and NEM. The difference is significant at $p < 0.05$ (paired t test). All cells were recorded with pipettes containing KCl and were voltage-clamped at -70 mV.

block DSI of sIPSCs; (3) DSI of evoked IPSCs seems to be similar to DSI of sIPSCs with respect to G-protein involvement; and (4) NEM can have presynaptic effects on GABA release. NEM increased sIPSC activity as it did the amplitude of evoked monosynaptic IPSCs. It also increased the frequency of TTX-insensitive mIPSCs in a manner independent of external Ca^{2+}, without affecting iontophoretic $GABA_A$ responses. These actions are clearly compatible with a presynaptic site of action on the GABAergic interneurons.

We had not previously determined whether the suppression of monosynaptic evoked IPSCs, which follows a DSI-inducing voltage step, is also susceptible to G-protein inhibition. DSI of sIPSCs had been studied mainly in the presence of carbachol, whereas DSI of evoked IPSCs is investigated in the absence of muscarinic receptor activation and therefore could conceivably have been caused by a different mechanism. The prevention of DSI of monosynaptic-evoked IPSCs by NEM thus supports the conclusion that the evoked IPSCs are indeed susceptible to DSI, like that of sIPSCs. Evidence that they are the same is also useful because it is often more convenient to study evoked rather than spontaneous IPSCs. The site of action of NEM in blocking DSI is not known. NEM does not inhibit DSI simply by increasing basal activity, because carbachol, which also increases sIPSC activity, promotes rather than reduces DSI. More strikingly, NEM enhanced TTX-resistant mIPSC frequency, even in the absence of extracellular Ca^{2+}, indicating an effect on the presynaptic release process independent of voltage-dependent Ca^{2+} channels. A presynaptic site for a G-protein-dependent role in DSI is supported by previous work showing that, although pertussis toxin-pretreated slices did not show DSI, manipulation of postsynaptic

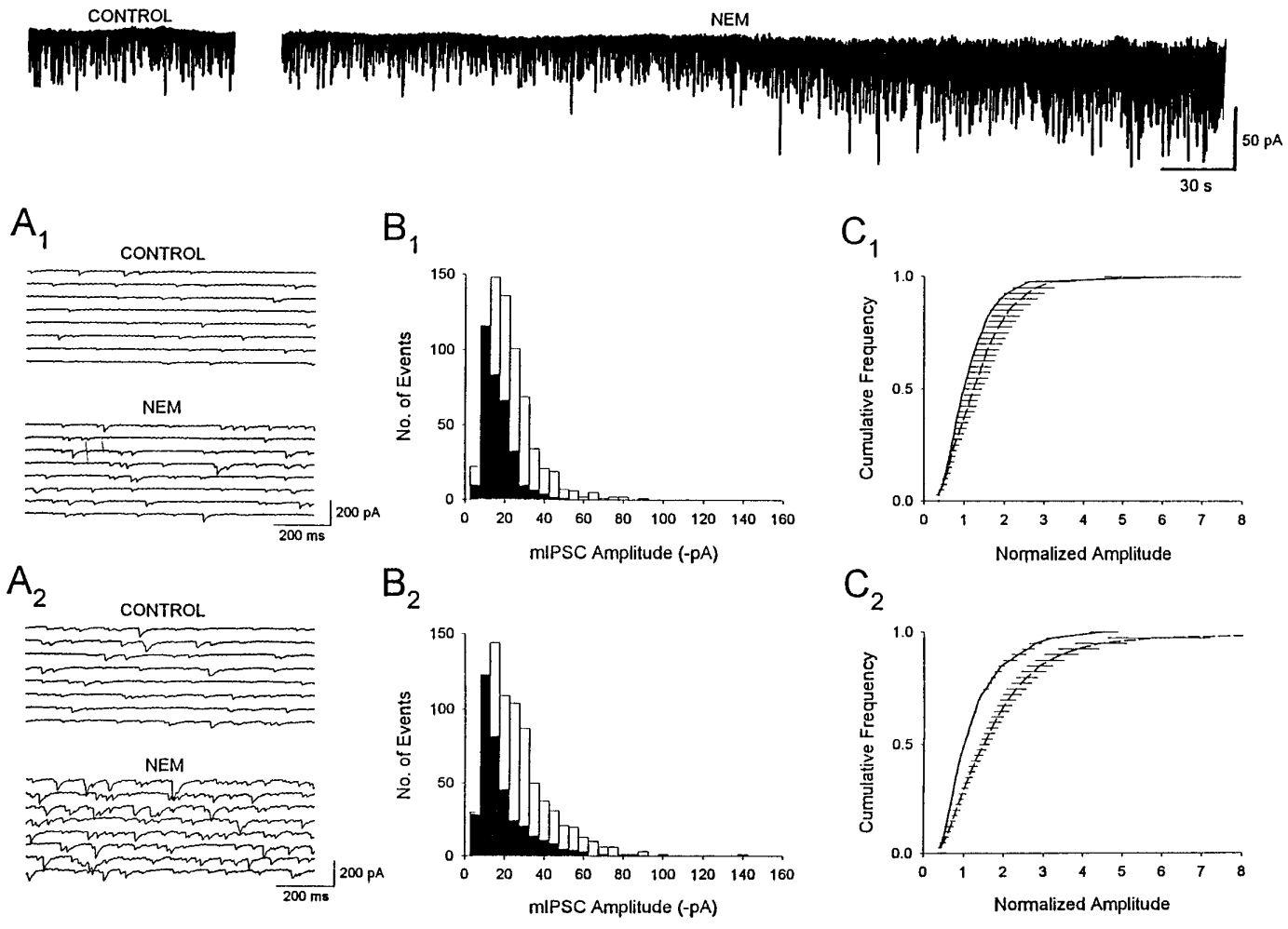

Figure 7. NEM induces an increase in mIPSC activity in the absence of extracellular Ca^{2+}. The *top trace* illustrates the onset of actions of 300 μM NEM on mIPSCs recorded from a CA1 pyramidal cell under conditions in which Ca^{2+} was omitted from the bathing solution and the concentration of Mg^{2+} was raised from 2 to 4 mM. Miniature IPSCs in NEM were recorded between the 4th and 10th minutes of NEM application. A 5 min gap separates control and NEM conditions. *A1*, Continuous traces of mIPSCs obtained from another cell are shown on an expanded time scale. *B1*, Corresponding amplitude histograms of mIPSCs collected over a 1 min interval in control (*filled bars*) and in the presence of NEM (*open bars*). *C1*, Averaged cumulative amplitude distributions obtained from six cells comparing mIPSCs recorded in control (*solid line*) with those recorded in NEM (*dashed line*). *A2*, Continuous traces of mIPSCs recorded in media containing 0 mM Ca^{2+}, 100 μM EGTA, and 8 mM Mg^{2+}. *B2*, Corresponding amplitude histograms of mIPSCs collected over a 30 sec interval in control (*filled bars*) and in the presence of NEM. *C2*, Averaged cumulative amplitude distributions from four cells comparing events recorded in control (*solid line*) with those recorded in the presence of NEM (*dashed line*). In all experiments, CNQX, APV, and TTX were present in the bathing solution. All cells were recorded with patch pipettes containing KCl and were voltage-clamped at −70 mV.

G-protein effectors GTP, GTPγS, and GDPβS did not alter DSI (Pitler and Alger, 1994). Finally, the decrease in variability of evoked IPSC transmission caused by NEM represents a presynaptic modification. NEM seemed to increase the reliability of evoked IPSC transmission by synchronizing IPSC components that in control conditions occur at variable latencies. This could occur if NEM improved the safety factor for conduction through branching axonal plexi or increased the excitability of presynaptic fibers generally. In any case, NEM clearly had presynaptic actions.

Nevertheless, despite evidence for presynaptic effects of NEM on DSI, the actual site of the putative presynaptic G-protein in blocking DSI is unknown. We cannot rule out a postsynaptic effect of NEM to decrease a Ca^{2+} current during the DSI-inducing pulse. NEM can reduce voltage-dependent Ca^{2+} currents in isolated SCG neurons (Shapiro et al., 1994) and in *Aplysia* neurons (Fryer, 1992; in *Aplysia*, however, the G-protein seems to be pertussis toxin insensitive). Note that if NEM does prevent DSI by blocking a postsynaptic Ca^{2+} current, this must be mediated by a Ca^{2+} channel different from the one(s) involved in GABA release from nerve terminals. Identification of the postsynaptic Ca^{2+} channels involved in DSI will be important.

NEM was applied for ~90 sec at a concentration of 50–100 μM by Shapiro et al. (1994). However, the SCG cells studied by this group were acutely isolated from other cells, and the drug could be applied rapidly at full strength. In the slice preparation, we found that 50 μM NEM did not affect any of the measures described in this report within 10–15 min. At 250 or 300 μM applied for 4–12 min, there were fairly marked effects, although they varied in magnitude. We adopted a protocol involving an 11 min application for uniformity, but the actual concentration of NEM in the center of the slice may not have equilibrated within the cells at that point. It is likely that the same effects could be obtained with lower concentrations if administered for a longer time.

At low concentrations the effect of NEM on isolated cells (Nakajima et al., 1990; Shapiro et al., 1994) or membrane preparations (Harden et al., 1982; Kitamura and Nomura, 1987) seems

to be relatively specific to decoupling of G-proteins from their associated membrane receptors. At high concentrations it can have other effects as well. At a concentration of 500 μM applied for 10 min to isolated membrane fragments, NEM blocked ^{3}H-labeled binding of G-protein-linked ligands to their receptors (Kitamura and Nomura, 1987; Shinoda et al., 1990). Iontophoretic $GABA_A$ receptor-mediated responses were not altered by our protocol, indicating that at least GABA binding to $GABA_A$ receptors was not altered in these experiments.

We do not know how NEM increased GABA release. The mIPSC frequency was clearly increased by a mechanism independent of external Ca^{2+}. In control solutions plus TTX, there was no increase in mIPSC amplitude in five of six cells. Hence, the increase in evoked IPSC amplitudes reflects the enhancement of the presynaptic release process implied by the increased mIPSC frequency. It is not clear how to interpret the increase in mIPSC amplitudes occasionally seen in the 0 Ca^{2+}/8 mM Mg^{2+}/100 μM EGTA experiments. It probably does not represent an increase in postsynaptic $GABA_A$ receptor responsiveness because there was no corroborating evidence from the iontophoretic experiments or from mIPSC experiments in control solution. Alternatively, the apparent amplitude increase could reflect a technical limitation in our ability to distinguish temporally among individual mIPSCs when they overlap because of a greatly increased frequency. Finally, in view of the demonstrated role of an *N*-ethylmaleimide-sensitive factor (NSF) in neurotransmitter release, it is possible that NEM has some novel effect that results in the occurrence of larger mIPSCs. Miniature $GABA_A$ IPSCs in hippocampus are thought to represent single quantal events (Collingridge et al., 1984; Edwards et al., 1990). When NEM increased mean mIPSC amplitudes, it evidently did so by increasing the positive skew in the distribution; there was less effect on modal mIPSC values. Although not understood, this positive skew is commonly seen in quantal analysis in CNS tissue. NEM could affect the process that produces larger mIPSCs.

A great deal of recent work has elaborated a scheme for the understanding of cellular vesicle fusion and release. A central factor in this process is NSF, an ATPase whose hydrolysis of ATP is required for membrane fusion (Whiteheart et al., 1994). Vesicle–membrane fusion in the Golgi apparatus is inhibited by NEM. Accordingly, we were initially concerned that NEM might simply inhibit synaptic transmission. Indeed, with prolonged application NEM did irreversibly inhibit transmission, but NEM had actually enhanced GABAergic transmission at the point at which DSI was blocked. There seems to be little information on the effects of NEM on physiologically measured synaptic transmitter release in general, so we do not know whether the changes in GABAergic transmission that we have seen are common to other transmitter systems. In a brief report, it was noted that NEM increased miniature endplate potential frequency at toad neuromuscular junction (Carmody, 1978). NEM also facilitated synaptic activation of the compound action potential in sympathetic ganglion (Sasaki et al., 1983) and increased radiolabeled norepinephrine release in hippocampal slices (Hertting and Allgaier, 1988). For the most part, previous work does not reveal the site of NEM action. In preliminary work, we have seen that NEM causes a slight increase (20–25%), followed by a decrease of field potential elicited by stimulation in st. radiatum in CA1 (Alger et al., 1995). Because these field potentials are induced by activation of glutamatergic synapses, it may be that glutamate transmission is transiently facilitated by NEM as well. Determining the mechanism of action of NEM on synaptic potentials may provide useful insights into both the DSI process and fundamental aspects of neurotransmitter release.

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