Activation of the Cellular Proto-Oncogene Product p21Ras by Addition of a Myristylation Signal

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The 21-kD proteins encoded by *ras* oncogenes (p21Ras) are modified covalently by a palmitate attached to a cysteine residue near the carboxyl terminus. Changing cysteine at position 186 to serine in oncogenic forms produces a nonpalmitylated protein that fails to associate with membranes and does not transform NIH 3T3 cells. Nonpalmitylated p21Ras derivatives were constructed that contained myristic acid at their amino termini to determine if a different form of lipid modification could restore either membrane association or transforming activity. An activated p21Ras, altered in this way, exhibited both efficient membrane association and full transforming activity. Surprisingly, myristylated forms of normal cellular Ras were also transforming. This demonstrates that Ras must bind to membranes in order to transmit a signal for transformation, but that either myristate or palmitate can perform this role. However, the normal function of cellular Ras is diverted to transformation by myristate and therefore must be regulated ordinarily by some unique property of palmitate that myristate does not mimic. Myristylation thus represents a novel mechanism by which Ras can become transforming.

CELLULAR *ras* GENES ENCODE 21-kD guanosine diphosphate/guanosine triphosphate (GDP/GTP)–binding proteins termed p21Ras (*1, 2*). The p21Ras can acquire the ability to transform cells if activated by point mutations that affect the binding or hydrolysis of guanine nucleotide to favor the active GTP-bound form of the protein (*2, 3*). Genes encoding activated forms of p21Ras are the most frequently identified oncogenes in human tumors (*4*). p21Ras is modified by a palmitic acid attached to a cysteine near its COOH-terminus (*5*). Because nonpalmitylated forms are found in cytosolic fractions, the palmitate is thought to promote or stabilize the interaction of p21Ras with cellular membranes (*6*). This interaction with membranes is important biologically, as nonpalmitylated mutants of activated p21Ras no longer transform cells (*7*). However, it is not clear whether the lipid acts in a general way to increase p21Ras hydrophobicity or whether the COOH-terminus and palmitate, in particular, are involved in more specific interactions between p21Ras and membrane-bound proteins or lipids.

Myristic acid is attached to the NH_2-terminus of a select group of proteins (*8*) and is involved in the association of p60v-Src and several viral structural (Gag) proteins with membranes (*9*). We reasoned that if the major role of the lipid on p21Ras was to promote nonspecific membrane-protein interactions, then a myristyl group might be capable of performing this same function even if attached at another site on the protein.

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A naturally occurring myristylated variant of H-Ras had already been described: the p29Gag-Ras protein encoded by the oncogene of Rasheed sarcoma virus (RaSV) (*10*). p29Gag-Ras is a fusion protein containing rat H-Ras sequences and a 59-residue NH_2-terminal extension derived from the Rasheed leukemia virus (RaLV) Gag protein (which is responsible for its myristylation) and viral sequences of unknown origin (*11*). New chimeric Gag-Ras proteins were constructed by linking DNAs encoding either the first 11 amino acids or all 59 residues of the NH_2-terminal sequences of RaSV (Fig. 1, legend) to the full 189 residues of either normal human cellular H-Ras (wild type, WT) or activated H-Ras (Leu^{61}) in which the glutamine residue at position 61 was replaced with a leucine (Fig. 1). In addition, palmitylated (Cys^{186}) and nonpalmitylated (Ser^{186}) forms of both proteins were analyzed. Immunoprecipitation of Ras from [^{35}S]methionine-labeled NIH 3T3 cells transfected with the DNA constructs confirmed that proteins of the correct expected sizes were present (Fig. 2). Specific incorporation of amide-linked [^{3}H]myristic acid was easily detected in all chimeric Gag-Ras (Fig. 2, lane 9) (*12*).

As with Ras containing only palmitate, more than 90% of a p29-Leu^{61}-Cys^{186} Gag-Ras bearing both myristate and palmitate was found in a 100,000*g* pellet (P100) (Fig. 2, lanes 1 and 2). The p21-Ser^{186} protein lacking any lipid was completely soluble (Fig. 2, lanes 3 and 4). When either the 11- (p22) or 59-residue (p29) myristylation signals were present, approximately 60% of myristylated Gag-Ras (Ser^{186}) proteins sedimented in a crude membrane-containing fraction (P100) (Fig. 2, lanes 5, 6, 7, and 8). Both lengths of the NH_2-terminal myristylation signal thus conferred the ability to bind to membranes on a soluble p21-Ser^{186}. Preliminary experiments in which immunofluorescence was used to localize p22-WT-Ser^{186}

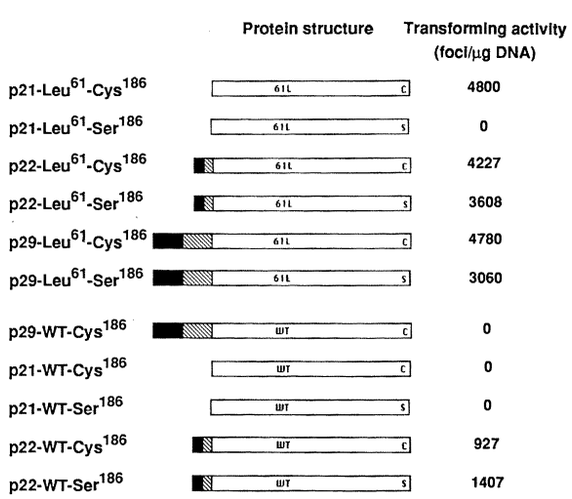

Fig. 1. Diagram of chimeric Gag-Ras and their transforming activities. Gag residues (black boxes) and viral- or vector-derived sequences (cross-hatched boxes) were linked (*24*) to the initiating methionine of full-length H-Ras possessing either a cysteine (C) or serine (S) at Ras position 186. The NH_2-terminal amino acid sequences of the chimeric Gag-Ras p29 [M]G*QSLTTPLSLTLDHWKDVRDRARDQSVEIKK^{32} GPLRRSGTVAPASGGAGAPGLAAPVEA^{59} (+189 amino acids of p21); and p22 [M]G*QSLT^6 KGGTI^{11} (+189 amino acids of p21) (*25*). The 248-residue chimeric Gag-Ras (p29) contains 59 NH_2-terminal non-Ras amino acids from Rasheed sarcoma virus (*11*) linked to the 189 residues of human H-Ras. The first 32 amino acids are derived from the rat leukemia virus p15Gag (underlined), whereas the following 27 residues stem from viral sequences unrelated to Gag or Ras. The 200-residue chimeric Gag-Ras (designated p22) contains the first six amino acids of Gag linked to five residues encoded by the pAT–H-Ras vector sequence (*26*) and the full 189 amino acids of human H-Ras. The initiating methionine (brackets) is removed and myristic acid is attached to the glycine (asterisk) that follows (*10*). The Ser^{186} mutation was introduced by oligonucleotide-directed mutagenesis to change Cys (TGT) to Ser (AGT). Both normal and activated versions of human H-Ras had been constructed previously (*27*). To quantify focus-forming activity, 10 ng of purified plasmid DNA was coprecipitated with 20 μg of calf thymus DNA by the calcium phosphate technique and added to 10^6 NIH 3T3 cells (*27*). Foci were counted after 14 to 18 days. The numbers presented are the average of from 5 to 15 separate dishes from at least three different transfection assays. 61L, Leu^{61}.

protein within the transformed cells also indicated that myristylation had reestablished binding of the nonpalmitylated protein to the plasma membrane (*12*). That two different fatty acids attached at distinct sites caused *ras*-encoded proteins to bind to membranes raised the possibility that myristate, and perhaps palmitate as well, provided a simple hydrophobic domain that enabled the protein to interact with membranes, but which had no specialized role in regulation of p21Ras activity.

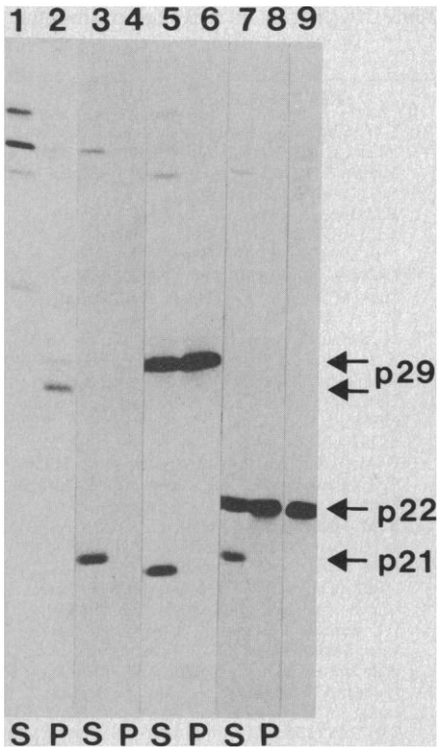

Fig. 2. Subcellular fractionation and acylation of Gag-Ras. Cells (2×10^6) derived from individual foci of transformed cells or mass cultures of G418-selected nontransformed p21-WT-Ser^{186} cells were labeled overnight with 100 μCi of ^{35}S-labeled methionine-cysteine (Trans-Label, ICN Pharmaceuticals) per milliliter, followed by a 1-hour chase in nonradioactive medium. Crude membrane-containing fractions (P) were separated from cytosol (S) by hypotonic lysis of cells and centrifugation at 100,000*g* for 30 min (*28*). p22-WT-Ser^{186} cells (lane 9) were also labeled with 1 mCi of [^{3}H]myristic acid per milliliter as described (*28*). p21Ras was isolated by immunoprecipitation with the H-Ras–specific antibody YA6-172 (*27*) and analyzed by SDS–polyacrylamide gel electrophoresis. Proteins were detected by fluorographic exposures of 8 to 15 days. The top arrow labeled p29 indicates p29-Leu^{61}-Cys^{186} (lane 2), a myristylated precursor that is synthesized in the cytosol, then associates rapidly with membrane-containing fractions and is converted to a species (p29, bottom arrow) with greater mobility that contains both myristate and palmitate. The minor ~21-kD proteins in lanes 5 and 7 arise from initiation at the (now internal) original initiating methionine of p21(Ser^{186}), lack both myristate and palmitate, and are thus cytosolic. Lanes 1 and 2, p29-Leu^{61}-Cys^{186}; lanes 3 and 4, p21-WT-Ser^{186}; lanes 5 and 6, p29-Leu^{61}-Ser^{186}; lanes 7 and 8, p22-WT-Ser^{186}; and lane 9, [^{3}H]myristate-labeled p22-WT-Ser^{186}.

To determine if the membrane binding promoted by the myristyl group mimicked in a functional way the association induced by palmitate, we compared the ability of palmitate- and myristate-containing Ras to cause focus formation on monolayers of NIH 3T3 cells. Both p22 and p29 forms of myristylated, nonpalmitylated Gag-Ras (Leu^{61}-Ser^{186}) transformed NIH 3T3 cells with efficiencies nearly as great as the very strongly transforming palmitylated versions (Fig. 1). This suggested that, for activated forms of p21Ras, association of the protein with membranes was crucial for transformation, but that the binding induced by myristate was equivalent functionally to that of palmitate.

Both myristylated but nonpalmitylated (Ser^{186}) Gag-Ras remained in the (upper) precursor form and were not processed to the more rapidly migrating mature form (*6, 13*), even though the proteins associated with membranes (Fig. 2, lanes 1, 2, 5, and 6). Recent studies have implied that the maturation of the Ras precursor is not due exclusively to palmitylation (*14*). The absence of a mature form for these myristylated membrane-bound Ras (Ser^{186}) proteins suggested that it is not membrane binding but rather the cysteine at position 186 that is required for this (as yet undefined) processing. More importantly, this demonstrated that neither palmitate nor COOH-terminal processing were required for transduction of signals that lead to transformation. Similar results have been obtained by Lacal *et al.* (*15*) by attaching a 15–amino acid myristylation signal derived from p60v-Src to a viral p21H-Ras. As the NH_2-terminal sequence of p60Src (Met-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-) (*16*) and p15Gag (Fig. 1) are quite different, the restored ability to cause transformation is likely to be a consequence of the myristyl group and not the particular amino acids used as a signal sequence for myristylation.

Unexpectedly, two forms of normal unactivated p21Ras carrying an 11–amino acid myristylation signal (p22-WT-Ser^{186} or Cys^{186}) also caused focus formation (Fig. 1). Both p22-WT-Ser^{186} and p22-WT-Cys^{186} had potent transforming activity, producing 900 to 1400 foci per microgram of DNA under conditions where Leu^{61} derivatives caused ~4000 focu per microgram and where p21-WT-Cys^{186} or p29-WT-Cys^{186} caused none. Cells derived from p22-WT-Ser^{186} and p22-WT-Cys^{186} foci could also grow in soft agar (*17*). The Ras portions of these proteins were confirmed to be completely normal by DNA sequence analysis.

Transformation by p22-WT-Ser^{186} did not result from either of the two mechanisms described previously for activation of Ras: altered GTP binding (*18*) or decreased guanosine triphosphatase (GTPase) activity (*2, 3*). Analysis of myristylated p22-WT-Ser^{186} in vitro revealed high-affinity GTP binding (concentration for half maximal binding, $10^{-8}M$) and the high GTPase activity characteristic of nontransforming Ras (Fig. 3, A and B). Both p22 and p29 myristylated normal Ras hydrolyzed bound ^{32}P-labeled deoxyguanosine triphosphate (dGTP) to GDP efficiently, whereas an activated Ras(Leu^{61}) version remained primarily in the GTP form. The in vitro GTPase activity and GTP binding of these myristylated proteins thus mirrored accurately the properties of the wild-type or activated Ras from which they were derived.

Cellular, nontransforming Ras have recently been shown to interact with GTPase-activating protein (GAP), a cytosolic protein that stimulates their intrinsic GTPase activity and maintains the protein in its inactive GDP-bound form (*19*). GAP does not stimulate the GTPase of activated Ras, which remain in the GTP-bound form (*19*). GAP is thought to interact with Ras in a region between residues 32 to 40 of the Ras protein and is proposed to be an effector protein through which normal Ras exerts its effects on the cell (*20*). However, it was possible that the membrane binding induced by myristylation prevented stimulation by GAP, or that the relatively large amount of p22-WT-Ser^{186} in the focus-selected cells overwhelmed the ability of GAP to keep the protein in its inactive GDP-bound state. We determined directly the guanine nucleotides bound to p22-WT-Ser^{186} by labeling cells with radioactive phosphate, isolating p22-WT-Ser^{186} by immunoprecipitation, and then releasing and separating the bound nucleotides by two-dimensional thin-layer chromatography (TLC). The p22-WT-Ser^{186} was found to be primarily in the GDP form (~90%), with the remaining 10% being GTP (Fig. 3C, a). The p29-WT-Cys^{186} (Fig. 3C, b), which is palmitylated, also bound mostly GDP. An activated p29-Leu^{61}-Ser^{186} protein contained >90% GTP (Fig. 3C, c). The extent to which bound GTP may have been converted to GDP during immunoprecipitation of the normal (wild-type) proteins, which have a high intrinsic GTPase activity, is likely to be small (*21*). Thus, if these data reflect accurately the nucleotide state of Ras within the cell, this implies that p22-WT-Ser^{186} not only continues to interact with GAP, but that it causes transformation even though predominantly in the GDP form.

The inability of the p29-WT-Cys^{186} pro-

tein to transform cells is a potentially important observation. This protein is myristylated and binds to membranes with the same efficiency as p22-WT-Ser[186], but has a 59– rather than 11–amino acid leader sequence. Two possible explanations for the lack of activity of p29-WT-Cys[186] are (i) the length or structure of the 59–amino acid leader of p29 hinders interactions with membrane-associated targets; or (ii) the presence of this particular leader disrupts the general structure of the protein and renders it inactive. This second model is not likely, as the Leu[61] version of p29 is functional biochemically and transforms cells efficiently. Thus, it appears that not merely the presence of a myristyl group and membrane association, but also the length of myristylation signal sequence is important to position the normal Ras optimally for interaction with proteins that trigger the transformation process.

These studies demonstrate that Ras must bind to membranes in order to transmit a signal for transformation (and presumably for normal function), but that such binding can be generated by either myristate or palmitate. Nevertheless, for the processes in which cellular Ras participates normally, only palmitate produces a specific (nontransforming) association of Ras with membranes. The surprisingly effective transforming ability of p22-WT-Ser[186] could arise because myristate addition is a permanent (*22*), rather than a reversible acylation like palmitate (*23*). This could disrupt what is ordinarily a transient association between Ras and its normal membrane-bound target proteins. Alternatively, the myristyl group could promote binding to an inappropriate target with which cellular Ras does not usually associate. In either case, the unexpected and efficient activation of cellular Ras by myristylation offers a new tool for the study of the sequence of events that leads to transformation by Ras. In addition, the attachment of a myristylation signal sequence is clearly a technique that can yield unexpected biological information by inducing or perturbing membrane association of proteins that are not myristylated ordinarily.

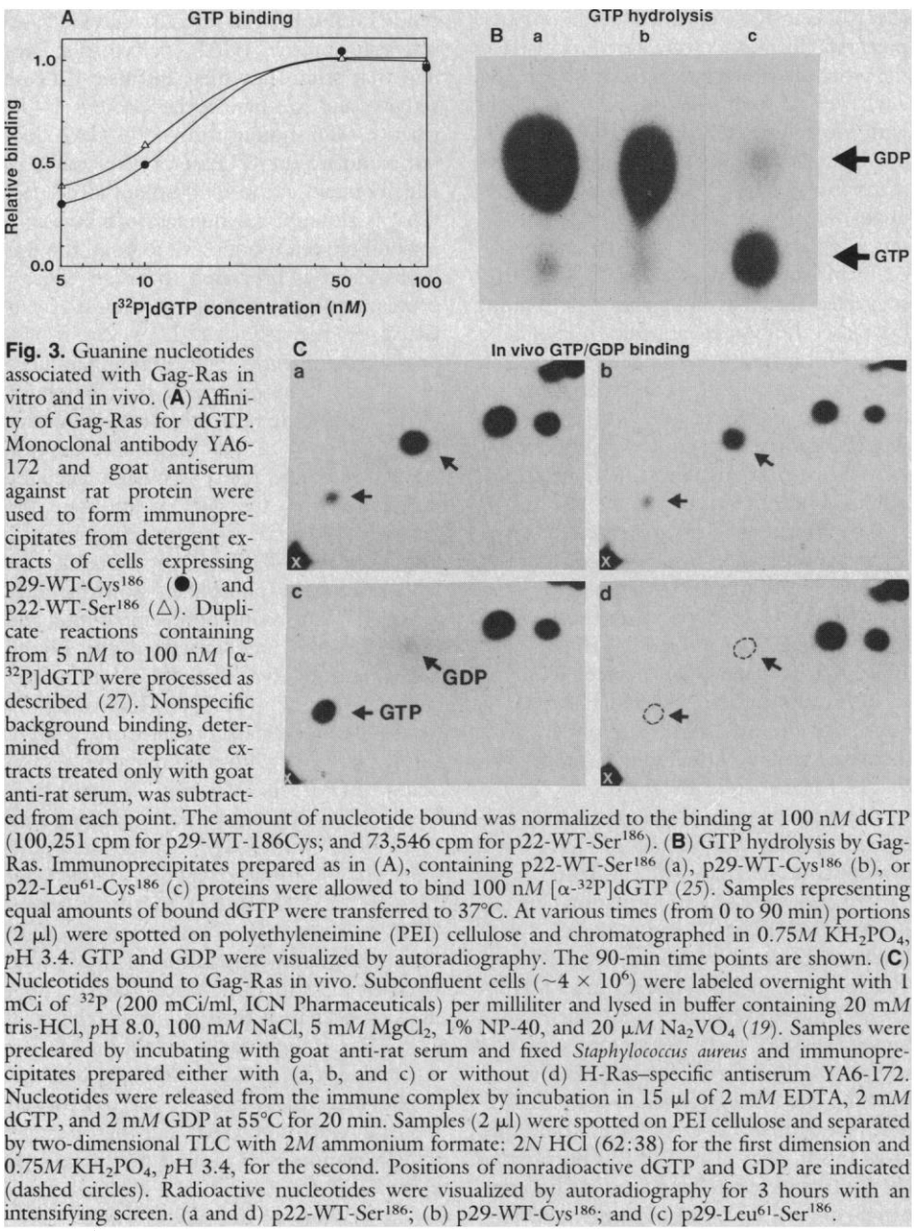

Fig. 3. Guanine nucleotides associated with Gag-Ras in vitro and in vivo. (**A**) Affinity of Gag-Ras for dGTP. Monoclonal antibody YA6-172 and goat antiserum against rat protein were used to form immunoprecipitates from detergent extracts of cells expressing p29-WT-Cys[186] (●) and p22-WT-Ser[186] (△). Duplicate reactions containing from 5 n*M* to 100 n*M* [α-^{32}P]dGTP were processed as described (*27*). Nonspecific background binding, determined from replicate extracts treated only with goat anti-rat serum, was subtracted from each point. The amount of nucleotide bound was normalized to the binding at 100 n*M* dGTP (100,251 cpm for p29-WT-186Cys; and 73,546 cpm for p22-WT-Ser[186]). (**B**) GTP hydrolysis by Gag-Ras. Immunoprecipitates prepared as in (A), containing p22-WT-Ser[186] (a), p29-WT-Cys[186] (b), or p22-Leu[61]-Cys[186] (c) proteins were allowed to bind 100 n*M* [α-^{32}P]dGTP (*25*). Samples representing equal amounts of bound dGTP were transferred to 37°C. At various times (from 0 to 90 min) portions (2 μl) were spotted on polyethyleneimine (PEI) cellulose and chromatographed in 0.75*M* KH_2PO_4, *p*H 3.4. GTP and GDP were visualized by autoradiography. The 90-min time points are shown. (**C**) Nucleotides bound to Gag-Ras in vivo. Subconfluent cells (~4 × 10^6) were labeled overnight with 1 mCi of ^{32}P (200 mCi/ml, ICN Pharmaceuticals) per milliliter and lysed in buffer containing 20 m*M* tris-HCl, *p*H 8.0, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1% NP-40, and 20 μ*M* Na_2VO_4 (*19*). Samples were precleared by incubating with goat anti-rat serum and fixed *Staphylococcus aureus* and immunoprecipitates prepared either with (a, b, and c) or without (d) H-Ras–specific antiserum YA6-172. Nucleotides were released from the immune complex by incubation in 15 μl of 2 m*M* EDTA, 2 m*M* dGTP, and 2 m*M* GDP at 55°C for 20 min. Samples (2 μl) were spotted on PEI cellulose and separated by two-dimensional TLC with 2*M* ammonium formate: 2*N* HCl (62:38) for the first dimension and 0.75*M* KH_2PO_4, *p*H 3.4, for the second. Positions of nonradioactive dGTP and GDP are indicated (dashed circles). Radioactive nucleotides were visualized by autoradiography for 3 hours with an intensifying screen. (a and d) p22-WT-Ser[186]; (b) p29-WT-Cys[186]; and (c) p29-Leu[61]-Ser[186].

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24. The p29Gag-Ras was constructed by ligating a 0.5-kb Bgl II–Hind III fragment of Rasheed sarcoma virus (coding for 63 amino acids, including the first 4 of RaSV Ras) to a 1.1-kb Hind III–Bam HI fragment that contains the human H-Ras sequences for residues 5 to 189. p22Gag-Ras was generated by introducing a 1.4-kb Bgl II fragment containing the entire coding sequence of RaSV *ras* into the Bgl II site of the H-Ras expression vector pAT–H-Ras (*26*). The resulting plasmid was then digested with Xba I to delete the COOH-terminal 236 amino acids of RaSV p29, treated with mung bean nuclease, and religated. Coding sequences of both p29 and p22 mutants, contained on the resulting 1.6-kb Bgl II–Bam HI fragment, were introduced into the Bam HI site of the pZIP-NeoSV(x)1 retrovirus vector plasmid (*26*). The sequences of all mutants were confirmed by dideoxy sequencing.
25. Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Activity-Dependent Enhancement of Presynaptic Inhibition in *Aplysia* Sensory Neurons

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Tail shock produces transient presynaptic inhibition and longer lasting presynaptic facilitation of the siphon sensory neurons in *Aplysia*. The facilitation undergoes activity-dependent enhancement that is thought to contribute to classical conditioning of the gill- and siphon-withdrawal reflex. Inhibition of the sensory neurons has now also been shown to undergo activity-dependent enhancement when action potential activity in the sensory neurons is paired with inhibitory transmitter. This effect appears to involve an amplification of the same cellular mechanisms that are involved in normal presynaptic inhibition. These results suggest that activity-dependent enhancement may be a general type of associative cellular mechanism.

SEVERAL DIFFERENT ASSOCIATIVE cellular mechanisms may underlie aspects of learning, including activity-dependent enhancement of facilitation (*1*), associative changes in excitability (*2, 3*), long-term potentiation (*4*), and long-term depression (*5*). Activity-dependent facilitation is thought to contribute to classical conditioning of the gill- and siphon-withdrawal reflex in *Aplysia* (*6, 7*). This mechanism appears to be an amplification of a cellular mechanism of sensitization, that is, presynaptic facilitation of the siphon sensory neurons by serotonin acting through adenosine 3′,5′-monophosphate (cAMP) (*8*). We wished to examine whether activity-dependent enhancement is a more general phenomenon which occurs for other modulatory effects. An opportunity to test this idea was provided by the finding that, in addition to sensitization and classical conditioning, the gill- and siphon-withdrawal reflex also undergoes transient behavioral inhibition (*9, 10*). The inhibition is thought to be due in part to presynaptic inhibition of the siphon sensory neurons by the peptide FMRFamide acting through the arachidonic acid cascade (*10, 11*). We now report that, like facilitation, inhibition of the sensory neurons undergoes an associative, activity-dependent enhancement when spike activity in the sensory neurons is paired with inhibitory transmitter.

To test whether inhibition of the siphon sensory neurons is modulated by paired action potential activity, we puffed FMRFamide onto the cell body of a sensory neuron either paired or unpaired with a brief train of action potentials in the neuron (Fig. 1A) (*12*). In the first set of experiments, we examined the effect of this training on the amplitude of the synaptic connection from the sensory neuron to a motor neuron. Unpaired training produced rather short-lived inhibition of the excitatory postsynaptic potential (EPSP), whereas paired training produced longer lasting inhibition (Fig. 1B). On average, the two training procedures produced similar inhibition 1 min after training, but whereas the EPSP then returned to near baseline 2 min after unpaired training, it remained inhibited for several minutes after paired training (Fig. 1C). There was a significant overall effect of training procedure [$F(1,19) = 6.72$, $P < 0.02$] and significant differences in planned comparisons between paired and unpaired training 2 min [$F(1,57) = 9.57$, $P < 0.01$] and 3 min [$F(1,57) = 4.52$, $P < 0.05$] after training (*13*).

These results suggest that action potential activity paired with FMRFamide ("paired action potential activity") enhances the FMRFamide-induced inhibition. However, because high-frequency action potential trains can produce posttetanic potentiation (PTP) at these synapses (*14*), an alternative interpretation is that FMRFamide paired with an action potential train ("paired FMRF") decreases the potentiation produced by the train. If the paired and unpaired training procedures both produce a mixture of inhibition (caused by FMRFamide) and potentiation (caused by the action potential train), a difference between the training procedures could be due to a difference in either process. To address this issue, we first determined whether the action potential train we used (five spikes at 10 Hz) produced potentiation. Toward this end, we ran a second set of experiments with a similar design but with two new training procedures: one with the action potential train alone and one in which the EPSP was simply tested at the usual times. Testing produced synaptic depression that continued at a rate similar to that seen before training (Fig. 1, C and D). Compared to this control procedure, the train of action potentials produced potentiation that was modest (approximately 120% of control on each trial after training) but statistically significant [$F(1,19) = 4.61$, $P < 0.05$].

To analyze if paired action potential activity enhances inhibition or if paired FMRFamide reduces potentiation, we ran a third set of experiments comparing the effects of paired training and training with the FMRFamide puff alone (Fig. 1D). Paired training produced significantly greater inhibition than FMRFamide-alone training [$F(1,19) = 4.83$, $P < 0.05$]. This result cannot be explained by reduction of potentiation by paired FMRFamide, because the FMRFamide-alone procedure includes no action potential train and thus no potentiation. Rather, the data provide support for the idea that paired training enhances inhibition. It is possible that unpaired training also modifies the effects of action potential activity or FMRFamide.

We next tested if the activity-dependent enhancement of inhibition by paired action potential activity occurred pre- or postsynaptically. We first tested a possible postsynaptic mechanism, a pairing-specific change in input resistance in the motor cell. To

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