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The Effects of Valproate and Phenytoin on the cAMP and cGMP Levels in Nervous Tissue (41997)

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Abstract. Earlier studies have demonstrated that valproic acid (VPA) and phenytoin (PHT) influence the excitability properties of crayfish axons through different mechanisms. PHT was found to antagonize the electrophysiologic effects of VPA. The purpose of the present study was to determine if the electrophysiologic effects of VPA and PHT are correlated with changes in the cellular levels of either cAMP or cGMP as these substances are known to influence membrane excitability. It was found that PHT (0.1 m*M*) has no effect on the levels of either cAMP or cGMP within crayfish neural tissue. VPA (4.0 m*M*) also has no effect on cAMP levels. However, it does significantly reduce the levels of cGMP. Pretreatment of neural tissue with PHT has been shown to eliminate the effects of VPA on membrane excitability. It was found that this pretreatment has no influence on VPA's ability to reduce cGMP levels. The effect of VPA on cGMP levels is observed in the absence of spontaneous activity. Therefore, it is concluded that the observed reduction in cGMP levels does not represent the modulation of cGMP levels that is known to accompany activity. Two experiments demonstrate that the 4-mV depolarization of membranes by VPA can not account for its effect on cGMP levels. In the first, pretreatment with PHT abolished the depolarizing effect on VPA but not its effect on cGMP. In the second, a concentration of ouabain which depolarizes crayfish neural tissue by 8–10 mV without producing spike activity had no effect on either cAMP or cGMP levels. These experiments effectively dissociate the electrophysiologic response to VPA and PHT from changes in cyclic nucleotide levels.

Valproic acid (VPA), a relatively new anticonvulsant agent, is particularly effective against absence seizures and severe, otherwise uncontrollable epilepsy (1). Phenytoin (PHT) is the pharmacologic agent most frequently used to control seizure activity. Earlier reports from this laboratory (2, 3) used standard electrophysiologic techniques to determine the effects of VPA and PHT, alone and in combination, on the bioelectric characteristics of an individual neuronal membrane, the crayfish medial giant axon. Both VPA and PHT depressed membrane excitability in this neuronal model but in different ways. Pretreatment with PHT was found to antagonize many of the membrane effects of VPA. These results help to explain the cooperative clinical action of VPA and PHT (4, 5).

The second messengers cAMP and cGMP have been linked to altered membrane excitability properties. Although it is not known whether it is a cause or an effect, increased cellular electrical activity is accompanied by an elevation of both cAMP and cGMP in brain tissue (6). Intraventricularly injected dibutyrl cAMP produces seizure activity in experimental animals that can be depressed in a dose-dependent fashion by PHT (7). The Ca^{2+} influx that normally takes place during the neuronal action potential (8) is dependent upon cyclic nucleotide levels. The K^+ conductance, and therefore the refractory period of the neuron, is directly related to the magnitude of this Ca^{2+} influx. Ca^{2+} influx is reduced by PHT (9). Kupferberg *et al.* (10) have shown that VPA lowers the brain level of cGMP as it elevates the level of the inhibitory transmitter GABA. These data suggest that modulation of the cellular cyclic nucleotide levels by VPA and PHT may account for part of their effects on membrane excitability. The following experiments were conducted to determine the effects of VPA and PHT, alone and in combination, on the cAMP and cGMP levels in crayfish nervous tissue in the hope of linking the level of these compounds to physiological function.

Materials and Methods. The brain and nerve cord from the crayfish *Procambarus clarkii* were used in all experiments. After the cord was carefully dissected, it was bathed in Van Harreveld's (11) crayfish solution containing NaCl, 190 m*M;* KCl, 5.4 m*M;* $CaCl_2$, 13.5 m*M;* $MgCl_2$, 2.6 m*M;* Na HCO_3,

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2.3 m*M*; Tris–HCl, 15 m*M*. The tris buffer system maintained the pH at 7.4. All experiments were conducted at room temperature (21–23°C). Control nerves were equilibrated in Van Harreveld's solution for 90 min. Nerves that were treated with ouabain, VPA, or PHT were transferred to a bathing medium containing the test compound for the final 30 min of the test period. Nerves that were pretreated with PHT remained in control medium for only 30 min. They were then bathed in medium containing PHT for 30 min followed by exposure to both PHT and VPA for the final 30 min of the experiment. The pH of all media was maintained at 7.4.

At the end of the 90-min experiment, all nervous tissue was lightly blotted on filter paper and rapidly frozen in aluminum clamps cooled in liquid nitrogen. The samples were stored in an ultrafreezer at −70°C. Frozen tissue samples were homogenized at 4°C in 1 ml of cold 6% trichloroacetic acid (TCA). TCA supernatants were extracted four times with 10 ml of ethyl ether saturated with water. The extracted aqueous phase was evaporated under a stream of air and the residue dissolved in 0.05 *M* sodium acetate buffer, pH 6.2. This extract was used directly in the cAMP and cGMP radioimmunoassay kits purchased from New England Nuclear.

The cyclic nucleotide assay was basically that described by Steiner *et al.* (12). The technique employs competitive binding between the cyclic nucleotide in the tissue sample and an iodinated antigen (the radioactive marker) to the nucleotide-specific antibody. Nucleotide levels were determined by adding the extract to the antigen–antibody complex. When the displacement reaction reached equilibrium, the antigen–antibody complex was precipitated from solution by a second antibody. The amount of marker remaining was determined by counting the activity of the precipitate in a gamma spectrometer (Packard Auto-Gamma Model 500C) that automatically corrects for quenching. The cyclic nucleotide level was measured from a standard curve relating percentage of the bound marker in the precipitate to the concentration of cyclic nucleotide added. This technique was sensitive enough to assay for both cAMP and cGMP in a single nerve cord. A quantity of tritiated nucleotide that yields a known number of counts on a beta spectrometer was added to each sample vial at the beginning of the assay. The number of counts measured at the end of the extraction procedure was compared with the counts added at the beginning of experiment to yield the percentage recovery. Nucleotide levels were normalized per milligram of protein measured by the standard method of Lowry *et al.* (13).

Data for each experimental condition were subjected to the Grubbs outlier test (14). Any data point thus determined to be taken from another population was eliminated from the data pool. The statistical significance of the differences in nucleotide levels between test conditions was evaluated by means of an unpaired *t* test.

Results. Table I presents the effects of various pharmacologic interventions on the cAMP and cGMP levels in crayfish nervous tissue. A concentration of 4 m*M* VPA was used in the first series of experiments because we previously found this to be the maximal dose that did not have toxic effects on cellular electrophysiologic characteristics (2). VPA had no significant effect on cAMP levels but significantly reduced cGMP by an average 33%. These results were compared with the effects of 0.11 m*M* PHT, the maximal water soluble concentration of this compound (2).

TABLE I. EFFECTS OF VARIOUS PHARMACOLOGIC INTERVENTIONS ON THE CYCLIC NUCLEOTIDE LEVELS IN THE CRAYFISH SPINAL CORD

	cAMP	cGMP
Control	36.1 ± 4.0 (18)	4.98 ± 0.57 (17)
VPA[a]	27.5 ± 2.8 (18)	3.35 ± 0.54* (15)
PHT[a]	30.2 ± 3.9 (18)	4.94 ± 0.56 (18)
PHT + VPA[a]	29.7 ± 3.4 (18)	3.21 ± 0.35* (13)
Ouabain[a]	30.9 ± 6.4 (8)	4.70 ± 1.20 (8)

Note. All data are presented as means ± SE. Units are pmole of nucleotide/mg protein. The number of spinal cords assayed for each experimental condition is indicated within the parentheses.

[a] Maximally effective concentrations of these compounds were used.

* Signifies a statistically significant ($P < 0.05$) difference between control and test levels.

PHT had no significant effect on either cAMP or cGMP levels. An earlier investigation of the effects of VPA and PHT on crayfish neural tissue demonstrated that pretreatment of giant axons with PHT prevented many of the electrophysiologic effects of VPA (2). Therefore, in the next series of the experiments, the nervous tissue was bathed for 30 min in a solution containing PHT before it was exposed for 30 min to VPA. Pretreatment with PHT did not influence the response of cyclic nucleotide levels to VPA; VPA had no effect on cAMP levels but reduced cGMP levels by an average of 35%.

Reports from other laboratories have demonstrated that PHT and VPA are effective in reducing cGMP and cAMP levels when they have first been elevated by depolarizing agents such as ouabain. A ouabain concentration of 0.5 m*M* blocks nearly 100% of the Na^+–K^+-ATPase activity in crayfish nervous tissue (15) and significantly depolarizes the resting membrane without causing spontaneous activity (3, 16). Table I shows that this concentration of ouabain was without effect on either cAMP or cGMP levels.

Discussion. Increased electrical activity and cellular depolarization have been shown to increase both cellular cAMP and cGMP levels (6, 17). These effects appear to be mediated through an increased release of neurotransmitters in the case of cAMP and through increased Ca^{2+} influx (18) and subsequent stimulation of guanylate cyclase in the case of cGMP (17, 19, 20). Ouabain is often used to elevate cyclic nucleotide levels by depolarizing the cell membrane through inhibition of the Na^+–K^+ pump. In crayfish neural tissue 0.5 m*M* ouabain blocks Na^+–K^+-ATPase activity (15) and depolarizes the cells by 8–10 mV (3, 16). Yet, when we measured the effect of ouabain on the cyclic nucleotide levels within crayfish neural tissue, we found that neither the levels of cAMP nor of cGMP were altered from control values. This lack of an effect on either cyclic nucleotide suggests that depolarization itself is not sufficient to influence cyclic nucleotide levels. It would appear that increased spike activity with its associated Ca^{2+} influx (21) and synaptic activity is required. The depolarization produced by ouabain is never associated with the production of spontaneous spike activity in crayfish axons (3).

Both VPA and PHT are known to inhibit depolarization induced increases in cyclic nucleotide levels (17, 22). These effects may be important in seizure control because elevated cyclic nucleotide levels have been implicated in the maintenance of sustained seizure discharge (23). The present study has focused on the effects of VPA and PHT on the normal resting cyclic nucleotide levels of crayfish neural tissue because these resting levels may be important for seizure initiation. For example, Levitan and Norman (24) have demonstrated that elevated cellular levels of cGMP can depolarize neuron R15 of *Aplysia* and produce bursting activity. We found that PHT has no effect on either resting cAMP or cGMP levels. VPA also has no effect on cAMP levels while it significantly lowers the amount of cGMP within the cell. Others have reported that PHT and VPA have no effect on the resting levels of the cyclic nucleotides in various brain areas (19, 26). The only region of the brain excepted is the cerebellum where high levels of spike activity normally occur (25). Both PHT and VPA were found to reduce cerebellar cyclic nucleotide levels (19, 26). Spike activity is not required for the effect of VPA on cGMP levels in crayfish neural tissue.

VPA and PHT influence the resting membrane properties and spike characteristics of crayfish giant axons in different ways. VPA depolarizes the cell by 4 mV while PHT does not, VPA reduces the magnitude of the action potential while PHT does not, and PHT reduces both the resting Na^+ and K^+ conductance of the membrane while VPA reduces only the K^+ conductance (2, 3). These differences in membrane bioelectric characteristics could be related to the different effects of these two compounds on cGMP levels. However, this does not appear to be the case. Pretreatment of axons with PHT prevents VPA from producing most of its effects on membrane excitability properties (2). Yet, pretreatment with PHT did not prevent VPA from producing a decrease in the cellular level of cGMP. These experiments do not allow us to speculate on the mechanism behind VPA's effect on cGMP levels; it could be through an inhibition of cGMP production or through a stimulation of cGMP breakdown. They do dissociate the effect of VPA on resting levels of cGMP from its

influence on the bioelectric properties of crayfish axons. They do not, however, discount the importance of resting cyclic nucleotide levels with respect to the propensity of neurons to develop bursting activity.

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