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Evidence that α-Dihydrograyanotoxin II Does Not Bind to the Sodium Gate

Yoshinori Soeda*, R. D. O'Brien, J. Z. Yeh, and Toshio Narahashi

Section of Neurobiology and Behavior, Cornell University, Ithaca, New York, 14853 and Department of Physiology and Pharmacology, Duke University Medical School, Durham, North Carolina 27710

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Summary. The basis for the ability of α-dihydrograyanotoxin II (α-2HG-II) to promote Na^+ conductance in axons was sought. The apparent binding of tritiated α-2HG-II to neural and other preparations was studied, using equilibrium dialysis, with lobster axon membranes, *Torpedo* electroplax, housefly head, and rat brain, liver and kidney. In every case the "binding" was nonsaturating and was suggested to involve nonspecific partitioning into the tissue. Supporting evidence was the similarity of extent of "binding" in all tissues and its relative insensitivity to neuropharmacological agents. α-2HG-II did not affect the Na^+ conductance of phospholipid bilayers, nor did it permit transport of ^{22}Na into a bulk organic phase. It was concluded that α-2HG-II did not bind to the sodium gate, but possibly to a sodium permease present at a frequency of less than one per μ^2 of cell membrane.

Recently, Seyama and Narahashi (1973) have shown that α-dihydrograyanotoxin II (α-2HG-II), which is a derivative of the toxic principle from leaves of plants of the family *Ericaceae*, affects the sodium permeability of squid axon. Whether applied internally or externally, 3×10^{-6} M α-2HG-II produced a half-maximal effect, which took the form of a depolarization which was exclusively related to sodium. The effect could be antagonized by tetrodotoxin in a mixed (competitive and noncompetitive) manner. The treated axon was still capable of sustaining a sodium action potential when the membrane potential was brought back to the original level by a hyperpolarizing current. The effect is not specific to squid axons, as the results below will show.

The above findings were compatible with an effect of α-2HG-II upon the sodium gate (*see* Discussion). If such were the case, α-2HG-II, which is relatively easy to tritiate with high specific activity (Soeda, 1974), would be an excellent tool for use in isolation of the sodium gate. The agent previous-

* *Present address:* Nisso Institute for Life Sciences, Oiso, Kanagawa-ken, Japan.

ly used has been tetrodotoxin; because its derivatives have poor physiological potency (Narahashi, Moore & Poston, 1967), only tritium exchange methods have been employed, and consequently, material of low specific activity (Henderson & Wang, 1972) or purity (Hafemann, 1972) has been obtained.

We therefore embarked upon a study of the binding of tritiated α-2HG-II to neural and other tissues, with the intent of determining whether it bound to the sodium gate.

Materials and Methods

Denburg (1972) has shown that the microsomal fraction derived from leg nerve of the lobster *Homarus americanus* contains most of the axonal membranes, and this was prepared as follows. Forty-nine ml of a 14% homogenate was prepared in a solution of 10 mM tris hydroxymethylaminoethane buffer (pH 7.8) in 10% sucrose (0.32 M), and the microsomal fraction (M_2 of Denburg) was suspended in 3.7 ml of the tris-sucrose buffer. Experiments with fresh rat brain, liver and kidney, or frozen electroplax from *Torpedo californica* or with frozen house fly head employed the same protocol.

The tritiated α-2HG-II was prepared as described by Soeda (1974) with a specific activity of 1.21 Ci/mmole. The nonradioactive α- and β-2HG-II were kindly donated by Professor J. Iwasa. The $^{22}NaCl$ was obtained from ICN, Chemical and Radioisotope Division, California.

Biological activity of the tritiated α-2HG-II was evaluated with frog's sartorius muscle preparations. The depolarization of the muscle membrane was taken as a measure of activity, and the potency was compared with that of nonradioactive α-2HG-II. This preparation was chosen because a large number of measurements could be made with a high degree of accuracy. Conventional microelectrode technique with 3 M KCl-filled glass capillaries was employed. The frog Ringer's solution used consisted of (mM): Na^+ 118; K^+ 2.5; Ca^{2+} 1.8; Cl^- 121.1; HPO_4^{2-} 2.15; $H_2PO_4^-$ 0.85, and final pH 7.2. The experiments were done at a room temperature of 22 °C.

The sensitivity of lobster nerves to α-2HG-II was evaluated by means of intracellular microelectrode technique. Changes of the resting membrane potential were followed on a strip chart recorder before and after application of α-2HG-II at a room temperature of 22 °C. Artificial sea water was used as the bathing medium and consisted of (mM): Na^+ 450; K^+ 10; Ca^{2+} 50; Cl^- 575; Tris 10, and final pH 8.0.

Equilibrium dialysis was carried out in 7 cm lengths of 1 cm-wide dialysis tubing (Union Carbide). In each length was placed 0.25 ml of the preparation, and it was dialyzed against 100 ml of lobster saline solution containing the radioactive ligand. Equilibrium was achieved within 12–14 hr at 25 °C, and then three 0.1 ml samples of the bath and three 0.1 ml samples from each bag were placed in counting vials, and to each was added 10 ml of a scintillation solution containing 18 grams of POP, 1.2 grams dimethyl POPOP, 340 ml of Beckman Bio-Solv, 450 ml of methyl cellosolve, and 3 liters of toluene. Radioactivity was counted with a Tricarb 3374 counter with external quench correction. The samples from each bag after dialysis were also assayed for protein by the method of Lowry, Rosebrough, Farr and Randall (1951).

The possibility that α-2HG-II might bestow sodium conductance upon bilayer membranes was studied by a procedure which was successful in the parallel case of the induction of potassium conductance in bilayers by valinomycin (Hilton *et al.*, 1973). The same apparatus

was used with the phospholipid bilayer formed from *Torpedo* electroplax phospholipids. The buffer solution in the two compartments was 0.05 M with respect to Na^+ and 0.025 M with respect to Cl^- and PO_4^{3-}.

The data to be presented will show the grayanotoxin "binds" in conformity with a simple partitioning between the medium and the tissue subfractions, without evidence for saturable binding sites. Given this situation, the question arises as to how many saturable binding sites could exist in addition, and yet escape detection because of the plethora of nonsaturable binding.

Suppose that a system contains a saturable high-affinity component and a component not saturable over the range studied; this latter component might be related to a very large number of low-affinity binding sites or to partitioning between the buffer and a lipid phase in the tissue preparation. Then the observed binding will be a sum of the binding to the saturable component, which is given by:

$$B_1 = \frac{a}{\frac{K_D}{L} + 1} \tag{1}$$

where B_1 is the amount bound, a is the number of binding sites, K_D is their dissociation constant, and L is the ligand concentration; and of binding to the nonsaturable component, which is given by:

$$B_2 = kL \tag{2}$$

where k is (for instance) a partitioning constant. The observed binding B will be the sum of B_1 and B_2:

$$B = \frac{a}{\frac{K_D}{L} + 1} + kL. \tag{3}$$

The plot of B as a function of L from this equation shows initially a fast-rising exponential curve, to which both B_1 and B_2 contribute, and then a linear part during which B_1 contributes a fixed amount (a, when $L \gg K_D$) and B_2 an amount proportional to L. Thus, a is obtained by the vertical difference between (*i*) the linear part of the plot of B as a function of L and (*ii*) the line which passes through the origin and has the same slope as (*i*). This value corresponds to the y intercept of the extrapolated plot of B as a function of L. It will be shown that the plot of the observed data appears to pass through the origin. The error associated with the observation that the plot passes through the origin will yield a statement about the number of saturable binding sites that might exist in addition, and yet escape detection.

Results

Biological Activity

The resting membrane potential of the frog sartorius muscle fibers was measured before and during applications of α-2HG-II at a concentration of 1×10^{-5} M. This concentration of the toxin caused a membrane depolarization quickly which in turn triggered muscle twitches. Because of this, measurements of the membrane potential could not be made until the twitches had ceased. The values of the resting potential before and 10

Table 1. Resting membrane potential of frog sartorius muscle before and during application of 1×10^{-5} M tritiated and cold α-2HG-II[a]

Type of α-2HG-II	Control (mV)	After α-2HG-II	
		10 min (mV)	30 min (mV)
Nonradioactive	-96.6 ± 0.67 (22)	-49.2 ± 3.73 (17)	-43.6 ± 1.04 (21)
Tritiated	-94.8 ± 0.80 (20)	-47.0 ± 0.86 (16)	-45.2 ± 0.86 (20)
P[b]	>0.05	>0.5	>0.2

[a] Data are the means ± SEM with the number of measurements in parentheses.
[b] P values were obtained with Student's t test.

and 30 min after application of the tritiated or cold α-2HG-II are given in Table 1. Both radioactive and nonradioactive samples caused a large depolarization of the membrane and were equally potent. Giant axons of the circumoesophageal connectives of the lobster were also depolarized by unlabelled α-2HG-II (Riccioppo and Narahashi, *unpublished observation*). However, the lobster axons were less sensitive than the squid axons reported by Seyama and Narahashi (1973). At concentrations of 2.5×10^{-5} M and 1×10^{-4} M, the depolarization amounted to 12 and 23 mV, respectively, in lobster axons in comparison with 45 and 55 mV in squid.

Binding to Lobster Nerve

Equilibrium dialysis studies were performed on the microsomal fraction from the lobster nerve. Binding of tritiated α-2HG-II was readily observed. Preliminary studies, using α-2HG-II at 10^{-7} M, dealt with the time to reach equilibrium, and it was established that at 5 °C equilibrium was reached after 16 hr, whereas at 25 °C equilibrium was reached after 12 hr. The amount of binding was temperature-dependent, amounting to 0.038 pmoles/mg protein at 5 °C and 0.435 pmoles/mg protein at 25 °C.

Binding studies were carried out over two ranges of concentration at 25 °C. One very wide range (4×10^{-4} to 5×10^{-9} M) showed (Fig. 1) proportionality between binding (B) and α-2HG-II concentrations (L) as shown by the constancy of the ratio B/L throughout this range of concentrations. We next wished to evaluate how many saturable sites in the physiologically important concentration range might have escaped detection. We selected the range in which it was possible to use the radioactive α-2HG-II without dilution with the nonradioactive form, i.e.

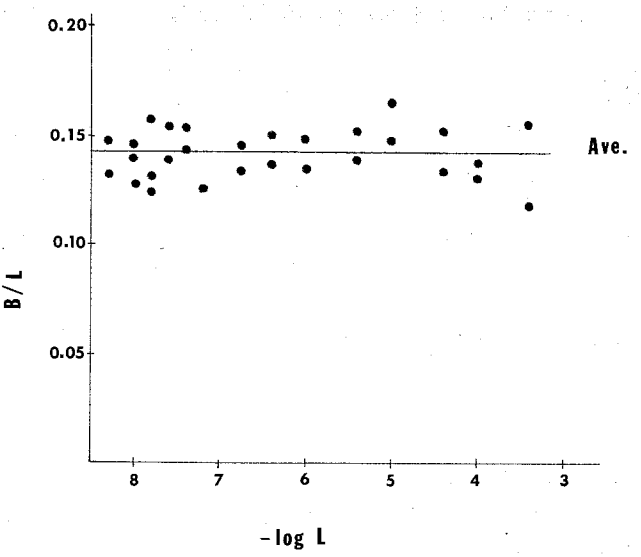

Fig. 1. Binding of α-2HG-II to lobster nerve microsomes over a wide range. *B*, pmoles α-2HG-II bound/mg protein; *L*, molar concentration of α-2HG-II ($\times 10^{-8}$ M)

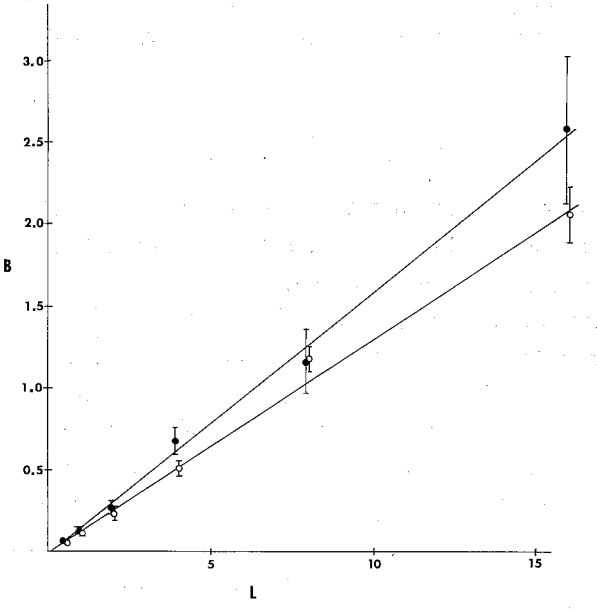

Fig. 2. Binding of α-2HG-II to lobster nerve (–●–) and rat liver (–○–) microsomes. *B*, pmoles α-2HG-II bound/mg protein; *L*, molar concentration of α-2HG-II ($\times 10^{-8}$ M); the vertical bars are the standard deviations of triplicate experiments. Regression equation:
–●– $B = 0.16084\,L - 0.02857$; –○– $B = 0.13079\,L - 0.01000$

1.6×10^{-7} to 5×10^{-9} M. Over this range, no saturation could be seen, as judged by the linearity of the plot of B as a function of L (Fig. 2). It was concluded that α-2HG-II binds in a nonspecific way to membrane components. Clearly, a small number of saturable sites could have escaped detection (*see* Materials and Methods). The computed value of the B intercept was -0.0286 ± 0.202 pmoles/mg potein. We can thus state with 95 % confidence that if saturable sites exist, they constitute no more than two standard errors from the mean, which (for a positive value) is 0.369 pmoles/mg, corresponding to 0.69 molecules per μ^2 of axon membrane; in this calculation we have used the estimate of Moore, Narahashi and Shaw (1967) that there are 7000 cm^2 per g of wet lobster leg nerve.

Binding to Various Tissues

The question was considered as to whether the binding of α-2HG-II was in any way specific to neural tissues. The binding of 5×10^{-8} M α-2HG-II to lobster axon, *Torpedo* electroplax, rat brain and housefly head (all of which are high in neural components) was compared with the binding to rat liver and rat kidney. The highest binding per gram of tissue was found for rat liver, and the amount bound per mg of protein was substantially the same for all six tissues ranging from 0.313 pmoles/mg for rat brain to 0.733 for lobster nerve.

A possibility was that α-2HG-II binding might at least be specific to some subcellular component, even though it was found in all tissues studied. Experiments on subcellular fractions derived from rat liver were performed; "binding" occurred in approximately equal amounts to mitochondrial and microsomal fractions, and significant binding was even obtained to the soluble proteins. With rat liver microsomes, a linear relation between the binding and ligand concentration was observed (Fig. 2), passing through the origin, as for lobster nerve. The data once more suggested that there were either no saturable sites, or else that the fraction of them was small enough to go undetected in the extensive nonspecific binding.

If the binding was to a specific target site, such as the sodium gate, the sodium pump, or a neurohumoral receptor, it would be anticipated that appropriate blockade of the binding by physiologically active drugs should occur. Studies were performed with microsomes from lobster nerve, using α-2HG-II at 10^{-7} M and appropriate drugs added to the dialysis bath at 10^{-5} M. The following agents inhibited to the percent indicated in parentheses: α-2HG-II (0); β-2HG-II (10); tetrodotoxin (5);

p,p-dichlorodiphenyldichloroethane (14); ouabain (25); procaine (23); tubocurarine (20). Two agents gave somewhat higher blocking activity; these were tetraethylammonium (32) and serotonin (42). These last two agents were also studied on rat liver microsomes under the same conditions; tetraethylammonium gave 24% inhibition and serotonin 41%. None of these findings indicated specificity or a marked sensitivity to neuropharmacological agents.

The Ion-Carrier Hypothesis

It was possible that α-2HG-II might induce sodium conductance by acting as an ion-carrier, in much the same way that valinomycin produces potassium conductance in both synthetic and natural membrane systems (Mueller & Rudin, 1967). Consequently, experiments were performed under conditions which successfully showed the induction of potassium conductance by valinomycin. A bilayer was formed from *Torpedo* electroplax phospholipids, and positioned between compartments containing buffered sodium solutions (*see* Materials and Methods). The membrane had the expected low conductance, due to its impermeability to cations; the value averaged 10^{-8} mho/cm^2. The conductance was not changed by adding α-2HG-II in concentrations ranging from 10^{-6} to 10^{-3} M. To insure that the membranes were active, 10^{-8} valinomycin and a small amount of potassium chloride were added at the end of each experiment; the expected valinomycin-induced conductance was observed.

It was concluded that the membrane system was quite appropriate for observing cation conductance induced by a carrier, but that α-2HG-II did not act as a carrier in such a system.

Another check on the ion carrier hypothesis was a study on bulk transport of ion into an organic phase. In the parallel case of K^+ transport by valinomycin, radioactive K^+ can be transported into an organic phase in the presence of the carrier (Pedersen, 1968). It was therefore decided to explore whether α-2HG-II would induce ^{22}Na to partition into an organic solvent.

The experiment would be vitiated if the α-2HG-II were to be extracted from the aqueous phase entirely in the course of the experiment, so it was first established with tritiated α-2HG-II that the carrier could coexist in both phases. Two ml of 5×10^{-7} ^{3}H-α-2HG-II was shaken with 2 ml of the indicated solvents, and the parenthetic percentage of α-2HG-II appeared in the organic layer; cyclohexane (0.12); toluene (3.0); monochlorobenzene (6.3); dichloroethane (30.3); chloroform (54.3). Further

experiments were carried out in a similar way with chloroform and water, adding 0.028 mM ^{22}Na to the aqueous phase, and six different concentrations of α-2HG-II ranging from 0.05 to 1 mM. No sodium was found in the presence or absence of α-2HG-II. It was concluded that the latter did not act as an ion carrier, at least in bulk solvent transport.

Discussion

The term "sodium gate" used in this paper means the entity to which tetrodotoxin binds, with consequent loss of Na^+ transport. It is not yet possible to determine unambiguously whether this entity is the true gate, or the sodium channel which is regulated by the gate. Since the gates are probably no more than three times as numerous as the channels, our findings imply that α-2HG-II binds to neither.

Two limitations in this study should be noted. (a) There is a remote possibility that tritiated α-2HG-II completely fails to bind to the putative gate and is completely inactive, whereas the unlabelled form does both. To exclude this possibility one would need to have material at least 10 times more radioactive, so that about half of its molecules would be tritiated, and one could clearly detect loss of physiological activity. Such labelling has rarely been achieved in probes of this type. (b) We conducted most of the binding studies at 25 °C. This temperature has the advantage of being closer to physiological temperatures than, say, 5 °C; and gave over ten times more binding than 5 °C; but the risk is that in 12 hr there could be denaturation of the putative gate.

The evidence of Seyama and Narahashi (1973) based on neurophysiological studies of the squid giant axon, clearly indicates a specific promotion of Na^+ influx by α-2HG-II in resting axons. Even though the sodium gate is usually conceived as being involved in the action potential rather than the resting potential, it was conceivable that the α-2HG-II effect was upon the sodium gate, thus making the toxin a suitable probe for the gate, because (1) the effect of α-2HG-II was profoundly reduced by 0.1 μM tetrodotoxin (Seyama & Narahashi, 1973), which is usually regarded as a specific reagent for the sodium gate; (2) 10^{-6} M tetrodotoxin reduces the ouabain-insensitive influx of Na^+ into resting squid axon by 59%, implying that "a significant proportion of the resting Na influx into squid nerve takes place through the Na-selective channels which are involved in the action potential" (Baker *et al.*, 1969); and (3) tetrodotoxin at 0.3 μM hyperpolarizes the resting membrane of the squid giant axon by about 5 mV (Freeman, 1971).

We therefore prepared tritiated α-2HG-II with a specific activity such that one could detect its binding to the sodium gate, which for lobster nerve was taken to be present in an amount equivalent to 13–36 gates per square micron (Moore *et al.*, 1967; Keynes, Ritchie & Rojas, 1971; Hafemann, 1972; Barnola, Villegas & Camejo, 1973), as measured by tetrodotoxin binding. However, we have been unable to substantiate the hypothesis that α-2HG-II combines with the sodium gate. It is apparent that the toxin is readily taken up by the tissues that we have studied, but there appear to be no true binding sites discoverable under our circumstances. Instead, the agent acts as if it partitions between the aqueous exterior and the apolar interior of the membranes explored herein. The view is further supported by the observations that binding is not specific to neural tissue, nor is it found in any particular subcellular fraction, nor is it sensitive to specific analogs.

Several lines of evidence compatible with the present view have recently been obtained using grayanotoxin I (GTX I), an analog which exerts the same physiological action as α-2HG-II on nerve membranes.

(a) The antagonism by tetrodotoxin of the increase in resting sodium conductance caused by GTX I is noncompetitive in nature (Narahashi & Seyama, 1974). Since the resting sodium conductance is the parameter that is primarily affected by GTX I, this observation is more reliable than the previous one (Seyama & Narahashi, 1973) in which the membrane potential was measured.

(b) The apparent dissociation constant of tetrodotoxin in blocking the sodium conductance increase (gate) during activity is 3×10^{-9} M (Cuervo & Adelman, 1970), whereas that of tetrodotoxin in inhibiting the grayanotoxin action is 4×10^{-8} M (Narahashi & Seyama, 1974).

(c) Whereas the resting sodium conductance increase by GTX I is dependent on the membrane potential, the potential range where a negative conductance occurs is about 50 mV more negative than that where the sodium conductance during activity exhibits a negative conductance (van den Bercken & Narahashi, *unpublished*).

(d) In both normal and GTX I-treated squid axons, the permeability ratio of resting sodium channels to various organic cations is considerably different from the ratio during activity (Hironaka & Narahashi, 1975).

A possibility is that α-2HG-II combines with a small subpopulation of sodium gates, or with a sodium permease site which is present to the extent of only 2% of the sodium gates. The basis of this statement is as follows. The sodium conductance of the resting squid axon is 0.016 mmho/cm^2 of axon membrane; this resting flux is increased 100-fold by the application

of α-2HG-II but increased 5000-fold in the course of the action potential of untreated nerve. Consequently, if the sodium permease combined with the toxin had a Na^+ conductance identical with an opened sodium gate, there must be about 50 times less sodium permease systems than there are sodium gates, even though the population of sodium gates is in turn extraordinarily small. The results with lobster axon showed that 0.69 α-2HG-II binding molecules per μ^2 could have escaped detection. Taking the average number of sodium gates in lobster axon as 25 per μ^2 (average of four values cited above), it follows that if the hypothetical permease systems were 2.8% or less in frequency than the gates, they would have escaped detection.

A quite different possibility is that the toxin affects the gate without binding to it, for instance by modifying the character of the lipid matrix within which the gate operates.

We conclude that α-2HG-II, under the conditions of our experiments, does not bind to the sodium gate; nor does it act as a valinomycin-like ion-carrier. It could combine with a site present at an incidence of about 0.69 per μ^2 or less, and have escaped detection by our methods.

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