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Mediation of Classical Conditioning in *Aplysia californica* by Long-Term Potentiation of Sensorimotor Synapses

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Long-term potentiation (LTP) is considered an important neuronal mechanism of learning and memory. Currently, however, there is no direct experimental link between LTP of an identified synapse and learning. A cellular analog of classical conditioning in *Aplysia* was used to determine whether this form of invertebrate learning involves *N*-methyl-D-aspartate (NMDA)–type LTP. The NMDA receptor-antagonist DL-2-amino-5-phosphonovalerate significantly disrupted synaptic enhancement after associative training but did not disrupt synaptic enhancement after nonassociative training. Thus, classical conditioning in *Aplysia* appears to be mediated, in part, by LTP due to activation of NMDA-related receptors.

LTP of *Aplysia* sensorimotor synapses, like LTP of synapses in the CA1 region of the mammalian hippocampus (*1*), requires strong postsynaptic depolarization and postsynaptic influx of Ca^{2+} (*2*, *3*). Furthermore, induction of LTP of *Aplysia* sensorimotor synapses also resembles LTP of CA1 synapses (*4*) in its requirement for activation of NMDA-type receptors (*5*) because it can be inhibited by the vertebrate NMDA receptor-antagonist DL-2-amino-5-phosphonovalerate (APV) (*3*). The finding (*3*) that LTP of the sensorimotor synapses can be induced in Hebbian (*6*) fashion raises the possibility that LTP might mediate classical conditioning of the siphon-withdrawal reflex of *Aplysia* (*7*). Tail shock, the unconditioned stimulus (US) for this form of associative learning, strongly depolarizes, and typically fires, the siphon motor neurons (*8*). Thus, paired presentation of the conditioned stimulus (CS)—weak tactile stimulation of the animal's siphon or mantle—and the US during conditioning should produce a pattern of neural activity within the nervous system of *Aplysia* like that used to induce Hebbian LTP of in vitro sensorimotor synapses (*3*): brief firing of the sensory neurons paired with strong depolarization of the motor neurons (*9*). Support for the idea that classical conditioning in *Aplysia* might involve LTP of sensorimotor synapses comes from the finding that infusing the postsynaptic motor neuron with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy) ethane-*N*,*N*-*N*′,*N*′-tetraacetic acid (BAPTA) blocks a cellular analog of classical conditioning (*10*).

In this study, we tested the hypothesis (*3*, *11*) that classical conditioning of the siphon-withdrawal reflex of *Aplysia* is mediated, in part, by NMDA receptor-type LTP. Accordingly, we examined whether the cellular analog of classical conditioning of this reflex (*12*) was disrupted when training was carried out in the presence of APV. We assessed the strength of the synapse between a siphon sensory neuron and a siphon motor neuron in the abdominal ganglion before, during, and after conditioning-related training (*13*). For this cellular analog of classical conditioning, the CS was brief intracellular stimulation of the sensory neuron and the US was extracellular stimulation of the tail (P9) nerves (Fig. 1A). Some preparations received the paired CS-US stimuli in artificial seawater (ASW) containing APV (100 μM) (*14*). We also included two groups of untrained preparations that received the test stimuli but not the paired stimuli. One untrained group (Test alone) received the test stimuli in normal ASW; the other untrained group (Test alone–APV) received the test stimuli in ASW containing APV. Furthermore, we carried out additional control experiments in which the CS and US were delivered in unpaired fashion in the presence and absence of APV (see below).

The monosynaptic sensorimotor excitatory postsynaptic potentials (EPSPs) in preparations that received only the test stimuli in ASW exhibited the homosynaptic depression characteristic of *Aplysia* sensorimotor synapses (Fig. 1, B and C) (*15–17*). The presence of APV did not significantly affect this homosynaptic depression, as indicated by the Test alone–APV data (*17*, *18*). The monosynaptic EPSPs in preparations that received paired presentations of the CS and US in normal ASW (CS^+ group) were significantly enhanced on both the 15- and 60-min posttests compared with the pretest EPSPs as well as with the EPSPs of Test alone preparations on the posttests (*18*). The presence of APV during training significantly disrupted the synaptic enhancement produced by paired stimulation. However, APV did not completely eliminate this synaptic enhancement; the mean amplitude of the CS^+–APV EPSPs was significantly greater than that of the Test alone–APV EPSPs on the 15-min posttest (Fig. 1, B and C; CS^+–APV data). The paired stimulation also produced strong enhancement of the amplitude of the potentially polysynaptic sensorimotor EPSPs evoked on the test trials during training. This enhancement was reduced in the presence of APV, albeit not significantly so (*19*).

A possible explanation for the reduced enhancement of the monosynaptic sensorimotor connection after training in APV is that the drug might have disrupted the activity, or efficacy, of endogenous facilitatory interneurons, which are activated in *Aplysia* by US-related stimuli (*8*, *20*, *21*). To test this possibility, we carried out experiments similar to those described above but with a specifically unpaired training protocol. Such a protocol induces presynap-

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tic facilitation of sensorimotor synapses (*12*, *22*). This nonassociative form of synaptic enhancement is thought to play a significant role in behavioral sensitization of the withdrawal reflex (*23*). During nonassociative training, preparations received five unpaired presentations of the CS and US in which the US preceded the CS by 2.5 min (Fig. 2A). These experiments were carried out in either normal ASW or in ASW containing APV. A group of untrained preparations that received only the test stimuli (Test alone group) was also included (*24*). As before, the Test alone group exhibited homosynaptic depression (Fig. 2, B and C) (*25*). Unpaired training in either normal ASW (CS^- group) or ASW containing APV (100 μM; CS^-–APV group) resulted in significant enhancement of the sensorimotor EPSP, compared with the Test alone group, when assessed 15 min after the last US (Fig. 2, B and C) (*26*). There was no significant difference between the amplitudes of the EPSPs for the CS^- and CS^-–APV groups on either the 15- or the 60-min posttest. Thus, APV did not disrupt enhancement of the monosynaptic sensorimotor EPSP because of unpaired stimulation. Unpaired training, like paired training, enhanced the test sensorimotor EPSPs evoked during training, but the presence of APV did not decrease the amplitude of these EPSPs (*27*). In summary, our data from the experiments involving unpaired stimulation indicate that the reduction of synaptic enhancement observed after paired training in APV cannot be attributed to disruption of facilitatory pathways (*28*).

The CS^+ and CS^- groups were directly compared (Fig. 2D). The CS^+ EPSP and the CS^- EPSP were significantly greater than the Test alone EPSP on the 15- and 60-min posttests (*29*). The CS^+ EPSP and the CS^- EPSP were statistically indistinguishable on the 15-min posttest, but on the 60-min posttest the CS^+ EPSP was significantly greater than CS^- EPSP. Thus, unpaired training produced significant nonassociative enhancement of the monosynaptic sensorimotor EPSP, as indicated by the significant difference between the CS^- and Test alone EPSPs on both posttests; also, paired training induced a long-term associative synaptic enhancement, which was evident 60 min after the last bout of paired stimulation (*30*).

These results support the hypothesis (*3*, *11*) that LTP-related enhancement of sensorimotor synapses plays a significant role in classical conditioning in *Aplysia*. A model for how the stimuli used for classical conditioning of the siphon-withdrawal reflex (*7*) induce LTP of the sensorimotor synapses (*2*, *3*) is shown (Fig. 3). Together with earlier results (*10*), our data indicate that the site of induction for associative, conditioning-related enhancement of the sensorimotor connections must be, in part, postsynaptic. Induction of this synaptic change cannot be exclusively presynaptic, as previously argued (*12*, *22*). It is unclear, however, whether the locus of expression for this synaptic enhancement is pre- or postsynaptic. Some evidence (*12*, *31*, *32*) is consistent with a presynaptic locus of expression. If conditioning-related enhancement of the sensorimotor connections does involve increased presynaptic release, then our results implicate a retrograde messenger analogous to that implicated in LTP of synapses in the CA1 region of the hippocampus (*33*) (Fig. 3). However—again by analogy with LTP of CA1 synapses (*34*)—even if classical conditioning in *Aplysia* does involve increased presynaptic release, postsynaptic changes may also contribute to the expression of conditioning-related enhancement of sensorimotor connections.

Another issue raised by these results is the role of serotonin [5-hydroxytryptamine (5-HT)], or other facilitatory neurotransmitters, in classical conditioning in *Aplysia*. Such modulatory neurotransmitters play a central role in a previous hypothesis about the cellular mechanism of this form of associative learning (*12*, *22*). Significant, albeit still indirect, evidence supports a role for 5-HT in classical conditioning in *Aplysia* (*12*, *21*, *31*, *32*, *35*, *36*). This suggests the possibility that US-stimulated release of 5-HT, and possibly other endogenous modulatory transmitters, might interact with NMDA-type LTP during classical conditioning, producing both pre- and postsynaptic cellular changes (Fig. 3).

Evidence from a variety of studies suggests that NMDA receptor-dependent synaptic plasticity is involved in associative learning in vertebrates (*37*). But the precise role of NMDA receptors in vertebrate

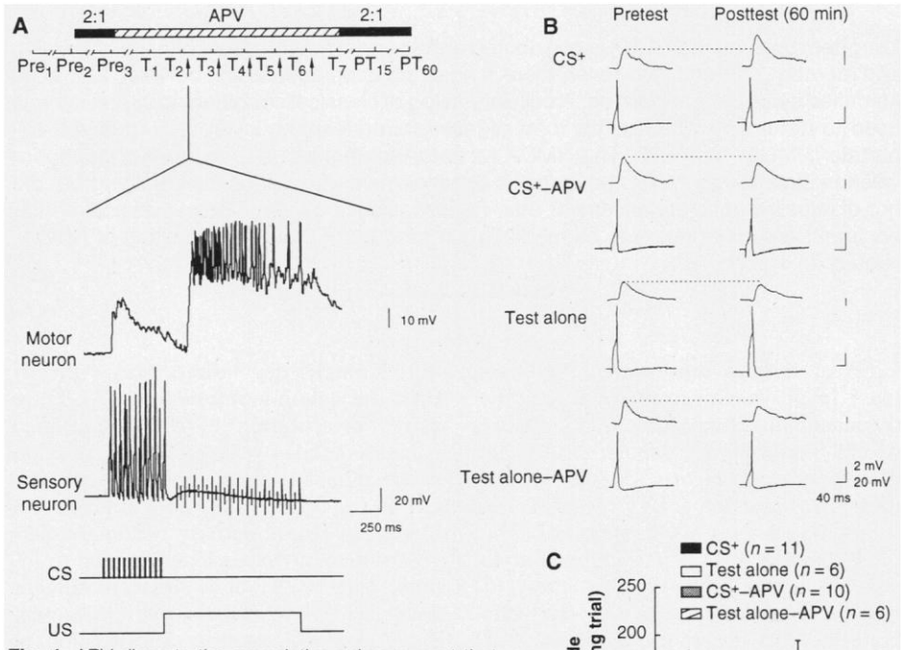

Fig. 1. APV disrupts the associative enhancement that results from repeated CS-US pairings. (**A**) Experimental protocol for the cellular analog of classical conditioning of siphon withdrawal. Training included seven test trials (T_1 to T_7) and five CS-US pairings (arrows). In addition, there were three pretests (Pre_1 to Pre_3) and two posttests. The three pretests, as well as Test 1, were separated by 15 min. Posttests (PT_{15} and PT_{60}) were conducted 15 and 60 min after the last CS-US pairing. Pretest 1 and Pretest 2 were carried out in normal ASW. After Pretest 2 the 2:1 ASW (black bars) (*13*) was perfused into the recording chamber, and Pretest 3 was performed. The 2:1 ASW was then washed out, and training proceeded in normal ASW or in ASW with APV (hatched bar). After Test 1 the 2:1 ASW was reintroduced and remained in the recording chamber during the posttests. Sample records from a sensory neuron and a motor neuron during a CS-US pairing from a CS^+–APV experiment are also shown. The US produced strong depolarization and firing of the motor neuron. (**B**) Representative records from pretest and 60-min posttests. (**C**) Effect of APV on the cellular analog of classical conditioning. The EPSP amplitude for the 15- and 60-min posttests was normalized to the amplitude of the EPSP of Pretest 3 (A).

learning is highly controversial (38). Our results indicate that NMDA-type synaptic plasticity is a phylogenetically ancient neural mechanism that mediates at least one kind of invertebrate associative learning. Thus, the siphon-withdrawal reflex of *Aplysia* and its underlying neural circuitry offer a relatively simple model system for rigorous analysis of the role of NMDA-type receptors in associative learning. Such an analysis should facilitate an understanding of the contribution of NMDA receptors to learning in more complex animals.

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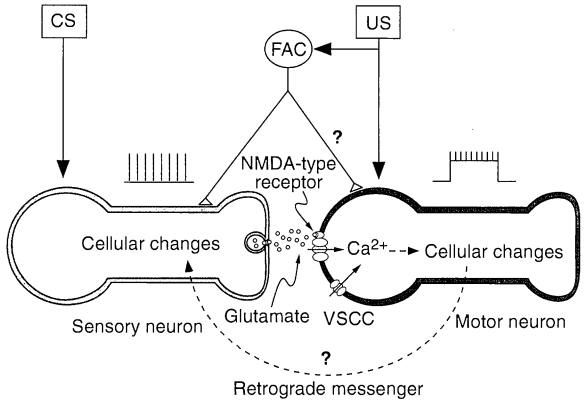

Fig. 3. Model of how the stimuli used for classical conditioning of the withdrawal reflex of *Aplysia* induce LTP of sensorimotor synapses together with other associative cellular changes. According to this model the CS, weak tactile stimulation of the siphon or mantle, activates mechanoreceptive sensory neurons. The US, tail shock, causes strong depolarization of siphon motor neurons. [This postsynaptic depolarization occurs indirectly by activation of excitatory interneurons (*8, 20*).] The release of the presynaptic neurotransmitter—glutamate (*5*) or a related excitatory amino acid (*39*)—together with the strong postsynaptic depolarization activates postsynaptic NMDA, or NMDA-type, receptors. Activation of postsynaptic NMDA-type receptors, and perhaps the opening of postsynaptic voltage-sensitive Ca^{2+} channels (VSCC) (*3, 40*), causes a postsynaptic influx of Ca^{2+}. This increase in intracellular Ca^{2+} initiates a biochemical cascade within the motor neuron—possibly involving activation of one or more postsynaptic protein kinases (*41*)—which results in LTP of the sensorimotor synapses. The US also activates facilitatory interneurons (FAC), some of which are serotonergic (*8, 20, 21, 35*). Activation of these facilitatory interneurons, paired with presynaptic activity, produces associative presynaptic changes (*12, 31, 32, 42*), which are believed to contribute to the strengthening of sensorimotor connections. Furthermore, the facilitatory transmitter (or transmitters) may modulate the cellular pathways activated by postsynaptically induced LTP. Finally, the postsynaptically induced LTP may cause release of a retrograde messenger, which may play a critical role in associative presynaptic changes, particularly enhanced presynaptic release (*31*).

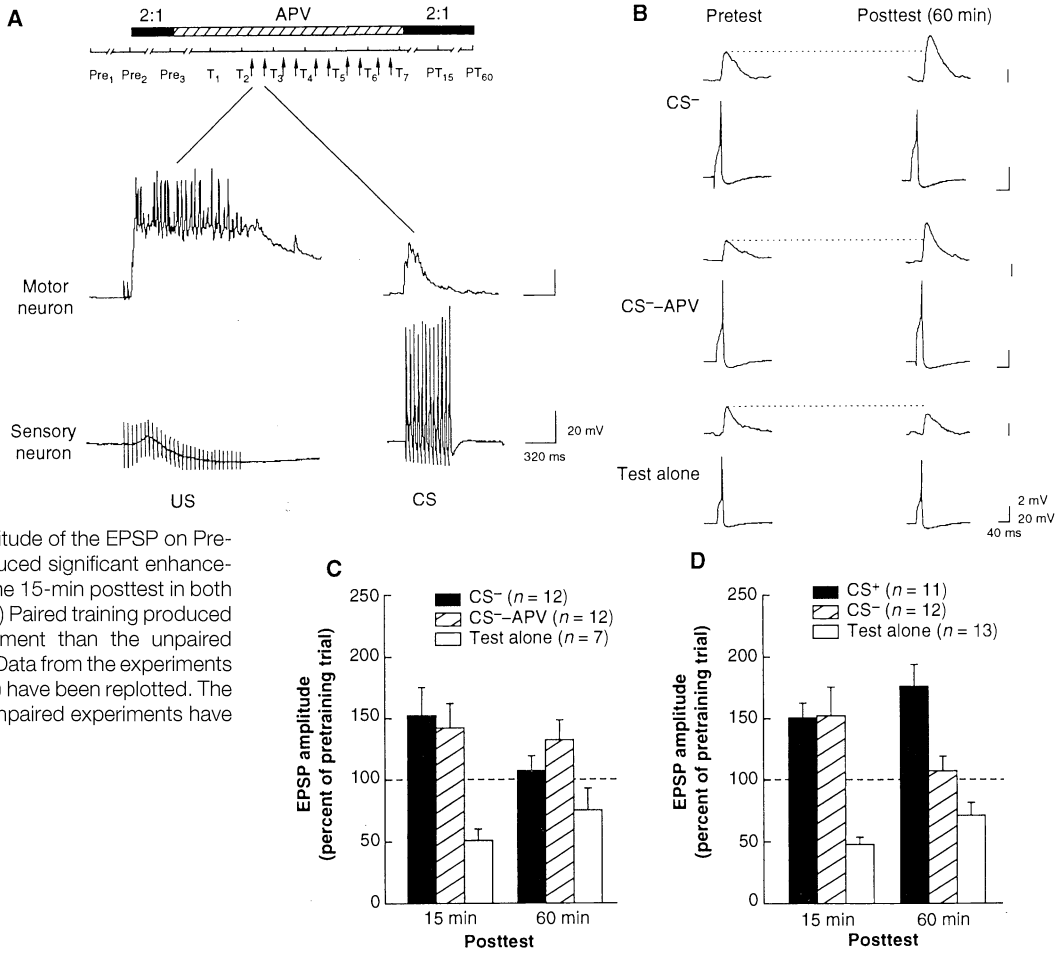

Fig. 2. Effect of APV on nonassociative synaptic enhancement and comparison of the effects of paired and unpaired training on sensorimotor connections. (**A**) Nonassociative training protocol. The protocol is identical to that for paired training (Fig. 1A) except that the onset of the US preceded the onset of the CS by 2.5 min. Presentations of the unpaired stimuli are indicated by arrows. Sample recordings from a sensory neuron and a motor neuron during the US stimulation (left trace) and during the subsequent CS stimulation (right trace) are also shown. (**B**) Representative records from pretest and 60-min posttests. (**C**) APV does not disrupt nonassociative enhancement. The amplitude of the EPSP on each of the posttests was normalized to the amplitude of the EPSP on Pretest 3. The unpaired stimulation produced significant enhancement of the monosynaptic EPSP for the 15-min posttest in both the CS^- and the CS^-–APV groups. (**D**) Paired training produced significantly more synaptic enhancement than the unpaired training protocol 60 min after training. Data from the experiments done in normal ASW (Figs. 1C and 2C) have been replotted. The test alone data from the paired and unpaired experiments have been combined (*29*).

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9. Although simultaneous pairing of presynaptic stimulation and postsynaptic depolarization was originally used for Hebbian LTP of sensorimotor synapses (*3*), significant LTP of sensorimotor synapses also results from a forward pairing protocol in which the presynaptic stimulation precedes the postsynaptic depolarization by 0.5 s [X. Y. Lin and D. L. Glanzman, *J. Neurophysiol.* **77**, 667 (1997)].
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13. The preparation and general experimental methods are described in (*10*). A single siphon sensory (LE) neuron [J. Byrne, V. Castellucci, E. R. Kandel, *J. Neurophysiol.* **37**, 1041 (1974)] and a single small siphon (LFS) motor neuron (*8*) were both impaled with sharp microelectrodes. Testing and training then proceeded as described (*10*). Briefly, during training there were five bouts of CS-US stimulation at a rate of one per 5 min. The paired stimulation began 1 min after the second test trial (T_2; Fig. 1A). The CS consisted of evoking 12 action potentials (at 25 Hz) in the sensory neuron by intracellular stimulation. The US consisted of 1 s of tail nerve shock (3-ms pulses at 25 Hz), the strength of which was set to three to six times the threshold intensity for evoking EPSPs in the motor neuron. Pretest 3 and both of the posttests (Fig. 1A) were carried out in modified ASW, which contained high concentrations of Mg^{2+} and Ca^{2+}. This 2:1 ASW [368 mM NaCl, 13.8 mM $CaCl_2$, 8 mM KCl, 101 mM $MgCl_2$, 20 mM $MgSO_4$, and 10 mM Hepes buffer (pH 7.6)] reduces the interneuronal contribution to the sensorimotor EPSP [L.-E. Trudeau and V. F. Castellucci, *J. Neurosci.* **12**, 3838 (1992)].
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16. Summary statistics are presented as mean ± SEM; *n* denotes number of preparations. Statistical significance for multiple group comparisons was assessed by either parametric or nonparametric [Kruskal–Wallis (KW)] analysis of variance (ANOVA). The choice of statistical test was determined by the outcome of Bartlett's tests for homogeneity of variances performed on the group data. Subsequent between-group comparisons were made with Student–Newman–Keuls tests (parametric) or Dunn's tests (nonparametric). Planned comparisons between two experimental groups were made with either unpaired *t* tests or nonparametric Mann–Whitney tests. Intragroup comparisons (between pre- and posttests) were made with Wilcoxon signed-rank tests. All reported levels of statistical significance represent two-tailed values.
17. The mean amplitude of the Test alone EPSP was 44.2 ± 8% for the 15-min posttest and 43.7 ± 11% for the 60-min posttest. Both of the posttest EPSPs were smaller than the Test alone EPSP on Pretest 3 ($P < 0.01$ for each comparison). The mean amplitudes of the Test alone–APV EPSP were 61.0 ± 10% for the 15-min posttest and 65.6 ± 7% for the 60-min posttest. Both of these posttest EPSPs were also smaller than the Test alone–APV EPSP on Pretest 3 ($P < 0.01$ for each comparison).
18. The mean amplitudes of the CS^+ EPSP were 150.5 ± 12% for the 15-min posttest and 176.0 ± 18% for the 60-min posttest. The mean amplitudes of the CS^+–APV EPSP were 110.2 ± 15% for the 15-min posttest and 115.4 ± 15% for the 60-min posttest. The differences among the groups were significant for both the 15-min posttest [$F(3,29) = 13.6$, $P > 0.0001$] and the 60-min posttest [$F(3,27) = 13.2$, $P < 0.0001$]. The EPSPs in the CS^+ group were enhanced on both the 15- and 60-min posttests compared with the Test alone group ($P < 0.001$ for each comparison) as well as with the CS^+–APV group ($P < 0.05$ for the 15-min posttest comparison, $P < 0.01$ for the 60-min posttest comparison). Furthermore, the EPSPs in the CS^+–APV group were larger than those in the Test alone–APV group on the 15-min posttest ($P < 0.05$), although not on the 60-min posttest ($P > 0.05$). The differences between the Test alone and the Test alone–APV groups were not significant on either posttest ($P > 0.05$ for each comparison). Finally, there were no differences among the mean raw EPSPs evoked on Pretest 3 in the four experimental groups [CS^+ EPSP = 5.2 ± 1.2 mV; CS^+–APV EPSP = 8.4 ± 1.6 mV; Test alone EPSP = 5.4 ± 0.7 mV; Test alone–APV EPSP = 7.1 ± 1.4 mV; $F(3,29) = 1.3594$, $P > 0.2$].
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24. Because there were no significant differences between the Test alone and the Test alone–APV groups in the paired-training experiments (Fig. 1, B and C) (*17, 18*), we did not include a Test alone–APV group in the unpaired-training experiments.
25. The mean EPSP for the Test alone group was 51.0 ± 9% on the 15-min posttest and 75.3 ± 18% on the 60-min posttest. The Test alone EPSP was smaller on the 15-min posttest than on Pretest 3 ($P < 0.04$). The difference between the EPSPs for Pretest 3 and the 60-min posttest was not significant ($P > 0.1$).
26. The mean EPSP was 152.2 ± 23% on the 15-min posttest for the CS^- group and 142.3 ± 20% for the CS^-–APV. The differences among the three groups on the 15-min posttest were significant (KW = 10.7, $P < 0.005$). The mean EPSPs for the CS^- and CS^-–APV groups were enhanced on the 15-min posttest compared with the Test alone group ($P < 0.01$ for CS^- versus Test alone; $P < 0.05$ for CS^-–APV versus test alone). The difference between the mean EPSPs in the CS^- and CS^-–APV groups on the 15-min posttest was not significant ($P > 0.05$). The mean EPSP on the 60-min posttest was 107.3 ± 12% for the CS^- group and 132.3 ± 16% for the CS^-–APV group. ANOVA indicated that the differences among the three groups on the 60-min posttest were not quite significant ($P > 0.06$). There were no differences among the mean raw EPSPs evoked on Pretest 3 in the three experimental groups (CS^- EPSP = 4.8 ± 1.0 mV; CS^-–APV EPSP = 4.0 ± 0.9 mV; Test alone EPSP = 4.4 ± 1.2 mV; $F(2,28) = 0.2094$, $P > 0.81$).
27. Data not shown.
28. Another possible explanation for disruption by APV of the pairing-induced enhancement of the sensorimotor EPSP (Fig. 1, B and C) is that the drug may have reduced the amount of postsynaptic depolarization caused by the US. This US-induced postsynaptic depolarization (see Fig. 1A) presumably results from the firing of excitatory interneurons that are normally activated by tail shock (*8*). Decreased postsynaptic depolarization by the US due to APV would be expected to reduce the likelihood of inducing LTP of sensorimotor synapses (*2, 3*). To assess the possibility that pedal nerve shock produced less postsynaptic depolarization in the presence of APV, we quantified the amount of US-induced excitatory drive on the motor neuron by integrating the area under the postsynaptic membrane potential during the US for each bout of paired stimulation for both the CS^+ and the CS^+–APV groups. The mean area under the postsynaptic membrane potential during the US for Pairing 1 was virtually identical for the two groups (CS^+ = 35.1 ± 1.9 mV · s; CS^+–APV = 35.2 ± 1.4 mV · s). For subsequent pairings (Pairings 2 to 5) the presence of APV produced a slight increase—not a decrease—in the amount of US-induced postsynaptic depolarization. The mean area under the postsynaptic membrane potential during the US, summed across all five pairings, did not differ statistically between the two groups ($P > 0.6$). These data further argue that our findings cannot be attributed to a nonspecific effect of APV. Consistent with the apparent specificity of the disruptive effect of APV in our experiments is a recent report [S. Schacher, F. Wu, Z.-Y. Sun, *J. Neurosci.* **17**, 597 (1997)] that APV blocks an associative form of long-term enhancement of in vitro sensorimotor synapses but does not affect sensitization-related long-term enhancement of these synapses.
29. The differences among the three groups were significant for both the 15-min posttest (KW = 22.1, $P < 0.001$) and 60-min posttest [$F(2,32) = 17.8$, $P < 0.001$]. Both the mean CS^+ and CS^- EPSPs were larger than the mean Test alone EPSP on the 15-min posttest ($P < 0.001$ for both comparisons), but there was no difference between the CS^+ and the CS^- EPSPs ($P > 0.05$). The mean CS^+ EPSP and CS^- EPSP were both larger than the mean Test alone EPSP on the 60-min posttest (CS^+ versus Test alone, $P < 0.001$; CS^- versus Test alone, $P < 0.05$). Unlike the 15-min posttest, the mean CS^+ EPSP was larger than the mean CS^- EPSP on the 60-min posttest ($P < 0.01$). The Test alone data in Fig. 2D represent the combined Test alone data from Figs. 1C and 2C. The mean Test alone EPSP for the combined data in Fig. 2D was 47.8 ± 6% for the 15-min posttest and 60.7 ± 11% for the 60-min posttest.
30. The time course of the associative effect differed from that described by Hawkins *et al.* (*12*), who found that paired training yielded significantly more enhancement than unpaired training 5 to 15 min after training. Several procedural differences between the two studies might account for this difference. First, the duration and intensity of the CS and US in our study differed from those in the study by Hawkins *et al*. Second, Hawkins *et al*. used a differential conditioning paradigm in which paired and unpaired training were performed on the same preparations. Nevertheless, our 60-min posttest results substantially resemble the posttest results of Hawkins *et al*. (see their Fig. 1D), which suggests that the training recruited similar underlying cellular mechanisms in the two studies.
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Inhibition of Phosphatases and Increased Ca^{2+} Channel Activity by Inositol Hexakisphosphate

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Inositol hexakisphosphate ($InsP_6$), the dominant inositol phosphate in insulin-secreting pancreatic β cells, inhibited the serine-threonine protein phosphatases type 1, type 2A, and type 3 in a concentration-dependent manner. The activity of voltage-gated L-type calcium channels is increased in cells treated with inhibitors of serine-threonine protein phosphatases. Thus, the increased calcium channel activity obtained in the presence of $InsP_6$ might result from the inhibition of phosphatase activity. Glucose elicited a transient increase in $InsP_6$ concentration, which indicates that this inositol polyphosphate may modulate calcium influx over the plasma membrane and serve as a signal in the pancreatic β cell stimulus-secretion coupling.

Depolarization-induced opening of voltage-gated L-type Ca^{2+} channels results in an increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and is one of the main features of the stimulus-secretion coupling in insulin-secreting cells (*1*). Under physiological conditions, depolarization is initiated by rapid uptake and phosphorylation of glucose, which result in the closure of adenosine triphosphate (ATP)–regulated K^+ channels. Insulin-secreting cells also have a number of receptors whose activation regulates the intracellular concentration of inositol polyphosphates (*2*). Although a large number of inositol polyphosphates have been identified in eukaryotic cells (*3*), except for the inositol 1,4,5-trisphosphate–induced mobilization of Ca^{2+} from intracellular stores, little is known about their roles in cell regulation. Protein phosphorylation modulates the activity of voltage-sensitive ion channels (*4*), and in insulin-secreting cells, the activity of voltage-gated L-type Ca^{2+} channels is increased by inhibition of serine-threonine protein phosphatases (PPases) (*5*, *6*).

We examined the inositol phosphates present in insulin-secreting cells after labeling them for 168 hours with [2-^{3}H]myo-inositol (Fig. 1), when all inositol phosphates are at isotopic equilibrium (*7*, *8*). $InsP_6$ was the dominant inositol phosphate in hamster insulin-secreting (HIT) cells, as it is in other mammalian cells (*9*). An inositol-containing compound that is more polar than $InsP_6$, most likely a pyrophosphate derivative (*9*), was also present. Enzyme assays and immunoblots of cell homogenates showed that the cells contained serine-threonine PPases that were inhibited by okadaic acid (OA), microcystin-LR, calyculin-A, and nodularin (*10*). Inhibition of the serine-threonine PPases type 1 (PP1), type 2 (PP2A), and type 3 (PP3) in insulin-secreting cells, which may enhance phosphorylation of the voltage-gated L-type Ca^{2+} channel or an associated protein, results in an increase in channel open probability, $[Ca^{2+}]_i$, and insulin release (*5*, *6*).

$InsP_6$, at concentrations similar to those present in insulin-secreting cells (40 to 54 μM) (*11*), suppressed the activities of PP1, PP2A, and PP3 in a concentration-dependent manner, with inhibition constant K_i values at or below ~10 μM (Fig. 2A and Table 1) (*12*). The inhibitory effects of $InsP_6$ on PPase activities were similar to those of OA (*13*). Two isomers of inositol pentakisphosphate ($InsP_5$), Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,6)P_5, were one-half to one-fifth as potent, depending on the particular combination of $InsP_5$ and PPase (Fig. 2, C and D). Ins(1,2,3,4,6)P_5, like $InsP_6$, includes a 1,2,3-trisphosphate array that binds Fe^{3+} and probably other cations (*14*). However, the fact that Ins(1,2,3,4,6)P_5 has a lower potency than $InsP_6$ indicates that this type of chelation is not the primary mechanism in PPase inhibition (*15*). Whereas PP3 was the most selective for $InsP_6$, PP1 was the least selective, and PP2A fell in between (Table 1). However, neither the dominant inositol tetraphosphate ($InsP_4$) in these cells, Ins(3,4,5,6)P_4, nor the other major $InsP_4$, Ins(1,3,4,5)P_4, had any inhibitory effect up to 100 μM (*16*). The fact that removal of one or two of the six phosphate groups from $InsP_6$ either reduced or abolished these effects indicates that PPase inhibition by $InsP_6$ is specific and is not simply an effect of a concentrated array of monoester phosphate groups. Inositol hexasulfate ($InsS_6$), which presents a charge array similar to that of $InsP_6$, was about one-fourteenth as potent an inhibitor of PP2A and PP3 (Fig. 2B and Table 1). $InsP_6$ is

Table 1. K_i values of various inositol polyphosphates for the inhibition of the three serine-threonine PPases. The values for K_i and SEM were obtained by analysis of the data by nonlinear regression, fitting the data to sigmoidal dose-response curves generated by software (Prism; GraphPAD, San Diego, California). Values were obtained by one-way ANOVA with *P* values corrected for multiple comparisons by the Bonferroni method (Instat). Significant differences between the K_i values of $InsP_6$ and other inositol derivatives for each PPase are shown (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). ND, not determined.

Inositol derivative	K_i(μM) ± SEM		
	PPase 1 ($n = 4$)	PPase 2A ($n = 6$)	PPase 3 ($n = 6$)
$InsP_6$	13.40 ± 1.27	8.54 ± 1.11	3.85 ± 1.02
Ins(1,3,4,5,6)P_5	6.47 ± 1.50*	12.69 ± 1.28	13.5 ± 1.18**
Ins(1,2,3,4,6)P_5	ND	40.5 ± 1.10***	21.5 ± 1.76***
$InsS_6$	29.9 ± 1.87***	116 ± 1.02***	55.0 ± 1.25***

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