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The CML-Specific P210 *bcr/abl* Protein, Unlike v-*abl*, Does Not Transform NIH/3T3 Fibroblasts

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The v-*abl* oncogene of the Abelson murine leukemia virus (A-MuLV) is known to efficiently transform NIH/3T3 fibroblasts in vitro and to cause an acute lymphosarcoma in susceptible murine hosts. The role of its relative, the *bcr/abl* gene product, in the etiology of human chronic myelogenous leukemia (CML) remains speculative. To assess the transforming properties of the *bcr/abl* gene product, complementary DNA clones encoding the CML-specific P210 *bcr/abl* protein were expressed in NIH/3T3 fibroblasts. In contrast to the v-*abl* oncogene product P160, the P210 *bcr/abl* gene product did not transform NIH/3T3 cells. Cell lines were isolated that expressed high levels of the P210 *bcr/abl* protein but were morphologically normal. During the course of these experiments, a transforming recombinant of *bcr/abl* was isolated which fuses *gag* determinants derived from helper virus to the NH_2-terminus of the *bcr/abl* protein. This suggests that a property of viral *gag* sequences, probably myristylation-dependent membrane localization, must be provided to *bcr/abl* for it to transform fibroblasts.

THE PHILADELPHIA CHROMOSOME, a result of reciprocal translocation of distal segments of autosomes 9 and 22, is found in greater than 90% of cases of human chronic myelogenous leukemia (CML) (*1*). The translocation juxtaposes c-*abl* protooncogene sequences on chromosome 9 with a gene of unknown function, denoted *bcr*, on chromosome 22 (*2*). A novel hybrid messenger RNA transcribed from this locus directs the translation of a 210-kD phosphoprotein (P210) carrying both *bcr* and *abl* antigenic determinants (*3*). The P210 *bcr/abl* protein resembles the v-*abl* oncogene product of Abelson murine leukemia virus (A-MuLV) in its high tyrosine-specific protein kinase activity (*4*). In addition, the P210 and v-*abl* proteins involve replacements of NH_2-terminal c-*abl* sequences with *bcr* and viral *gag* sequences, respectively (*5–7*). To examine whether the CML-specific *bcr/abl* protein functions like the *gag*/v-*abl* protein of A-MuLV to transform fibroblasts in vitro, we tested various constructs which encode P210 *bcr/abl*. These constructs do not transform fibroblasts, suggesting that *bcr/abl* functions differently from the v-*abl* oncogene product.

Previously, we isolated overlapping complementary DNA (cDNA) clones that define the complete coding sequence for the P210 *bcr/abl* gene product (*8*). Using these clones, as well as cDNA clones of the murine c-*abl* gene (*9*), we have made several constructs for use in expression studies in tissue culture cells (Fig. 1). The plasmid pJW-TX (Fig. 1A), containing the cDNA for the full-length coding sequences of P210 and the SV40 origin of replication, directs the transient expression in COS cells of a 210-kD phosphoprotein that is immunoprecipitated with antisera to *abl* and co-migrates with the P210 *bcr/abl* protein of K562 cells (Fig. 2A, lanes 1 and 2). The cDNA thus encodes a protein with the properties of the CML-specific P210.

To isolate cell lines that expressed the P210 protein in a stable manner, we attempted to directly transfect P210 constructs, but few transformants were found. Knowing that *abl*-expressing constructs can be toxic to cells upon transfection (*10*), we co-transfected a construct that expressed P210 (pcEX6.5, Fig. 1C) with one expressing resistance to the antibiotic G418 (pSV2Neo) (*11*). Presence of the P210 construct resulted in a fivefold decrease in the number of G418-resistant colonies. Likewise, there was a strong negative selection against a transfected P210 construct bearing a co-selectable G418 resistance marker (pJW-RX in Fig. 1B). The apparent toxicity of P210-expressing constructs is similar to that of the v-*abl* gene product (*10*). To decrease the cytocidal effect of transfection of the *bcr/abl* cDNA, we attempted retroviral transduction of single copies of the toxic gene. Upon co-transfection of the pJW-RX construct with a construct encoding Moloney helper virus pZAP (*12*) and subsequent selection with G418, cell lines were isolated that stably expressed high levels of the P210 protein (Fig. 2B, lanes 1 and 2). The level of P210 kinase activity in these cell lines was comparable to or greater than P160 v-*abl* kinase activity in A-MuLV–transformed cells (Fig. 2B, lane 5). In a separate experiment, transfection of the retroviral-packaging cell line ϕ-2 (*13*) with pJW-RX and subsequent selection with G418 gave helper-free retroviral producer cell lines, which were screened for production of high levels of virion RNA (*14*). Cell lines selected in this manner expressed particularly high levels of P210 kinase activity (Fig. 2C, lane 1). Supernatants from these ϕ-2 producer cell lines transferred the P210 *bcr/abl* gene product by retroviral infection (Fig. 2C, lane 3). P210-expressing cell lines all appeared morphologically flat and were readily distinguished from cells transformed by the v-*abl* oncogene product (compare C and D of Fig. 3).

During experiments in which the pLTR6.5 construct (Fig. 1D) was co-transfected with the replication-competent Moloney helper virus pZAP (*12*) onto NIH/3T3 cells, a low frequency of transformed foci arose (less than 1% of control v-*abl* constructs). These transformed foci resembled those of v-*abl* (compare B and D of Fig. 3), and cell lines established from these foci produced high titers of virus capable of transforming NIH/3T3 cells at a frequency comparable to A-MuLV (*15*). To determine the nature of the transforming protein or proteins responsible for the transformation

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activity of these recovered viruses, we immunoprecipitated extracts of these cell lines with antisera against *abl*, *bcr*, and viral *gag* determinants (*3, 16, 17*). Cell lines from several transformed foci each expressed a 220-kD phosphoprotein that could be immunoprecipitated with antisera against *abl* determinants and was clearly distinguishable from P210 (a representative immunoprecipitation is shown in Fig. 4A, lanes 5 to 8). Immunoprecipitation demonstrated the presence of both *gag* and *bcr* determinants on the P220 protein as well (Fig. 4A, lanes 6 and 8). Mapping of the proviral structure in these transformed cells demonstrated that recombination had occurred within the *gag* sequence of the helper virus and the *bcr* sequences of the *bcr/abl* viral construct, resulting in a 220-kD protein consisting of NH_2-terminal *gag* sequence and approximately 60% of the *bcr* coding capacity (*15*). The *gag/bcr/abl* protein is a novel transforming variant of the *abl* protooncogene.

To verify that the pLTR6.5 construct expressed the expected P210 protein, NIH/3T3 cells co-transfected with pLTR6.5 and a construct encoding the Moloney helper virus pZAP (*12*) were screened without regard to their morphologic phenotype. Cell lines were isolated that expressed the P210 protein but were morphologically normal. Immunoprecipitation with several antisera showed that the P210 protein in the cell line examined carried *bcr* and *abl* determinants but was not recognized by the *gag* antiserum (Fig. 4B, lanes 5 to 8).

Our studies on the expression of the P210 *bcr/abl* protein in NIH/3T3 cells lead us to conclude that P210 does not transform NIH/3T3 fibroblasts, in contrast to the P160 *gag/v-abl* protein which readily induces morphologically transformed NIH/3T3 cells. Because P210 lacks the consensus sequence for an NH_2-terminal myristylation or any hydrophobic stretches suggesting a transmembrane domain (*8, 18*), a simple hypothesis to explain our results is that P210 *bcr/abl* is a cytoplasmic protein and cannot transform NIH/3T3 fibroblasts in culture because of an inability to bind to cellular membranes. Several studies suggest that membrane localization is critical for transformation by both the *abl* and *src* proteins (*19–21*). The *gag* portion of v-*abl*, like v-*src*, contains an NH_2-terminal myristyl fatty acid moiety thought to determine membrane association (*22*). NH_2-terminal mutants of v-*abl* and v-*src* that lack myristylation no longer localize to membranes and are transformation-defective (*21, 23*). Thus, lacking a potential myristylation site or a hydrophobic domain to anchor it to cellular membranes, *bcr/abl* should be cytoplasmic and unable to transform. In this regard, it is significant that spontaneous recombinants, in which NH_2-terminal viral *gag* sequences have fused to the *bcr/abl* protein, result in a *gag/bcr/abl* protein that readily transforms NIH/3T3 cells much like v-*abl*. The P220 *gag/bcr/abl* protein should be membrane-bound as a consequence of the myristylation of its NH_2-terminus. Only a fraction of *gag/v-abl* protein is ever recovered with membrane (*24*), however, making membrane at-

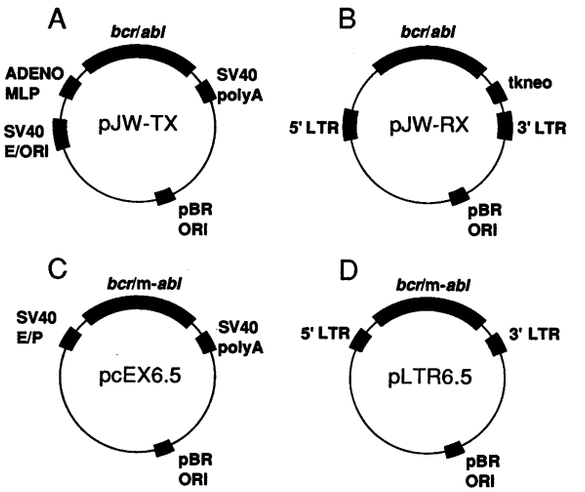

Fig. 1. Constructs encoding the P210 *bcr/abl* protein. (**A**) The pJW-TX is a derivative of p91023B (provided by R. Kaufman, Genetics Institute) (*31*). The cDNA sequence defining the complete coding region for the P210 protein (*8*) was cloned into the unique Eco RI restriction site of p91023B. Human c-*abl* sequence from cDNA clone 215 was truncated at the 3′ Hind III site by the addition of Eco RI linkers, thus removing 1.5 kb of 3′ untranslated sequence. The truncated clone 215 was ligated to the *bcr/abl* junctional clone 104 at the unique Hinc II site. The 104/215 clone was ligated at the unique Hind III site to 5′ *bcr* sequences derived from either clone 172 (which includes 120 bp of 5′ untranslated sequence) or clone 189 (which includes 445 bp of 5′ untranslated sequence). ADENO MLP, major late promoter of adenovirus; E/ORI, enhancer/origin of replication derived from SV40 (*31*). (**B**) The pJW-RX is a derivative of pMV6tkNeo (provided by B. Weinstein, Columbia University) (*32*). The cDNA fragment 172/215 was cloned into the unique Eco RI site of pMV6tkNeo. (**C**) The pcEX6.5 is a derivative of pcEXV-1 (provided by R. Germain, National Institutes of Health) (*33*). The cDNA encoding a P210 *bcr*/murine-*abl* protein was cloned into the unique Eco RI site of pcEXV-1. The 5′ human *bcr* sequence from the *bcr/abl* junctional cDNA clone 189 was ligated to 3′ murine c-*abl* cDNA sequence at the Xmn I site common to both murine and human c-*abl* cDNA (*5, 9*). E/P, enhancer/promoter region derived from SV40 (*33*). (**D**) The pLTR6.5 is a derivative of pUCLTR (provided by B. Mathey-Prevot, Whitehead Institute) (*34*). The *bcr*/murine-*abl* P210 cDNA was cloned into the unique Eco RI site of pUCLTR.

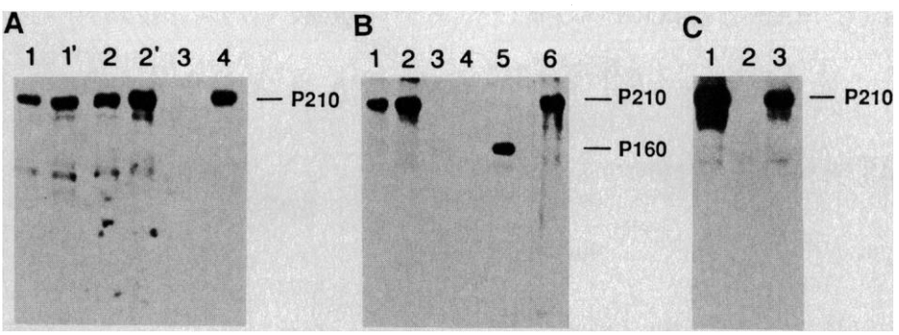

Fig. 2. Expression of the P210 *bcr/abl* protein in COS cells and NIH/3T3 fibroblasts. (**A**) Transient expression of P210 *bcr/abl* in COS cells. Ten micrograms of pJW-TX plasmid carrying either cDNA 172/215 (lanes 1 and 1′) or 189/215 (lanes 2 and 2′) was transfected onto 5×10^5 COS cells (lanes 1 and 2) or 1×10^6 COS cells (lanes 1′ and 2′). As a control, 10 μg of the parent plasmid p91023b was transfected onto 1×10^6 COS cells (lane 3). Cells were harvested 48 hours after transfection. Cell extracts were immunoprecipitated with 10 μl of v-*abl* antisera αpEX5 (*16*) and processed for in vitro immune complex kinase assay as described (*35*). ^{32}P-labeled proteins were displayed by electrophoresis on 8% SDS-polyacrylamide gels and visualized by autoradiography for 30 minutes at room temperature. Extracts of the CML cell line K562 (*36*) immunoprecipitated as above served as a positive control (lane 4). (**B**) Stable expression of P210 *bcr/abl* in NIH/3T3 fibroblasts. One microgram of pJW-RX was co-transfected with 1 μg of pZAP (*12*) onto NIH/3T3 cells. Two days later, cells were split and further incubated in G418 at 500 μg/ml. Individual colonies were picked at 3 weeks and expanded into cell lines. Cell extracts were immunoprecipitated as in A. Lanes 1 and 2, two independent NIH/3T3 cell lines from co-transfection with pJW-RX and pZAP; lanes 3 and 4, control NIH/3T3 cell lines transfected with the parent plasmid pMV6tkNeo; lane 5, NIH/3T3 cell line co-transfected with v-*abl* retroviral construct pAB160 (*37*) and pZAP (*12*); lane 6, K562 cell line. Immunoprecipitations were performed on equivalent numbers of cells for each line. Exposure time was 30 minutes at room temperature. (**C**) Transfer of P210 *bcr/abl* by retroviral infection. Lane 1, NIH/3T3 fibroblast cell line ϕ-2 (*13*) transfected with pJW-RX. This cell line was selected for high level virion RNA production by RNA slot blot analysis of cell culture supernatants (*14*). Lane 2, mock-infected ϕ-2 cell; lane 3, NIH/3T3 cell line infected with cell culture supernatant of cell line in lane 1. Cells were selected in G418 (500 μg/ml) prior to immunoprecipitation as in A. Exposure time was 2.5 hours at room temperature.

tachment difficult to directly examine.

Membrane localization may be necessary but it is not sufficient for transformation of fibroblasts by *abl* protein variants. Deletion of critical NH_2-terminal c-*abl* sequences also contributes to the activation of c-*abl* transforming potential. This is evident because one of the normal c-*abl* cDNA types [type IV (*9*) or type 1B (*25*)] encodes a glycine at amino acid position 2, which by functioning as a site for myristylation, should direct its membrane localization. Deletions in c-*abl* type IV that remove sequences near the NH_2-terminus but preserve the glycine at position 2 produce transforming c-*abl* proteins (*26*). When fused to *abl*, *bcr* deletes the entire NH_2-terminal first exon of *abl*, thus apparently activating the transforming potential but requiring a myristylation site for fibroblast transformation.

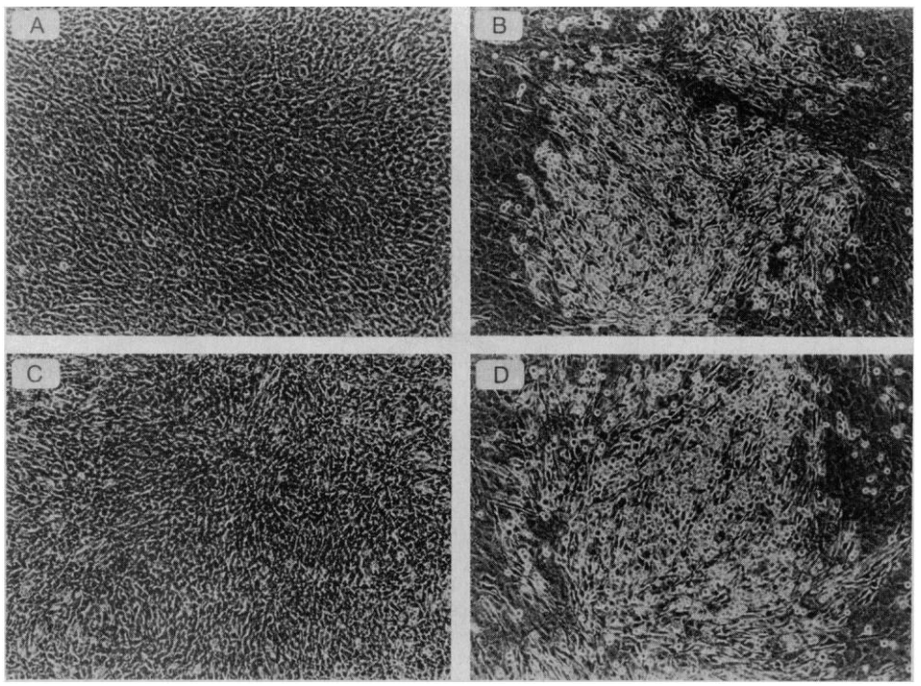

Fig. 3. Light micrographs of NIH/3T3 cell lines expressing *abl* protein variants (magnification, ×100). (**A**) Normal NIH/3T3 cell line. (**B**) Focus of transformed NIH/3T3 cells expressing P220 *gag*/*bcr*/*abl* protein. (**C**) NIH/3T3 cell line expressing P210 *bcr*/*abl* protein. (**D**) Focus of transformed NIH/3T3 cells expressing P160 *gag*/v-*abl* protein.

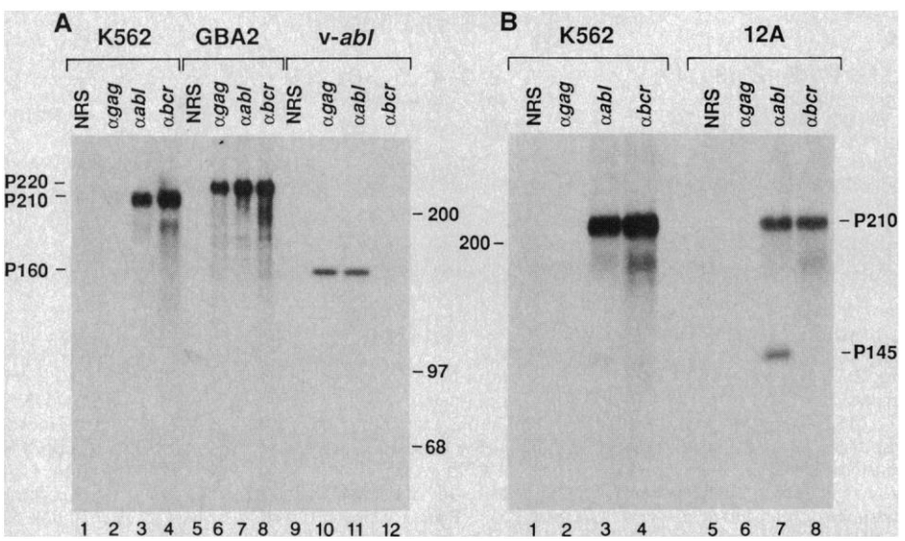

Fig. 4. Immunoprecipitation of *abl*-related proteins with antisera against *gag*, *abl*, and *bcr* determinants. (**A**) Lanes 1 to 4, K562 cell line; lanes 5 to 8, GBA2 (a transformed NIH/3T3 cell line expressing P220 *gag*/*bcr*/*abl* recombinant protein); lanes 9 to 12, v-*abl* (a transformed NIH/3T3 cell line expressing P160 *gag*/v-*abl* protein). NRS, normal rabbit serum control (10 μl); α*gag*, goat anti-murine AKR p15 *gag* antiserum (10 μl) (*17*). α*abl*, a mixture of αpEX4 and αpEX5 v-*abl* antisera (2 μl) (*16*); α*bcr*, antiserum against βgal-*bcr* fusion protein (10 μl) (*3*). (**B**) Lanes 1 to 4, K562 cell line; lanes 5 to 8, 12A (NIH/3T3 cell line expressing P210 *bcr*/murine-*abl* protein of construct pLTR6.5). Cytosol extracts were reacted with a mixture of the v-*abl* antisera αpEX4 and αpEX5 (*16*) and protein A–Sepharose immunoprecipitates were processed for in vitro immune complex kinase assay (*35*). The precipitated proteins were denatured by boiling in 2% SDS with β-mercaptoethanol buffer; diluted 1:30 in buffer containing 1% Triton X-100, 20 m*M* tris (*p*H 7.4), 50 m*M* NaCl and bovine serum albumin (1 mg/ml); and re-precipitated with different antisera as indicated. Immunoprecipitated proteins were displayed by SDS–polyacrylamide gel electrophoresis on 6% gels and visualized by autoradiography for 7 hours at −70°C with an intensifying screen.

Although P210 *bcr*/*abl* appears not to transform NIH/3T3 fibroblasts, preliminary data from Whitlock/Witte culture systems (*27*) suggests that the P210 *bcr*/*abl* protein can transform cells of hematopoietic lineage (*28*). Further definition of the transformation spectrum of P210 *bcr*/*abl* for in vitro cultures or animal models is needed to support the role of this protein in the pathogenesis of CML. Interestingly, a novel variant of *abl* (P185-190) has been implicated in Philadelphia chromosome–positive acute lymphocytic leukemia (Ph^+-ALL) (*29*), a malignancy reminiscent of murine Abelson disease (*30*). P185-190 may be more akin to v-*abl* in transforming activity than is P210. The distinct nature of these two proteins may underlie the different clinical presentations of Ph^+-ALL and Ph^+-CML.

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Full-Wave Rectification from a Mixed Electrical-Chemical Synapse

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Electrical and chemical synapses usually reinforce one another, but the pyloric late–to–lateral pyloric (PL-to-LP) neuronal connections in lobster stomatogastric ganglia create an inverted U-shaped transfer function between the two neurons: regardless of whether the PL membrane voltage swings positive or negative, the postsynaptic LP voltage will go negative. When the presynaptic cell voltage goes negative, the effect on the LP voltage is due to electrical coupling. During positive presynaptic voltages, the strong contribution of graded chemical inhibition from the PL to the LP neuron overrides the positive electrical coupling to produce net negativity.

AT CERTAIN TYPES OF SYNAPSES, long-lasting presynaptic voltage changes create equally long-lasting postsynaptic voltage responses. Although electrical coupling is the most familiar example of this, chemical synapses are often capable of maintained release of neurotransmitter in response to long-lasting changes in presynaptic voltage. Neurons that do not spike must use such graded chemical synaptic transmission (*1*), but some spiking neurons use it in addition to spike-evoked transmitter release (*2–5*). Like spike-evoked chemical synaptic transmission, graded chemical synaptic transmission may be excitatory or inhibitory.

In the stomatogastric ganglion of the spiny lobster, *Panulirus interruptus,* many neurons use a mixture of graded and spike-evoked chemical synaptic transmission (*3–5*). Of the 30 identified neurons of which the stomatogastric ganglion is comprised, 14 are involved in the central pattern generation for the pyloric rhythm used in processing food from stomach to gut (*6*). There are no excitatory chemical synapses in this system (Fig. 1, A and B): only electrical coupling and chemical inhibition are utilized. In our experiments, we studied the transfer function between lateral pyloric (LP) and pyloric late (PL) neurons from lobster stomatogastric ganglia. We now describe a property that emerges from a combination of electrical coupling and strong inhibitory chemical transmission: an input-output function that is shaped like an inverted U (*7*), not unlike the input-output function of a full-wave rectifier (for example, $y = -k|x|$).

The maintained effect of this mixed synapse is most easily seen (Fig. 1C) when tetrodotoxin is added to the tissue bath to block neuronal spikes (*8*). When the PL neuron is hyperpolarized with intrasomatically injected current, a similar (though attenuated) hyperpolarizing waveform is recorded in the soma of the LP neuron. This is typical of electrical coupling (*9*), and with pure electrical coupling a depolarization of the presynaptic neuron normally causes a parallel postsynaptic depolarization. As shown in trace D of Fig. 1C, however, depolarizing the PL neuron also causes a postsynaptic hyperpolarization. Trace D is not as rectangular as trace H from the hyperpolarizing stimulus; instead it has the peak-plateau waveform that characterizes graded chemical inhibition in the stomatogastric ganglion (*3, 10, 11*).

Exploration of a wide range of presynaptic stimuli of both polarities showed that the resulting input-output curve is an inverted U shape (Fig. 1D). Only for positive voltage changes of less than 12 mV does the positive output expected from electrical coupling exceed the small (near-threshold) contribution of chemical inhibition, creating a small range of PL voltages that have little effect on LP voltages. Otherwise, the sign of the output is insensitive to the polarity of the input.

For graded chemical inhibition to cancel positive electrical coupling potentials requires a particularly strong graded connection. There are several PL cells that make connections to LP cells; generally only one (and sometimes none) forms an inhibitory connection sufficiently strong to overcome the electrical coupling, resulting in an input-output curve such as that shown in Fig. 1D. Weaker connections may result in graded inhibition exactly canceling electrical coupling for positive stimuli, resulting in a diode-like curve providing half-wave rather than full-wave rectification.

The synaptic transmission between a cell pair can be modulated by activating a synaptic input to the presynaptic cell. The LP neuron is presynaptic to, and strongly inhibits, pyloric dilator (PD) neurons. Polarization of the PL neuron affects transmission from LP to PD neurons (Fig. 2, A and B): either depolarization or hyperpolarization of the PL neuron reduces the LP-to-PD neuronal response. Polarizing the PL neuron alone has no effect on PD neurons (since the LP neuron does not inhibit the PD neuron at rest with tetrodotoxin in the bath).

Although the pyloric rhythm can be generated and coordinated without neuronal spikes, by means of graded transmission alone (*5*), it typically operates with a combination of spike-evoked and graded synaptic transmission (*4*); Fig. 2, C and D, demonstrates the power of the PL neurons in modulating the pyloric rhythm (in saline without tetrodotoxin in the bath to block spikes). Inhibitory postsynaptic potentials

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