29. In a limited study, melatonin administration to individuals with free-running circadian rhythms who were subjected to fractional desynchronization reinforced the entraining strength of a low intensity light-dark cycle for the circadian rhythm of self-rated fatigue or alertness (*8*). Also, administration of melatonin to volunteers can phase shift the endogenous melatonin rhythm in some individuals (*8*), presumably by an action on a biological clock.
30. A function of melatonin in mammals that breed seasonally is regulation of the dramatic changes in reproduction that occur throughout the course of the year [B. D. Goldman and J. M. Darrow, *Neuroendocrinology* **73**, 386 (1983); F. J. Karsch *et al.*, *Recent Prog. Horm. Res.* **40**, 185 (1984); L. Tamarkin, C. J. Baird, O. F. X. Almeida, *Science* **227**, 714 (1985)]. Melatonin has been suggested as having a role in the initiation or timing of puberty in humans, although firm evidence of seasonal reproductive rhythms or an effect of melatonin on gonadotropin secretion in humans is lacking (*4*) [F. Waldhauser *et al.*, *Neuroendocrinology* **46**, 125 (1987)]. It is possible that melatonin binding sites are expressed in other hypothalamic nuclei at specific development stages (for example, puberty) or at extrahypothalamic sites not examined in the present study.
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34. Melatonin treatment alleviated the symptoms of jet lag after eastward travel over eight time zones in all subjects examined (*9, 10*), although only a small number of individuals was studied. The melatonin-treated individuals had decreased sleep latency and better sleep quality and were more alert than placebo-treated subjects (*10*). Melatonin also facilitated reentrainment of the endogenous melatonin and cortisol rhythms, implicating an effect of the hormone on a biological clock (*10*).
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Postsynaptic Calcium Is Sufficient for Potentiation of Hippocampal Synaptic Transmission

ROBERT C. MALENKA, JULIE A. KAUER, ROBERT S. ZUCKER, ROGER A. NICOLL*

Brief repetitive activation of excitatory synapses in the hippocampus leads to an increase in synaptic strength that lasts for many hours. This long-term potentiation (LTP) of synaptic transmission is the most compelling cellular model in the vertebrate brain for learning and memory. The critical role of postsynaptic calcium in triggering LTP has been directly examined using three types of experiment. First, nitr-5, a photolabile nitrobenzhydrol tetracarboxylate calcium chelator, which releases calcium in response to ultraviolet light, was used. Photolysis of nitr-5 injected into hippocampal CA1 pyramidal cells resulted in a large enhancement of synaptic transmission. Second, in agreement with previous results, buffering intracellular calcium at low concentrations blocked LTP. Third, depolarization of the postsynaptic membrane so that calcium entry is suppressed prevented LTP. Taken together, these results demonstrate that an increase in postsynaptic calcium is necessary to induce LTP and sufficient to potentiate synaptic transmission.

INTRACELLULAR FREE Ca^{2+} ACTIVATES an array of cellular processes, and thus Ca^{2+} functions as a critical and ubiquitous second messenger. Increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]$) occur either by activation of voltage-dependent Ca^{2+} channels (*1*) or by release from intracellular stores (*2*). In neurons, Ca^{2+} passes through a special type of ligand-gated ion channel linked to the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor (*3, 4*). NMDA receptors are found in high concentrations in a variety of brain regions (*5*) and appear to mediate the initiation of several forms of synaptic plasticity (*6*). The induction of LTP in the hippocampus, the most extensively studied model for memory in the vertebrate brain, requires NMDA receptor activation (*7*). LTP is an enhancement of synaptic transmission lasting many hours in response to brief repetitive activation of excitatory synapses. It has been suggested that Ca^{2+} entering through the NMDA channel acts as a second messenger to trigger LTP (*8*). Despite the fundamental importance of this step to our understanding of LTP, and perhaps other forms of synaptic plasticity, the evidence linking Ca^{2+} to LTP is remarkably limited (*9, 10*). To address this issue directly we have performed three types of experiment in hippocampal pyramidal cells, two of which have relied on nitr-5, a photolabile nitrobenzhydrol tetracarboxylate Ca^{2+} chelator (*11*).

We used standard procedures to prepare and maintain hippocampal slices (*12*). Microelectrodes were filled with nitr-5 (100 m*M*) loaded with Ca^{2+} (50 m*M*) dissolved in 1*M* CsCl to block K^+ conductances that might be activated upon raising intracellular $[Ca^{2+}]$. We impaled CA1 pyramidal cells in

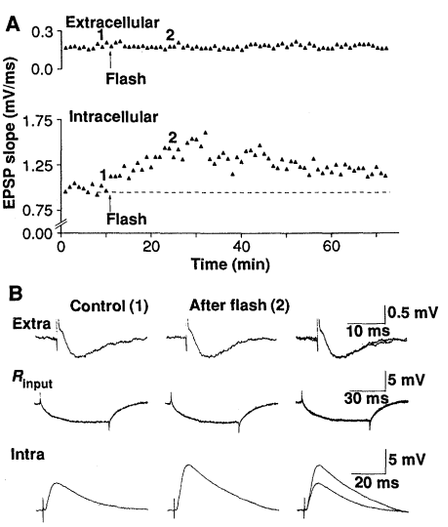

Fig. 1. Photolysis of intracellularly injected nitr-5 enhances synaptic transmission. (**A**) Graphs of the slope of the extracellular EPSP recorded in a stratum radiatum (upper) and the slope of the simultaneously recorded intracellular EPSP (lower) (*12*). Each point represents the average of six slope measurements. The cell was penetrated 15 min before time 0 on the graph. At the time marked by the arrow (flash) the slice was exposed to ultraviolet light for 25 s. (**B**) Sample records obtained at the times indicated by the numbers 1 and 2 in (A) (records are the average of six sweeps). (Upper records) The extracellularly recorded EPSP (Extra). (Middle records) The response to a constant current hyperpolarizing pulse (0.11 nA) used to monitor the input resistance (R_{input}). (Bottom records) The intracellularly recorded EPSP (Intra). The right-hand column shows superimposed records before and after the flash.

R. C. Malenka, J. A. Kauer, R. A. Nicoll, Departments of Pharmacology and Physiology, School of Medicine, University of California, San Francisco, CA 94143.
R. S. Zucker, Department of Physiology and Anatomy, University of California, Berkeley, CA 94720.

*To whom correspondence should be addressed.

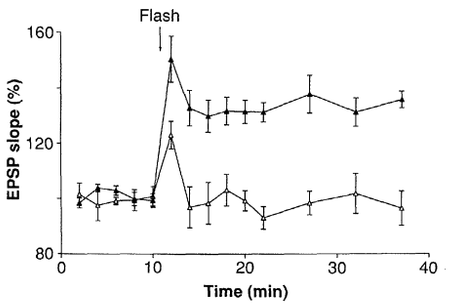

Fig. 2. Comparison of the photolysis of Ca^{2+}-loaded and unloaded nitr-5 on synaptic transmission. (▲) The mean EPSP slope recorded with electrodes filled with Ca^{2+}-loaded nitr-5 (n = 12). (△) The mean EPSP slope recorded with electrodes filled with unloaded nitr-5 (n = 6). The duration of the flash varied from 10 to 45 s depending on depth of the cell in the slice. The bars represent ±SEM. Each point was normalized to the average value of 20 EPSP slope measurements immediately prior to the flash.

hippocampal slices within 225 μm of the surface and studied effects on synaptic transmission. Cells were allowed to stabilize for at least 15 min after impalement to permit filling of the cell with Ca^{2+}-loaded nitr-5. Photolysis of nitr-5, which decreases the Ca^{2+} affinity by a factor of 40, was accomplished with a modified ILC Technology (Sunnyvale, California) 150-W Cermax xenon arc lamp fitted with a liquid filter to eliminate infrared and far-ultraviolet radiation. The intensity of this lamp at 360 nm was calibrated by determining its photolytic efficiency on dilute solutions of nitr-5 (*11*).

Photolysis of Ca^{2+}-loaded nitr-5 produced a large increase in the amplitude and initial slope of the excitatory postsynaptic potential (EPSP) (10 of 12 cells) (Figs. 1 and 2). No change in input resistance (R_{input}) was seen after the light pulse (Fig. 1B). Simultaneous recording of the extracellular population EPSP demonstrated that this was not a nonspecific effect of the light pulse on slice physiology, as no long-lasting effects on this EPSP were observed. To control for possible effects of photolyzed nitr-5 itself, we repeated the experiment with microelectrodes filled with nitr-5 that was not loaded with Ca^{2+} (n = 6). Photolysis of unloaded nitr-5 had no persistent effect on synaptic transmission, indicating that the release of Ca^{2+} from loaded nitr-5 is responsible for the enhancement of the EPSP (Fig. 2, open triangles) (*13*).

The enhancement of the EPSP after nitr-5 photolysis lasted from 20 to 70 min. However, the duration is unlikely to be significant because it is unknown how long intracellular [Ca^{2+}] remains increased after photolysis of nitr-5. Taking into account the concentration of nitr-5, the transmission of light through the slice, the buffering capacity of the cell, and passive diffusion of Ca^{2+}, we estimate that after photolysis of nitr-5 intracellular free [Ca^{2+}] was increased from the resting level to the low micromolar range (*14*). This [Ca^{2+}] may be maintained for a considerable time because the nitr-5 buffering would be expected to retard the lowering of cytoplasmic Ca^{2+}.

Ca^{2+} is thought to trigger LTP because intracellular injection of EGTA, a Ca^{2+} chelator, can block LTP induction (*9*) and transiently raised extracellular [Ca^{2+}] can persistently enhance EPSPs (*10*). Before photolysis, nitr-5 is a potent and more rapid chelator of Ca^{2+} than EGTA (*11*), and therefore it should be capable of quickly buffering any physiologically induced rise in intracellular [Ca^{2+}]. Thus, we examined the effects of nitr-5 on LTP. Tetanic stimulation that elicited LTP in the extracellular field EPSP only evoked posttetanic potentiation in the cell filled with nitr-5 (Fig. 3, A and B). The results with nitr-5 electrodes were compared to the results obtained when recording with a microelectrode filled with CsCl but without nitr-5. This was a particularly important control because intracellular Cs^+ has been reported to block LTP (*15*). Cells filled with Cs^+ exhibited robust LTP, in marked contrast to those cells filled with Cs^+ plus nitr-5 (Fig. $3B_2$). Simultaneous recordings of the field EPSPs (Fig. $3B_1$) indicate that the magnitude of LTP evoked in the two groups of slices was essentially identical. This set of experiments thus confirms the earlier observation concerning the effects of EGTA on LTP (*9*) and lends additional support to the hypothesis that a rise in intracellular Ca^{2+} is crucial for the induction of LTP. However, experiments in which intracellular Ca^{2+} was buffered do not distinguish increases in intracellular [Ca^{2+}] due to Ca^{2+} influx across the cell membrane from those due to release of Ca^{2+} from intracellular stores.

In cultured neurons of the central nervous system, holding the membrane potential at values more positive than about +20 mV suppresses the influx of Ca^{2+} through the NMDA channel (*4*). To test whether the influx of Ca^{2+} across the membrane is required for LTP we hypothesized that, whereas pairing single afferent stimuli with moderate depolarization will cause potentiation by removing the Mg^{2+} block of the NMDA channel (*16, 17*), strong depolarization should prevent potentiation by reducing Ca^{2+} influx through the channel. Therefore we paired EPSPs with strong (>4 nA) and moderate depolarization in the same cell (Fig. 4). When low-frequency stimuli were paired with membrane depolarization far beyond the EPSP reversal potential by means of large (4.5-nA) intracellular current

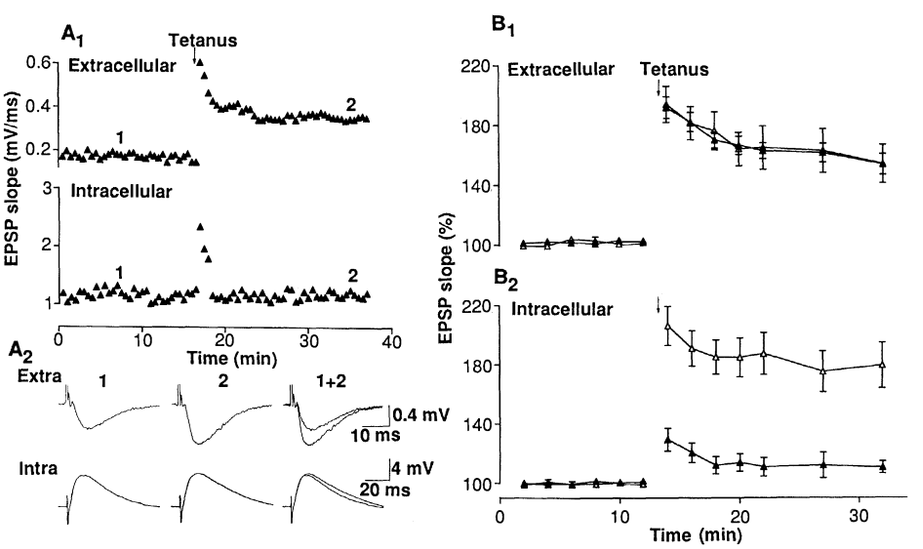

Fig. 3. Chelating intracellular Ca^{2+} with nitr-5 blocks LTP. (**A**) (A_1) Graphs of the slope of the extracellular EPSP recorded in stratum radiatum (upper) and the slope of the intracellularly recorded EPSP (lower) (*12*). The cell was penetrated 15 min before time 0 on the graph. At the time denoted by the arrow, the pathway was tetanized for 1 s at 100 Hz, two times, 20 s apart. After the tetanus the extracellular EPSP remained potentiated, while the intracellular EPSP showed only posttetanic potentiation. (A_2) Sample records obtained at the times indicated by the numbers 1 and 2 in (A_1) (records are the average of six sweeps). The right-hand column shows superimposed records of EPSPs before and after the tetanus. (**B**) Summary graphs comparing the results from nitr-5–filled cells (n = 26) to the results from CsCl-filled cells (n = 15). (B_1) shows that there is essentially no difference in the magnitude of LTP in the two populations of slices. (B_2) shows that although robust LTP was evoked in cells loaded with CsCl (△), little LTP was evoked in cells filled with CsCl plus nitr-5 (▲). The bars represent ±SEM. Each point was normalized to the averaged values of 20 EPSP slope measurements immediately prior to the tetanus.

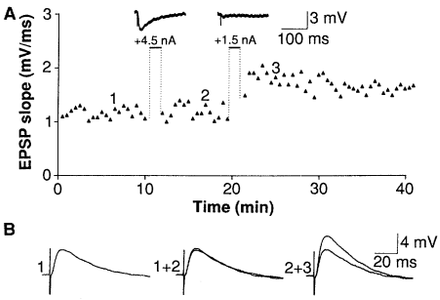

Fig. 4. Effect of membrane depolarization on the induction of LTP. (**A**) Graph of EPSP slope as a function of time of recording. Afferents were continuously stimulated at 0.1 Hz throughout the experiment. Strong depolarization of the cell with +4.5 nA (during bar) reverses the EPSP (see inset trace) but does not cause potentiation after the depolarization. More modest depolarization of the cell with +1.5 nA to approximately the reversal potential of the EPSP (see inset trace) is followed by a substantial potentiation. Little change in input resistance was observed after the depolarizations in this cell. Each point is the mean of three successive slope measurements. (**B**) EPSP records were taken at the times indicated by the numbers 1, 2, and 3 in (A) (records are the average of six sweeps).

injection, no enhancement of the EPSP was observed. In contrast, subsequent pairing of identical low-frequency stimuli with a more modest membrane depolarization to the EPSP reversal potential (which is ~0 mV) resulted in a marked potentiation. Potentiation 5 min after strong depolarization was 99.1 ± 2.3% of control and after moderate depolarization was 140.2 ± 10.0% of control (n = 5). We also examined the effect of large depolarizing current injection on tetanus-induced LTP and found that we were unable to block completely potentiation evoked in this manner (n = 5). This is not surprising because the large conductance increases during the summated EPSPs of the tetanus would tend to drive the membrane potential close to the reversal potential for the EPSP and thus permit Ca^{2+} entry.

The increase in the EPSP after photolysis of Ca^{2+}-loaded nitr-5 provides the first direct demonstration that a rise in postsynaptic intracellular [Ca^{2+}] is sufficient to enhance synaptic transmission. The finding that potentiation is blocked either by buffering changes in intracellular Ca^{2+} with nitr-5 or by retarding Ca^{2+} influx with a large membrane depolarization indicates that a rise in intracellular [Ca^{2+}], either directly from influx or indirectly by release from intracellular stores induced by the Ca^{2+} influx, is also necessary for the induction of LTP.

We have previously proposed that potentiation after a tetanus consists of two postsynaptically induced components, a decremental component that can be mimicked by NMDA receptor activation and a long-lasting, nondecremental component that in addition to requiring activation of NMDA receptors, requires stimulation of presynaptic afferents (*18*). The duration of the rise in [Ca^{2+}] elicited by nitr-5 photolysis is unknown and may last for tens of minutes, and therefore the duration of the EPSP enhancement cannot be used to determine which component is mimicked by the Ca^{2+} increase. Because application of NMDA only produces a short-term potentiation, one would predict that a transient rise in [Ca^{2+}] should only produce the short, decremental form of potentiation. A test of this hypothesis must await the development of a completely reversible Ca^{2+} chelator. However, the finding that both chelation of Ca^{2+} and suppression of Ca^{2+} entry prevented any increase in the EPSP suggests that both forms of potentiation depend on Ca^{2+} entering the postsynaptic cell, presumably through NMDA receptor channels on dendritic spines.

How might a postsynaptic rise in [Ca^{2+}] trigger an enhancement of synaptic transmission? One initial step might be the activation of one or more Ca^{2+}-dependent protein kinases. Type II Ca^{2+}-calmodulin–dependent kinase is found in high concentrations at the postsynaptic density in mammalian brain (*19*), and its activity becomes independent of Ca^{2+} after autophosphorylation (*20*). However, as yet there is little physiological evidence demonstrating a role for this kinase in LTP. In contrast, several lines of evidence suggest that activation of protein kinase C may play a role in LTP (*21*). In addition to activating protein kinases, Ca^{2+} stimulates proteases that may play a role in LTP (*22*). Although the specific events triggered by Ca^{2+} remain to be determined, our results demonstrate that a rise in postsynaptic Ca^{2+} is an essential step in the generation of LTP.

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13. The transient potentiation seen in each plot in Fig. 2 is likely to reflect a nonspecific effect of the light (presumably a rise in temperature), because potentiations with identical time courses were also often recorded simultaneously in the extracellularly recorded EPSP fields.
14. Slices were exposed to light for a duration sufficient to photolyze 60 to 80% of the nitr-5, calculated by estimating the light intensity after passing through the slice to the depth of the injected cell. By using the model outlined in detail elsewhere (L. Landò and R. S. Zucker, *J. Gen. Physiol.*, in press), we estimate that photolysis elevated intracellular [Ca^{2+}] from a resting level of 140 n*M* to between 2 and 4 μ*M*.
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Muscarinic Depression of Long-Term Potentiation in CA3 Hippocampal Neurons

STEPHEN WILLIAMS AND DANIEL JOHNSTON

Behavioral studies have suggested that muscarinic cholinergic systems have an important role in learning and memory. A muscarinic cholinergic agonist is now shown to affect synaptic plasticity in the CA3 region of the hippocampal slice. Long-term potentiation (LTP) of the mossy fiber–CA3 synapse was blocked by muscarine. Low concentrations of muscarine (1 micromolar) had little effect on low-frequency (0.2 hertz) synaptic stimulation but did significantly reduce the magnitude and probability of induction of LTP. Experiments under voltage clamp showed that muscarine blocked the increase in excitatory synaptic conductance normally associated with LTP at this synapse. These results suggest a possible role for cholinergic systems in synaptic plasticity.

LONG-TERM POTENTIATION (LTP) IS a form of use-dependent synaptic plasticity that is an attractive candidate as a substrate for learning and memory (*1*). LTP is a prominent feature of all excitatory synapses studied so far in the hippocampus, an area of critical importance for certain types of memory processing (*2*). Lesion of the cholinergic afferents to the hippocampus, the fimbria of the fornix (*3*), results in learning deficits in animals, an impairment that can be mimicked by cholinergic antagonists (*4*). Furthermore, memory deficits occurring with certain senile dementias have an associated diminishment of cholinergic function (*5*). We have investigated the effects of the cholinergic agonist muscarine on LTP in the voltage-clamped mossy fiber–CA3 pyramidal cell synapse of the hippocampus, a synapse particularly suitable for detailed biophysical analysis (*6*).

Transverse hippocampal slices (450 μm) were prepared from adult rats and maintained in vitro, at 32° to 34°C, with standard techniques (*6–9*). Conventional single-electrode voltage-clamp experiments were performed with low-resistance microelectrodes (20 to 40 megohms). Picrotoxin (10 μ*M*) was added routinely to the bathing medium to block inhibitory postsynaptic currents, thereby allowing unambiguous measurement of the excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) (*6, 8*); to prevent epileptiform discharges, 3 m*M* Ca^{2+} and 3 m*M* Mg^{2+} were present in the bathing medium (*8, 9*). Measurements of EPSPs were made between −70 and −90 mV (constant for any given cell) to prevent action potential generation. At this hyperpolarized holding potential, muscarine had little effect on input resistance, in contrast to potentials close to and positive to the resting potential (*10*), thereby minimizing consequent changes in EPSP amplitude. Data are based on 105 stable impalements (mean input resistance, 52 ± 2 megohms), 31 of which were maintained for at least 20 min after tetanic stimulation. LTP was defined as >20% increase in the synaptic response maintained for at least 15 min after (post) tetanic stimulation (15′ PT) (*7*). Comparisons between data sets were made with an appropriate *t* test, and data both in the text and figures are presented as mean ± SEM. Significance level was set at $P < 0.05$.

The CA3 pyramidal neuron of the hippocampus receives three major excitatory synaptic inputs: the mossy fiber, commissural, and recurrent pathways. It has been shown by extracellular measurement that LTP of the putative mossy fiber synaptic response is not blocked by 2-amino-5-phosphonovalerate (APV) (*11*), an *N*-methyl-D-aspartate (NMDA) antagonist, but LTP of the commissural-associational input to CA3 is blocked by APV (*11, 12*). As a test of our criteria for identifying mossy fiber responses (*6, 8*) (which were the location of stimulating electrode and EPSC kinetics), several experiments were performed in the presence of APV. We found that APV (10 μ*M*, $n = 5$; 50 μ*M*, $n = 2$) had no significant effect on the control EPSP or EPSC amplitude, and LTP could still be evoked (mean EPSP increase +71 ± 17%, $n = 4$). There was no significant difference between LTP obtained in control cells compared to that in cells tetanized in the presence of APV, confirming that the observed LTP is not dependent on NMDA receptor activation.

In control cells, synaptic responses were constant over time (up to 30 min); after

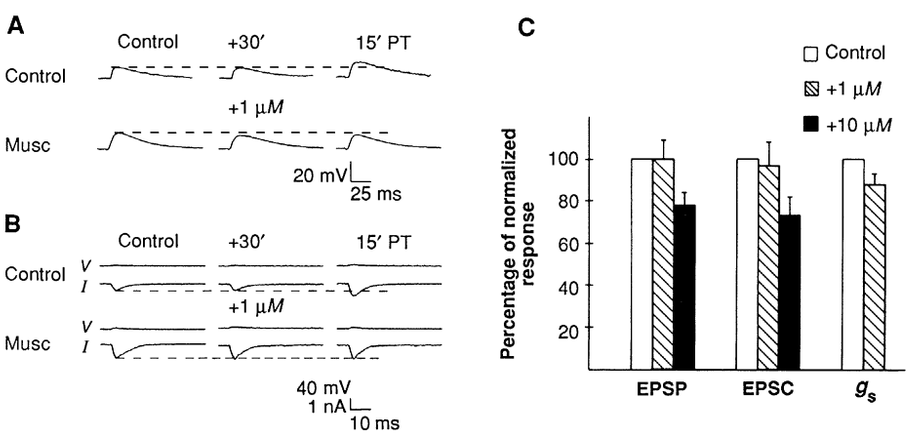

Fig. 1. The effects of muscarine on synaptic transmission and LTP. (**A**) Upper traces show that in a control cell the EPSP is stable for 30 min (30′), but that 15 min after tetanus (15′ PT) an increase in amplitude is seen. The lower traces (musc, different cell) show that 1 μ*M* muscarine produced a small depression of the EPSP before tetanus and prevented the development of LTP at 15′ PT. (**B**) In a similar experiment under voltage clamp (*V*, membrane potential; *I*, clamp current), LTP in the control cell was seen as an increase in the EPSC, but in the muscarine-treated cell no increase was apparent. (**C**) Summary of the actions of muscarine (1 and 10 μ*M*) on synaptic transmission in the absence of tetanus. All data were normalized to control. EPSP: 1 μ*M*, $n = 10$; 10 μ*M*, $n = 6$. EPSC: 1 μ*M*, $n = 8$; 10 μ*M*, $n = 5$. g_s: 1 μ*M*, $n = 5$.

Program in Neuroscience, Section of Neurophysiology, Department of Neurology and Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.