Synaptic Mechanism of Pentylenetetrazole: Selectivity for Chloride Conductance

Abstract. *In the neurons of* Aplysia californica *pentylenetetrazole (2 millimolar) greatly reduced chloride-dependent responses to the iontophoresis of putative transmitters. At the same concentration, pentylenetetrazole caused less attenuation of the other iontophoretic responses and had minimal membrane effects. Several convulsants have been observed to have a similar selectivity for the chloride conductance. A common mechanism of convulsant action—reduction of transmitter-induced chloride conductances—is hypothesized.*

Pentylenetetrazole (PTZ, metrazole), a clinically used convulsant agent, has been studied in several systems in an effort to determine its mechanism of epileptogenesis. It is well documented that PTZ has dramatic effects on membrane properties of neurons. In cells which were isolated from synaptic input, PTZ (10 to 70 m*M*) was shown to cause slow-wave potential oscillations and a burst pattern of firing (*1*, *2*). Bursting induced by PTZ is accompanied by the development of a negative resistance region in the current-voltage (*I-V*) relationship of the neurons (*3*) which is characteristic of the *I-V* curve of bursting neurons of *Aplysia* (*4*). However, synaptic actions of PTZ have also been indicated. At 10 m*M* PTZ, Wilson and Escueta (*5*) observed an attenuation of cholinergic inhibitory postsynaptic potentials without marked effects on excitatory postsynaptic potentials in the same neurons.

In the work reported here we further investigated the synaptic actions of PTZ, using the iontophoretic responses to putative transmitters in neurons of *Aplysia californica*. Cells were impaled with a 1.5*M* KCl or a 0.5*M* K_2SO_4 electrode and voltage-clamped by the single electrode method of Wilson and Goldner (*6*). A second, iontophoretic electrode was positioned extracellularly. This electrode contained one of the four neurotransmitters used: acetylcholine (ACh), 0.5*M*; serotonin (5-hydroxytryptamine, 5-HT), saturated; γ-aminobutyric acid (GABA), 1*M* (adjusted to *p*H 3.5); and dopamine (DA), 1*M*. Adequate bias current was applied to prevent leakage from the tip. Pulses of positive current (100 to 500 na, 500 to 2000 msec) were used to eject the transmitter and elicit a response. Iontophoretic pulses were applied at a constant frequency throughout the experiment at a rate which would not result in desensitization. Under control conditions [artificial seawater (ASW) at *p*H 7.8], it was ensured that the amplitude of the response was constant and that manipulation of membrane potential and the perfusion of ASW solutions would not alter the response [methods are described in greater detail in (*7*)].

To study the synaptic effects, PTZ (2 to 10 m*M* in ASW at *p*H 7.8) was tested on the three basic responses found in the neurons of *Aplysia*: fast excitatory responses due to an increase in sodium conductance, fast inhibitory responses due to an increase in chloride conductance, and slow inhibitory responses due to an increase in potassium conductance (*8*). Since the cells used in this study were voltage-clamped, the excitatory responses were seen as an inward current, while the inhibitory responses, at potentials more depolarized than the reversal potential, were seen as an outward current.

The effects of PTZ on a sodium-dependent response elicited by the iontophoresis of GABA is shown in Fig. 1A. In 2 m*M* PTZ, the response amplitude is approximately 80 percent of the control amplitude. In 10 m*M* PTZ (not shown) there was an attenuation of about 50 percent. Figure 1B is a plot of response amplitude against membrane potential for a sodium response elicited by GABA in another cell. The extrapolated reversal potential (*9*) of the response did not change in the presence of PTZ. When the neuron was washed with normal seawater, the response returned to control levels. An identical pattern of action by PTZ was observed for 5-HT, ACh, and DA excitatory responses (see Fig. 2A).

The effects of PTZ on the chloride-dependent inhibitory responses are much more potent (Fig. 1, C and D). The GABA response shown is reduced to approximately 50 percent of the control amplitude in 2 m*M* PTZ. This effect was independent of the neurotransmitter used to elicit the response (*10*). Chloride responses evoked by DA and by ACh were also attenuated about 50 percent in 2 m*M* PTZ (*11*). The chloride responses in 10 m*M* PTZ were greatly reduced to only 20 percent of the control amplitude (see Fig. 2B). Figure 1D shows a plot of membrane potential against response amplitude for a chloride response to GABA in another neuron. In this cell, as in all others studied, the reversal potential of the response was unaffected by PTZ.

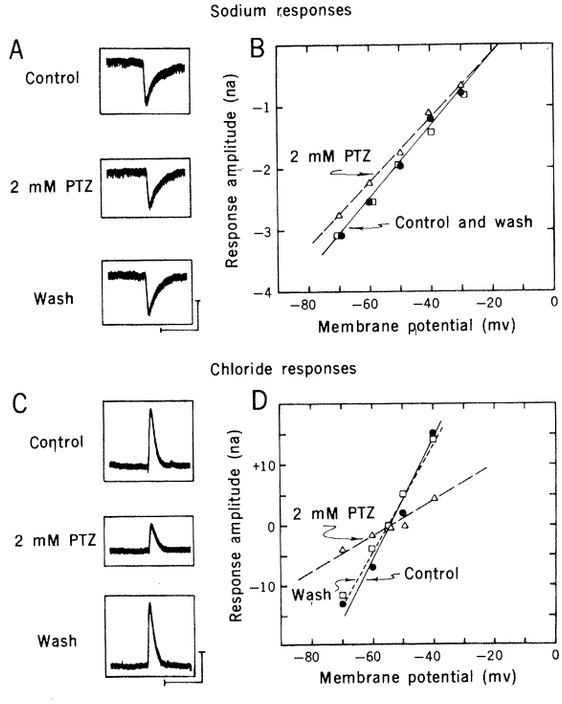

Fig. 1. Effects of 2 m*M* PTZ on typical iontophoretic responses. (A) Excitatory response due to the iontophoresis of GABA (500-na, 2000-msec pulses) in an unidentified neuron of the pedal ganglion. The neuron was voltage-clamped to a membrane potential of −60 mv. The downward deflection represents inward current. Pentylenetetrazole (2 m*M*) slightly attenuated the response, which returned to control levels after washing. Calibration: 24 seconds, 2 na. (b) Plot of response amplitude against membrane potential for an excitatory GABA response in another unidentified cell of the pedal ganglion. At 2 m*M* PTZ minimally reduced the response. Washing the neuron with ASW returned the response to control levels. (●) Control; (△) 2 m*M* PTZ; (□) wash. (C) Chloride-dependent inhibitory responses to the iontophoresis of GABA (500-na, 800-msec pulses) in a medial cell of the pleural ganglion. The membrane potential was held at −40 mv. The upward deflection represents outward current. At 2 m*M* PTZ greatly attenuated the response. Calibration: 24 seconds, 2 na. (D) Plot of response amplitude against membrane potential for a chloride inhibitory response evoked by GABA in R2 of the abdominal ganglion. At 2 m*M* PTZ potently reduced the amplitude of the response without affecting the reversal potential. Washing returned the response nearly to control levels. Symbols are the same as in (B). In (A), (B), and (D) KCl electrodes were used to record the responses; a K_2SO_4 electrode was used in (C).

The potassium-dependent inhibitory responses to ACh and DA were studied. On the average, 2 m*M* PTZ reduced these responses to only 92 percent of the control amplitude. Although the effectiveness of PTZ varied from cell to cell, in no experiment was the response reduced to less than 75 percent of the control value.

A summary of the data is presented in Fig. 2. Figure 2A shows that the most potent action of PTZ is on the chloride responses, which, regardless of the transmitter, are reduced to 40 to 50 percent of the control amplitude. Sodium and potassium responses are considerably less sensitive to PTZ. Figure 2B illustrates the pooled data for all neurotransmitters at 2 and 10 m*M* PTZ. Chloride responses are the most sensitive to PTZ at both concentrations.

At the concentrations of PTZ which greatly reduced the chloride response, membrane actions were not dramatic. At 2 m*M* PTZ had no effect on the *I/V* relationship of the neurons or on the action potential amplitudes. In agreement with the results of other studies (*2, 12*), however, 10 m*M* PTZ attenuated action potential overshoots and undershoots and in some cells induced a region of nonlinearity in the *I-V* curve. It would appear, therefore, that the most potent effect of PTZ in the neurons of *Aplysia* is a selective block of the chloride channels opened by the neurotransmitters (*13*). If PTZ has this effect in the mammalian central nervous system, increased excitability would occur as a result of reduced inhibition. Some studies (*14*) indicate that postsynaptic inhibition is unaffected by PTZ. However, there is also evidence (*15*) in mammalian neurons that inhibition produced by GABA is attenuated. Only small reductions in inhibition may be necessary to produce seizure foci, and these small reductions could be indetectable in test systems.

A variety of convulsants are used to induce epileptic seizures, and many of these agents are presumed to work by different mechanisms. It now appears that many of these agents may have a common effect. We report elsewhere (*7*) that penicillin selectively blocked the chloride responses to a number of neurotransmitters in the neurons of *Aplysia*. There are also data (*16*) that bicuculline and picrotoxin have similar effects, a selective block of the chloride responses. Other convulsant agents, strychnine (*17*) and curare (*18*), also attenuate both chloride and sodium responses in these neurons, and do so without regard to the neurotransmitter and at concentrations which minimally affect membrane properties. The observation that all of these convulsant agents antagonize the chloride response in *Aplysia*, without regard to the type of neurotransmitter, indicates an interaction with the chloride conductance rather than the receptor.

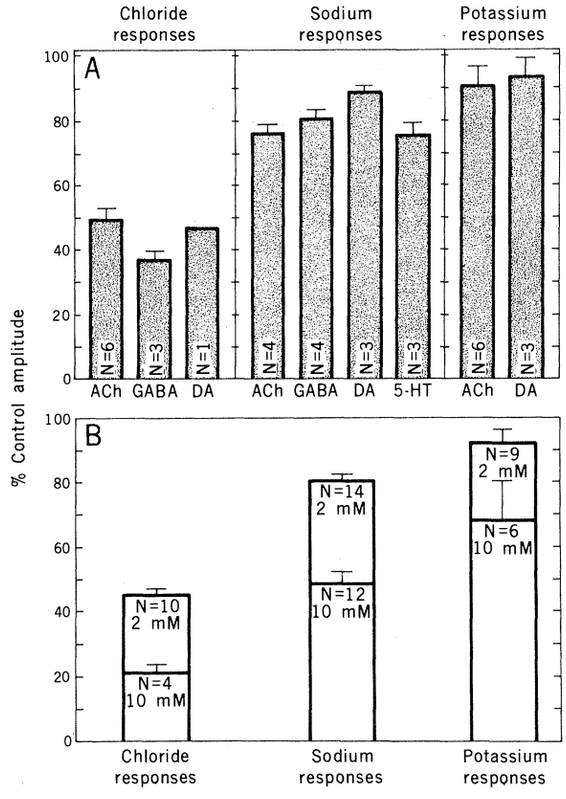

Fig. 2 (A) Summary of all the data at 2 m*M* PTZ. The amplitudes of the chloride, sodium, and potassium responses in 2 m*M* PTZ (expressed as percentages of control amplitudes) were averaged for each neurotransmitter. Chloride responses are greatly reduced regardless of the neurotransmitter used to elicit the response. Sodium and potassium responses are less affected. The degree of attenuation does not vary greatly with the neurotransmitter. (B) Data at 2 and 10 m*M* PTZ are illustrated. For each type of response, the results for all neurotransmitters were pooled and the average percentages of control amplitudes were plotted. The most potent effect of PTZ at both concentrations is on the chloride responses. In (A) and (B) bars indicate standard errors and *N* indicates the number of experiments.

This conclusion is further supported by studies in other systems. The attenuation of GABA inhibition by penicillin at the crab neuromuscular junction led Hochner *et al.* (*19*) to hypothesize a block of chloride channels. In a binding study on the synaptic membrane fractions of rat spinal cord, Young and Snyder (*20*) found that glycine and strychnine bind to distinct sites on the membrane. These authors also found (*21*) that anions which could substitute for chloride synaptically, inhibited strychnine binding, while anions which could not pass through the chloride channel did not alter strychnine binding. This led them to conclude that strychnine binding was associated with the chloride ionophore (*21*). Further support of this conclusion comes from Davidoff *et al.* (*22*), who found that in the spinal cord of the cat inhibition by glycine and GABA was noncompetitively attenuated by strychnine. Similarly, both picrotoxin (*23*) and bicuculline (*24*) produce a noncompetitive reduction of the GABA response at the crayfish neuromuscular junction. The finding that low chloride concentrations enhanced the action of picrotoxin in this system led Takeuchi (*25*) to hypothesize that the agent was interfering with the synaptic chloride channels. This is not to say that every drug will block every chloride response in every instance. Effectiveness may change with experimental technique or physiological preparation. But all these drugs, under one condition or another, have a powerful and selective effect on chloride-mediated processes. On the basis of these observations, we hypothesize that many convulsant agents may act through a similar mechanism: a selective reduction of transmitter-induced chloride conductance.

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9. Although the excitatory responses are thought to be due solely to an increase in sodium conductance on the basis of ionic substitution experiments by several investigators, an extrapolated reversal potential between − 20 and 0 mv is consistently observed (*8*). The reason for the discrepancy between the extrapolated reversal potential and the sodium equilibrium potential is not clear.
10. We attempted to find chloride responses for all four neurotransmitters tested. However, chloride inhibitory responses due to 5-HT and DA are extremely rare in the neurons of *A. californica*.
11. In one unidentified neuron, a response elicited by ACh and presumed to be due to an increase in chloride conductance was attenuated by only 26 percent. The other six cholinergic chloride responses were elicited in identified neurons (medial cells of the pleural ganglia and L2 of the abdominal ganglion) and behaved as illustrated, as did all of the chloride responses to the other neurotransmitters.
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Relaxin: A Disulfide Homolog of Insulin

Abstract. *Relaxin, a peptide hormone responsible for the widening of the birth canal in mammals, has been purified from the ovaries of pregnant hogs. The amino acid sequences of its constituent A and B chains were determined, and the positions of the disulfide cross-links were established. Relaxin was shown to be identical to insulin with respect to its disulfide bond distribution, but significant homology was lacking in other positions. These findings suggest that relaxin and insulin were derived from a common ancestral gene. Since the intrauterine mode of propagation is synonymous with the development of mammals, the genetic distance between insulin and relaxin should therefore permit an estimate of the earliest possible time of commitment of one evolutionary branch to the development of mammals. This event was estimated to have occurred about* 5×10^8 *years ago.*

Relaxin is a peptide hormone produced in the corpora lutea of pregnant mammals and acts in conjunction with estrogen on the structures of the birth canal to provide for the passage of the offspring (*1*). The major target organs include the uterus, uterine cervix, and vagina, as well as the pubic and sacroiliac joints.

The amino acid sequences of the two constituent chains of porcine relaxin (Fig. 2) were determined as reported (*2*, *3*). We have now secured information concerning the disulfide cross-linking pattern in relaxin and have thus demonstrated the existence of the first disulfide homolog of insulin.

The relaxin used for the disulfide cross link study was purified from pregnant hog ovaries (*4*). Tryptic fragments were prepared (under nonreducing conditions) by incubating 5 mg of the purified hormone with 50 μg of trypsin in 500 μl of 0.2*M* *N*-ethylmorpholine buffer (*p*H 8.5) and digested for 4 hours at 37°C. The resultant digest was lyophilized and then dissolved in 0.05*M* ammonium bicarbonate; this solution was injected onto the top of a high-pressure liquid chromatography (HPLC) column (μBondapak C_{18}, Waters Associates) and eluted with a stepwise gradient of acetonitrile in ammonium bicarbonate (0.05*M*). The result is shown in Fig. 1, and the tryptic fragments (outlined and designated Nos. 2 and 3 in Fig. 2) were identified by their amino acid composition as given in Table 1.

Table 1. Amino acid analyses of tryptic peptides derived from unreduced relaxin.

Amino acid	Peptide	
	HPLC No. 2	HPLC No. 3
Aspartic acid		
Threonine		
Serine		1
Glutamic acid	1	1
Glycine	2	1
Alanine	1	
Cysteine	4	2
Valine	1	2
Isoleucine	1	1
Leucine		2
Lysine		
Arginine	2	
Tryptophan*		2

*Determined spectrophotometrically.

In order to determine which cysteine residue (A8 or A9) was linked to residue B10, peptide No. 2 (Fig. 2) was subjected to one cycle of Edman degradation in the absence of reducing agents. Only phenylthiohydantoin alanine was obtained from the degradation cycle. The remaining peptide was recovered from the sequencer cup, dried, and redissolved in 200 μl of 0.2*M* *N*-ethylmorpholine buffer (*p*H 8.5). By means of thin-layer chromatography, it could be demonstrated that the remaining peptide still yielded only one spot when sprayed with ninhydrin or phenanthrenequinone (test for arginine). After treatment with mercaptoethanol to reduce its disulfide bonds the peptide yielded two spots in the same thin-layer chromatography system as described (*3*) for the separation of tryptic fragments derived from the B chain of relaxin. This result indicated that the relaxin A and B chains were linked through cysteine A9 and cysteine B10. Had the cross-link involved cysteines A8 and B10, the Edman step would have released the intrachain disulfide loop (A9 through A13) which would have been seen as a second peptide prior to reduction.

Evidence for a cross-link between the cysteines at A22 and B22 could be deduced from another tryptic peptide obtained from the total digest of relaxin. The tryptic peptide designated HPLC No. 3 in Fig. 1 contained the residues A21 leucine, A22 cysteine, and B17 leucine through B26 serine. The amino acid analysis of this peptide is also shown in

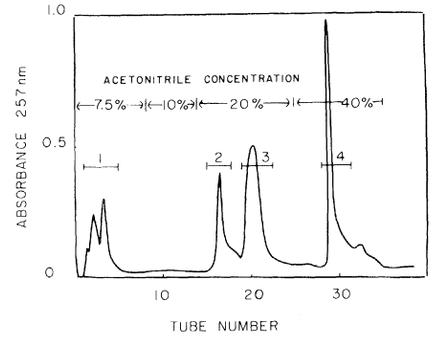

Fig. 1. Elution record of the separation by high-pressure liquid chromatography of tryptic peptides derived from unreduced relaxin. The acetonitrile gradient increased stepwise and peaks were collected as indicated in the figure. Peptides Nos. 2 and 3 were found to contain the disulfide linkages.