Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6467–6471, August 1991
Neurobiology

N-Methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca^{2+} channel activity in area CA1 of hippocampus

(long-term potentiation/modulation/Schaffer collateral/adenylyl cyclase/second messengers)

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Communicated by Eric R. Kandel, April 16, 1991

ABSTRACT **Tetanic stimulation of the Schaffer collateral inputs into area CA1 of the hippocampus causes *N*-methyl-D-aspartate (NMDA) receptor activation, an effect that contributes to the induction of long-term potentiation (LTP) in this region. The present studies demonstrate that LTP-inducing tetanic stimulation in rat hippocampal area CA1 elicited increased levels of cAMP. The elevation of cAMP was blocked by the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV). Bath application of NMDA also resulted in an increase in cAMP in CA1, an effect that was blocked by both APV and removal of extracellular Ca^{2+}. These findings suggest that activation of NMDA receptors elicits a Ca^{2+}-dependent increase in cAMP, and taken together with the data from tetanic stimulation, suggest that NMDA-receptor-mediated increases in cAMP could play a role in the induction of LTP in area CA1. One role for cAMP may be to increase Ca^{2+} influx through voltage-gated Ca^{2+} channels, as it was observed that application of either 8-bromo-cAMP or NMDA increased the fractional open time of high-threshold Ca^{2+} channels in CA1 pyramidal cells. Our results raise the possibility that a positive-feedback loop for Ca^{2+} influx in area CA1 exists. In this model, NMDA receptor-mediated Ca^{2+} influx leads to an enhancement of further Ca^{2+} influx via intermediate steps of increased cAMP and subsequent increased voltage-gated Ca^{2+} channel activity.**

Long-term potentiation of synaptic transmission (LTP) is a robust form of synaptic plasticity that is a strong candidate mechanism for learning and memory in the mammalian brain (for reviews, see refs. 1 and 2). The induction of the most commonly studied form of LTP in area CA1 of the hippocampus requires activation of *N*-methyl-D-aspartate (NMDA) receptors (3, 4). NMDA receptor activation allows the influx of Ca^{2+} into neurons, an effect that has been causally linked to the induction of LTP (5–8). The biochemical sequelae of the NMDA-receptor-mediated Ca^{2+} influx, however, are not well characterized. Available evidence suggests that the activation of Ca^{2+}-dependent protein kinases contributes to the induction of LTP (9–12). Ca^{2+} is a pleuripotent second messenger, and many biochemical effects of Ca^{2+} are well documented in the central nervous system, including activation, through calmodulin, of adenylyl cyclase (13, 14). In the experiments reported here, we tested the hypothesis that NMDA receptor activation increases cAMP in area CA1 of the hippocampus and that a physiological consequence of increased cAMP is an increase in the activity of voltage-gated Ca^{2+} channels.

MATERIALS AND METHODS

LTP Measurements. Male Sprague–Dawley rats (approximately 125 g) were decapitated and either hippocampus was removed. Slices 400 μm thick were prepared with a McIlwain tissue chopper (Brinkmann). The slices were perfused with a standard saline solution (124 mM NaCl/4.4 mM KCl/26 mM $NaHCO_3$/10 mM D-glucose/2 mM $CaCl_2$/2 mM $MgCl_2$, gassed with 95% O_2/5% CO_2, pH 7.4) in a Fine Science Tools (Belmont, CA) slice chamber at 32°C. Test pulses and high-frequency stimulation were delivered by a Masscomp (Concurrent Computer Corporation, Tinton Falls, NJ) 5400 computer with software written in the Basic-68 language (Division of Neuroscience, Baylor College of Medicine). Recording electrodes were made from glass filament microcapillaries with a Narishige (Greenvale, NY) horizontal electrode puller. Electrodes were filled with 1.2 M NaCl, and had a dc resistance of 1–5 MΩ. Stimuli were delivered through bipolar Teflon-coated platinum wires at constant currents ranging from 2 to 70 μA. Responses to Schaffer collateral stimulation (1) in the CA1 region were monitored for 20 min to assure a stable baseline. Tetanic stimulation consisted of three one-s trains of stimuli (100 Hz) every 5 s at the minimum stimulus current needed to elicit a maximal population excitatory postsynaptic potential (EPSP). This stimulus was sufficient to induce LTP in >90% of slices (20 of 22). LTP was defined as a 20% or greater increase in initial EPSP slope, or in initial experiments it was defined as a 30% or greater increase in population spike amplitude. When present, DL-2-amino-5-phosphonovaleric acid (APV) was 50 μM in the bath, a concentration that was found to block LTP induced by this stimulus paradigm (n = 6).

Measurement of cAMP. Slices were allowed to adjust to the recording chamber for 1.5–2 hr to allow cAMP levels to stabilize (15). Slices were removed from the chamber and rapidly frozen either after the test pulses (control) or 1 min after tetanic stimulation (tetanus). The CA1 region of the frozen slices was dissected and homogenized in 5% (wt/vol) trichloroacetic acid. cAMP was measured in duplicate determinations by using an RIA kit purchased from New England Nuclear. Protein was measured by a modified Bradford assay with bovine serum albumin as the standard (16).

Drug Application. Slices were prepared as above and exposed to either control saline or bath-applied drug for 10 min and then immediately frozen and dissected, and cAMP and protein content were assayed as described. Experiments with EGTA in the bath were performed identically with the exception that 1 mM EGTA replaced $CaCl_2$ in the bath.

Single-Channel Recording. The recordings were made from acutely exposed hippocampal CA1 pyramidal neurons from adult guinea pigs (17). The cells were bathed in isotonic K saline (bath saline, see below) to zero their membrane potential so that the potential across the patch of membrane could be known (18), and voltage steps were applied every 3 s to activate Ca^{2+} channels. Bath saline for most experiments contained (in mM) 140 potassium methylsulfate, 2 $MgCl_2$, 20

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Abbreviations: APV, DL-2-amino-5-phosphonovaleric acid; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate.
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D-glucose, 10 Hepes, and 10 EGTA (pH adjusted to 7.35 with KOH). The patch pipette saline contained (in mM) 100 $BaCl_2$ and 10 Hepes (pH adjusted to 7.4 with tetraethylammonium hydroxide). Tetrodotoxin (1 μM) and 3,4-diaminopyridine (100 μM) were added to the pipette saline at the time of the experiment to block Na^+ and K^+ channels. All recordings were made at room temperature (22–24°C). Drugs were applied by pressure ejection through a puffer pipette placed near the cell body, or were added to the bath. In patches with few channels, the fraction of time that channels were open during the step voltage command was calculated for each trace before and after drug applications; for many-channel patches the current traces were integrated to provide an indication of channel activity. All drug effects were evaluated for statistical significance at the 95% confidence level using a two-tailed Mann–Whitney *U* test.

RESULTS

Tetanic stimulation of the Schaffer collateral inputs to CA1 activates NMDA receptors and leads to LTP of these synaptic connections. In the present studies tetanic stimulation of the Schaffer collateral inputs into area CA1 induced LTP in greater than 90% of tetanized slices. The same tetanic stimulation paradigm was found to elicit increases in cAMP in area CA1, assayed 1 min after tetanus (146% ± 10% of control, $n = 20$) (Fig. 1*A*). The NMDA receptor antagonist APV (50 μM) blocked the increase in cAMP seen with tetanic stimulation (106% ± 8% of control, $n = 5$). These results suggest that LTP-inducing tetanic stimulation can cause an elevation in cAMP in area CA1 of the hippocampus through the activation of the NMDA subtype of excitatory amino acid receptors.

If the tetanus-induced rise in cAMP is mediated by NMDA receptor activation, then application of NMDA receptor agonists should also produce elevations in cAMP (19). We found that bath application of NMDA to hippocampal slices produced concentration-dependent elevation of cAMP in area CA1 (Fig. 2). A maximally effective concentration of NMDA, 1 mM, gave a large increase in cAMP (397% ± 41% of control, $n = 4$). The NMDA-induced increase in cAMP was half-maximal at 50 μM. Furthermore, application of APV (50 μM) blocked the increase in cAMP elicited by 100 μM

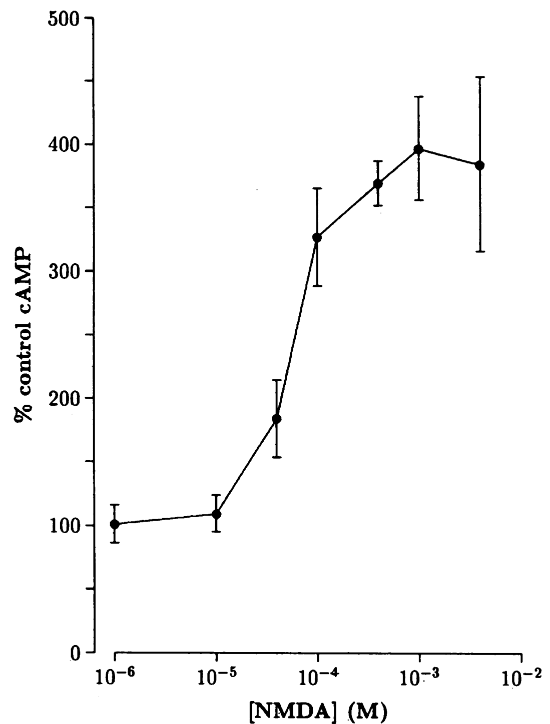

FIG. 2. NMDA receptor activation leads to an increase in cAMP in area CA1. NMDA was applied in the bath at the concentrations indicated. Points represent the average of four to six experiments in which the mean cAMP content of three drug-exposed slices was normalized to the mean cAMP content of three control slices from the same hippocampus. SEM is shown. In control experiments we observed that after washout of NMDA (400 μM), slices exhibited apparently healthy electrophysiological responses (data not shown).

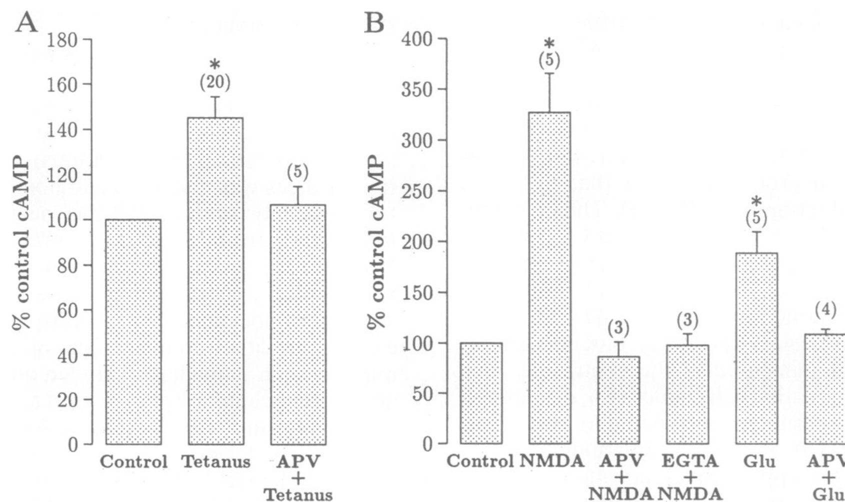

FIG. 1. cAMP levels are increased with LTP-inducing tetanic stimulation or NMDA application. (*A*) One experiment involved four slices from a single hippocampus. Two slices were given 10–60 min of test pulses (control), and two slices were given tetanic stimulation (tetanus). The cAMP levels in the slices were normalized to protein content and averaged for the two slices given similar treatments. These values were then normalized to the control slices from the same hippocampus; the number of experiments in each group is indicated in parenthesis. Error bars represent the SEM for the number of experiments in each group. Actual cAMP content for the slices was (in pmol of cAMP per mg of protein): control, 8.9 ± 0.6; APV control, 8.9 ± 0.9; tetanus, 12.7 ± 0.7, and APV + tetanus, 9.4 ± 1.0. The * indicates values significantly different from control ($P < 0.01$) by Student's *t* test. (*B*) Effects of APV and EGTA on NMDA-mediated increases in cAMP. EGTA (1 mM), APV (50 μM), NMDA (100 μM), and glutamate (Glu, 5 mM) were added as indicated. Actual cAMP content for the slices was (in pmol of cAMP per mg of protein): control, 11.7 ± 0.8; NMDA, 37.6 ± 3.8; APV control, 15.3 ± 1.0; APV + NMDA, 13.0 ± 2.6; EGTA control, 30.7 ± 4.4; EGTA + NMDA, 30.2 ± 5.7; Glu control, 8.8 ± 0.9; Glu, 15.9 ± 0.9; APV control, 10.8 ± 1.6; and APV + Glu, 12.0 ± 2.1. A single experiment consisted of the average cAMP content of three drug-exposed slices normalized to that of three control slices from the same hippocampus.

NMDA (87% ± 15% of control, $n = 3$) (Fig. 1*B*). In additional experiments, APV (50 μM) attenuated increases in cAMP elicited by 1 mM NMDA [397% ± 41% of control in the absence of APV ($n = 4$), and 238% ± 46% of control in the presence of APV ($n = 3$)], as would be expected for a competitive antagonist in the presence of a high concentration of agonist. We also observed that high concentrations of glutamate (5 mM) produced similar increases in cAMP (189% ± 21% of control, $n = 5$), which were also blocked by 50 μM APV (109% ± 5% of control, $n = 4$) (Fig. 1*B*). These data strongly suggest that activation of NMDA receptors leads to increases in cAMP in area CA1 of the hippocampus.

It is possible that NMDA-induced depolarization may result in release of norepinephrine from terminals in the slice. To rule out the possibility that the increase in cAMP might arise from β-adrenergic receptor stimulation, NMDA (400 μM) was added to slices in the presence of the β-adrenergic receptor antagonist timolol (2 μM). Timolol did not block the NMDA-induced increase in cAMP [NMDA (400 μM), 370% ± 18% of control cAMP, $n = 5$; NMDA (400 μM), + timolol (2 μM), 359% ± 32% of control cAMP, $n = 4$]. In contrast, timolol was able to block increases in cAMP elicited by the β-adrenergic receptor agonist isoproterenol [isoproterenol (10 μM), 198% ± 14% of control cAMP, $n = 4$; isoproterenol (10 μM), plus timolol (2 μM), 81% ± 3% of control cAMP, $n = 2$].

It has been postulated that a major role of the NMDA receptor in neurons is to allow influx of calcium (5–8). To test whether Ca^{2+} is necessary for the NMDA receptor-mediated increase in cAMP, we added NMDA (100 μM or 1 mM) to slices in the absence of extracellular Ca^{2+} and in the presence of 1 mM EGTA. We found that removal of extracellular Ca^{2+} completely blocked NMDA-elicited increases in cAMP; this blockade was independent of NMDA concentration (Fig. 1*B*). These results illustrate the necessity of extracellular Ca^{2+} in the elevation of cAMP by NMDA. The dependence of NMDA-induced increases in cAMP on extracellular Ca^{2+} suggests that the effect of NMDA receptor activation on cAMP levels is not a result of direct receptor/G-protein coupling to adenylyl cyclase, but rather is a consequence of the influx of Ca^{2+} through the NMDA receptor/ion channel. One means by which increased intracellular Ca^{2+} could produce an increase in cAMP is through Ca^{2+}/calmodulin stimulation of the adenylyl cyclase catalytic unit (13, 14), although other mechanisms cannot be ruled out.

The increase in cAMP in area CA1 after activation of NMDA receptors could have numerous physiological consequences (20–28). In previous studies we have shown that both β-adrenergic agonists and cAMP enhance the activity of high-threshold Ca^{2+} channels in dentate granule cells (29) and CA3 pyramidal neurons (30, 31). Furthermore, high-threshold Ca^{2+} channels have been suggested to play a role in LTP in area CA3 and more recently in area CA1 (31, 32). We therefore hypothesized that the increased cAMP resulting from NMDA receptor activation would increase the activity of Ca^{2+} channels in CA1 pyramidal neurons. To test for the sensitivity of high-threshold Ca^{2+} channels to cAMP, we made cell-attached patch-clamp recordings of Ba^{2+} currents through single Ca^{2+} channels before and after extracellular application of the membrane-permeant cAMP analog 8-bromo-cAMP. Ba^{2+} was used as the charge carrier because it produces larger currents through high-threshold Ca^{2+} channels than does Ca^{2+}. We found that 8-bromo-cAMP caused an increase in the fractional open time of Ca^{2+} channels recorded from CA1 pyramidal neurons (Fig. 3) (251% ± 86% of control channel activity, $n = 5$, statistically significant

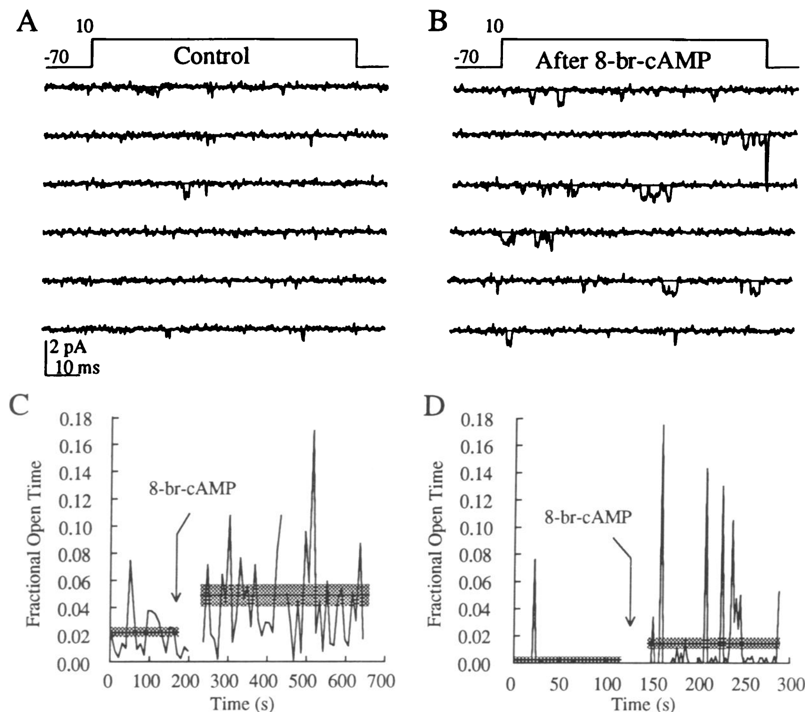

FIG. 3. Effect of 8-bromo-cAMP application on Ca^{2+} channels recorded from CA1 pyramidal neurons. The top trace schematically illustrates the duration of the voltage step. (*A*) Single-channel current traces in response to consecutive voltage steps from −70 to 10 mV. (*B*) Consecutive traces from the same cell-attached patch after puffer pipette application of 8-bromo-cAMP (5 mM). (*C*) Plot of fractional open time (probability of channel opening) during the voltage step vs. time for the experiment in *A* and *B*. (*D*) Plot of fractional open time vs. time from a different patch before and after a brief (1-s) application of 8-bromo-cAMP to the cell body through a puffer pipette. As pressure application of a membrane-permeant analog of cAMP was used, the final concentration of analog that reached the cell surface and entered the cell would be considerably lower than that contained in the puffer pipette. The arrow indicates the time of drug application. Gaps in this and subsequent plots indicate times for puffer pipette placement or measurements at other command voltages. Each plotted point is the average from three consecutive traces. Horizontal lines and shaded areas indicate the mean ± SEM during each segment.

increases in four of five patches). We next coapplied NMDA (1 mM) and Ca^{2+} (2 mM) to the outside of the neuron while recording the activity of single Ca^{2+} channels, using a cell-attached patch configuration. We found that brief coapplication of NMDA and Ca^{2+} caused an increase in Ca^{2+} channel activity (Fig. 4) (231% ± 36% of control channel activity, $n = 5$, statistically significant increases in five of five patches). Because the applied NMDA and Ca^{2+} did not have direct access to the Ca^{2+} channels under the patch pipette, this is strong evidence for the involvement of a second messenger in the NMDA-induced modulation of voltage-gated Ca^{2+} channels.

DISCUSSION

Our results demonstrate that LTP-inducing tetanic stimulation in area CA1 of the hippocampus leads to increased cAMP, an effect mediated by NMDA receptor activation. In addition, we have observed that NMDA-receptor-mediated increases in cAMP in area CA1 are blocked by removal of extracellular Ca^{2+}. Thus, Ca^{2+} influx through the NMDA receptor appears to be necessary to elicit NMDA-receptor-mediated increases in cAMP. cAMP and NMDA receptor activation also increased the probability of opening of voltage-gated Ca^{2+} channels in CA1 pyramidal neurons. Thus, NMDA receptor activation and subsequent increased cAMP levels may initiate a positive feedback loop for Ca^{2+} influx through increased activation of voltage-gated Ca^{2+} channels. The resulting increase in the Ca^{2+} signal may serve to enhance Ca^{2+}-dependent biochemical processes involved in LTP induction, such as the activation of Ca^{2+}-dependent protein kinases (9–12), or to enhance changes in neuron excitability that are associated with some forms of LTP (33).

It has been reported previously that NMDA can elicit sustained dendritic Ca^{2+} gradients in isolated CA1 neurons, and a single application of the excitatory agonist primes the neuron to produce a longer-lasting increase in intracellular Ca^{2+} in response to a second application (34). The existence of a cAMP-mediated positive-feedback loop for Ca^{2+} entry in neurons could contribute to this priming effect. Along these lines, these sustained gradients are blocked by treatment with sphingosine, a nonspecific inhibitor of both protein kinase C and calmodulin-dependent enzymes (35, 36).

We have observed that NMDA receptor activation elicits increased cAMP and increased activity of voltage-gated Ca^{2+} channels. In addition, a cAMP analog can mimic the effect of NMDA on Ca^{2+} channels. Therefore the most parsimonious explanation for NMDA causing increased Ca^{2+} channel activity is that NMDA acts on Ca^{2+} channels by elevating cAMP. However, our results cannot eliminate possible contributions of other second-messenger systems to the effects of NMDA on Ca^{2+} channels.

While we have observed that NMDA elicits an increase in Ca^{2+} channel activity in CA1 pyramidal neurons, it was recently reported that NMDA receptor agonists reduced a portion of the macroscopic Ca^{2+} current in CA1 pyramidal neurons isolated from young rats (37). In that study, however, the neurons were dialyzed with cAMP, ATP, and GTP, which would have occluded any NMDA-mediated increases in cAMP and subsequent changes in Ca^{2+} channel activity. Additionally, in our cell-attached recordings NMDA did not have access to the Ca^{2+} channels under the patch pipette, so we would not have seen any direct effect of NMDA on Ca^{2+} channel activity.

The cAMP-induced changes in Ca^{2+} channel activity may play a role in increasing the probability of induction of LTP in area CA1. This phenomenon has also been postulated to occur in area CA3 of the hippocampus, where isoproterenol and cell-permeant analogs of cAMP modulate voltage-dependent Ca^{2+} channel function (30, 31) and increase the likelihood of induction of LTP at mossy fiber synapses (38, 39). Thus, induction of NMDA-receptor-mediated LTP in area CA1 may have elements in common with induction of LTP at synapses not dependent upon NMDA receptor activation. In addition, cAMP has been implicated as playing an important role in learning and memory in invertebrates. At the crayfish "opener" neuromuscular junction, the importance of cAMP has been documented in serotonin-induced facilitation (40). In *Drosophila*, loss of cAMP phosphodiesterase activity and loss of sensitivity of adenylyl cyclase to

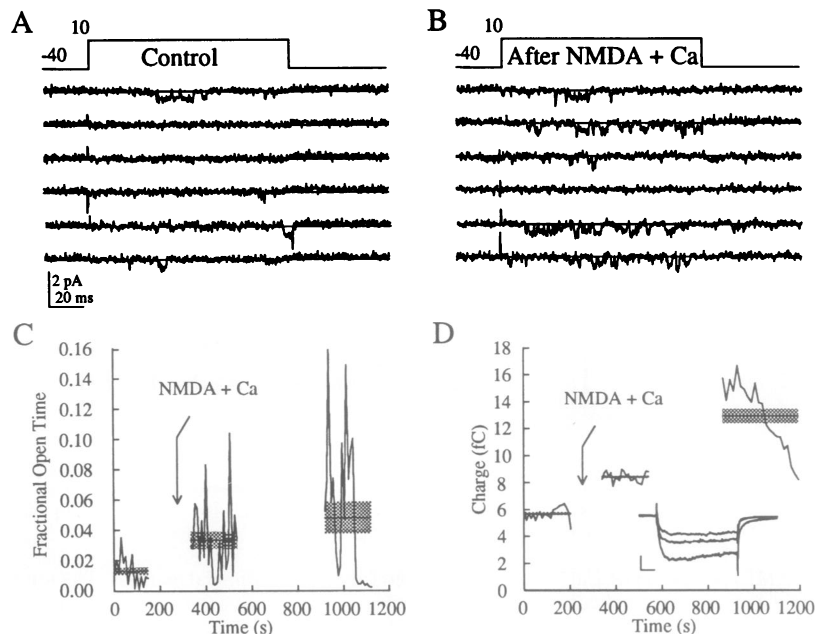

FIG. 4. Effect of coapplication of NMDA and Ca^{2+}. Consecutive current traces before (*A*) and after (*B*) two 500-ms pressure co-applications of NMDA (1 mM) and Ca^{2+} (2 mM) are illustrated. (*C*) Plot of fractional open time during the voltage step for the experiment in *A* and *B*. (*D*) Plot of the integral of the current from a different patch that contained several Ca^{2+} channels, before and after a 10-s coapplication of NMDA and Ca^{2+}. (*Inset*) Ensemble averaged currents for the three stretches of data plotted. Each trace is the average of 71 single-channel records. Calibration bars for *Inset*: 4 pA and 10 ms.

Ca^{2+}/calmodulin stimulation seem to be the predominant biochemical defects in the learning mutants dunce and rutabaga, respectively (41, 42). In *Aplysia*, the role for cAMP in synaptic plasticity and behavioral sensitization has been well characterized. In this system, neurotransmitter-induced increases in cAMP lead to an alteration of ion-channel function, contributing to synaptic facilitation (43). Thus, regulation of cAMP metabolism may be a common element in various forms of neuronal plasticity in mammals and in lower organisms.

We thank John Dani, Lutz Birnbaumer, and Jim Patrick for their useful comments on earlier versions of this paper. This work was supported by a C. Bell Pearce Fellowship in Neuroscience (D.M.C.), U.S. Public Health Service Grants RR-05425 (R.G. and J.D.S.) and MH-44754 (D.J.), and funds from the American Federation for Aging Research and the McKnight Foundation (J.D.S.).

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