GRAYANOTOXIN OPENS Na CHANNELS FROM INSIDE THE SQUID AXONAL MEMBRANE

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ABSTRACT External application of α-dihydro-grayanotoxin II (α-H_2-GTX II) to squid giant axon under nonperfused condition caused substantial membrane depolarization. Intracellular perfusion of the fibers retarded this depolarization appreciably. Tritium-labeled α-dihydro-grayanotoxin II ([^{3}H]α-H_2-GTX II) in the external medium can permeate through the cell membrane, but permeation of α-H_2-GTX II does not occur either with the carrier-mediated system or through the pores of the Na channel. The finding that the most hydrophilic grayanotoxin analogue, desacyl asebotoxin VII, is effective only when applied internally, strongly suggests that the receptor for grayanotoxin does not exist on the external surface of the membrane. The linear relationship between the concentration of [^{3}H]α-H_2-GTX II in the external medium and the count in the effluent from the perfused axon indicates that GTX II diffuses through the cell membrane's lipid phase and reaches the site of action only approached from the internal medium.

INTRODUCTION

Since the breakthrough of Noda et al. in elucidating the primary structure of the Na channel of *Electrophorus electricus* by cloning and sequencing complementary DNA (12), the focus of attention has shifted to unraveling the tertiary structure of the Na channel (1, 2, 6). Among the powerful tools available for studying the functional structure of the Na channel are biological toxins, which specifically act on the channel. For example, α-dihydro-grayanotoxin II (α-H_2-GTX II), batrachotoxin (BTX), veratridine, and aconitine bind to the Na channel and increase the membrane's permeability to Na ions by shifting both activation and inactivation kinetics in the hyperpolarizing direction (5, 7, 14, 15, 16). At an advanced stage of intoxication, the modified Na channel opens with much larger time constants and at more hyperpolarized membrane potentials than normal channels. This feature is particularly useful in confirming the proper incorporation of the isolated Na channels into the artificial membranes (4, 13, 17). Use of a specific toxin as a pharmacological tool for elucidating the tertiary structure of the Na channel requires an exact determination of the site of action of the toxin. By using both internal perfusion and tracer methods, the present experiments have clarified that a site of action for Na channel toxin is accessible from the internal membrane surface.

MATERIALS AND METHODS

Giant axons isolated from either *Loligo edulis* or *Sepioteuthis lessoniana* were internally perfused by the roller method of Narahashi and Anderson (10). The axons were mounted in a Plexiglas chamber, and perfused externally with chilled artificial seawater (ASW) and internally with standard internal solutoin (SIS). ASW had the following compositions (in mM): Na^+, 450; K^+, 10; Ca^{2+}, 50; Cl^-, 590; and Tris buffer 30. Temperature-corrected pH was adjusted to 8.0 SIS contained (in mM): Na^+, 50; K^+, 350; glutamate 320; F^-, 50; sucrose, 333; and phosphate buffer, 15; pH was adjusted to 7.3. A glass capillary electrode filled with 0.6 M KCl was inserted longitudinally into the axon for monitoring the membrane potential. In order to obtain a steady flow of perfusate, the application of constant hydrostatic pressure to the SIS reservoir was achieved by virtue of mariotte's bottle. All experiments were conducted at 5°C. Batrachotoxin was a generous gift from Dr. J. W. Daly, National Institutes of Health, Bethesda, MD. Aconitine was kindly supplied by Professor Sakai, School of Pharmacy, Chiba University, Japan. Veratridine was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Tritium-labeled α-H_2-GTX II (3) was prepared as follows. Tritium labeling of α-H_2-GTX II was performed in a completely sealed glass manifold. A mixture of 15.8 mg (0.045 mmol) of grayanotoxin II and 35 mg of 5% palladium charcoal catalyst in 1.2 ml of tetrahydrofuran was stirred vigorously under 30 mCi (0.13 ml) of tritium and 2.37 ml of hydrogen for 30 min.

Unreacted tritium and hydrogen were absorbed by active carbon granules under cooling. After opening the manifold, the crystals obtained were dissolved again in a few milliliters of methanol, and the solution was evaporated to remove residual unreacted tritium.

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The yield of the crystals was 11.7 mg, and total radioactivity was 1.9 mCi. From thin layer chromatography (TLC), more than 90% of the crystals were α-H_2-GTX II, one of the most potent grayanotoxins (8), and a small amount of grayanotoxin II remained unlabeled. The specific activity of the crystals was 57 mCi/mmol, and the radiochemical purity of [^{3}H]α-H_2-GTX II was ~95% from TLC-radio scanner.

The biological activity of [^{3}H]α-H_2-GTX II was tested on frog skeletal muscle fibers. Since [^{3}H]α-H_2-GTX II at a concentration of 3.4×10^{-6} M, which is the same as the critical concentration to induce a prolonged depolarization (8), gave rise to a similar phenomenon, [^{3}H]α-H_2-GTX II was considered to be equipotent, as compared with cold α-H_2-GTX II.

[^{14}C(U)]Sucrose (671.0 mCi/mmol) was purchased from New England Nuclear Boston, MA).

RESULTS AND DISCUSSION

External application of four toxins, as described above, caused a marked depolarization of the axonal membrane of the squid. However, as shown in Fig. 1, this was true only in nonperfused axons (internal perfusion off). When the axons were internally perfused with toxin-free solution, much less depolarization occurred. Since the toxins were applied externally in all cases, the difference in the drug action could be explained by supposing that after the toxins pass through the membrane, they may reach a site only accessible from the internal surface of the membrane. In order to confirm this supposition, it was examined whether [^{3}H]GTX II in the external medium can actually penetrate the membrane and appear in the intracellular solution. Fig. 2 *A* clearly demonstrates the uptake of labeled α-H_2-GTX II. The axon was soaked in a medium containing 200 μM [^{3}H]α-H_2-GTX II (bar in Fig. 2 *A* indicates period of application of [^{3}H]α-H_2-GTX II). Samples of the internal perfusate were collected and counted at intervals. Counts of the effluent due to the entry of externally applied [^{3}H]α-H_2-GTX II increased gradually to a steady state in ~10 min. After an initial delay of ~1 min, the membrane also started to depolarize toward its steady-state value of 30 mV. This depolarization is much smaller than that

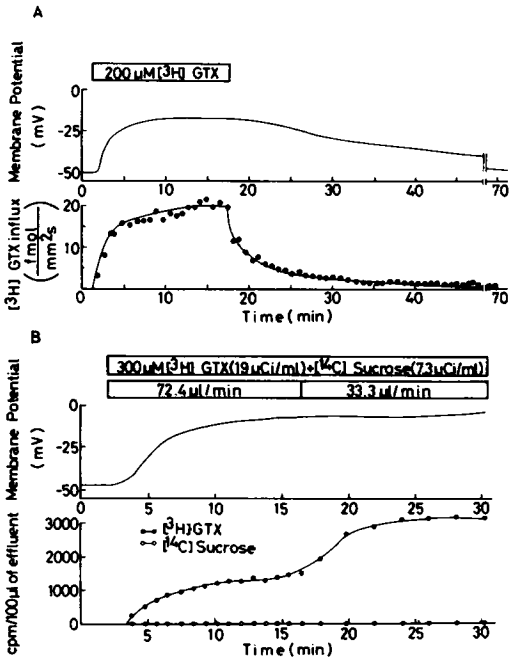

FIGURE 2 (*A*) Time course of [^{3}H]GTX II entry. Bar, external application of [^{3}H]α-H_2-GTX II (200 μM). Membrane of an internally perfused axon (*Loligo edulis*) (*top*). Two drops of perfusate (~80 μl) were collected per point and the tritium in the sample was counted (*bottom*). GTX II influx is expressed as fmol/mm^2 s. (*B*) Time course of the [^{3}H]α-H_2-GTX II entry (●) and sucrose inflow (○) in a perfused fiber exposed to [^{3}H]α-H_2-GTX II (300 μM).

which would have occurred if toxins of the same concentration were directly applied in the internal medium (13). When ASW containing [^{3}H]α-H_2-GTX II was switched to isotope-free ASW, counts in the effluent began to decline rapidly and fell within an hour to a marginal value. A significant retardation of the repolarization of membrane potential suggests that α-H_2-GTX II, which was once

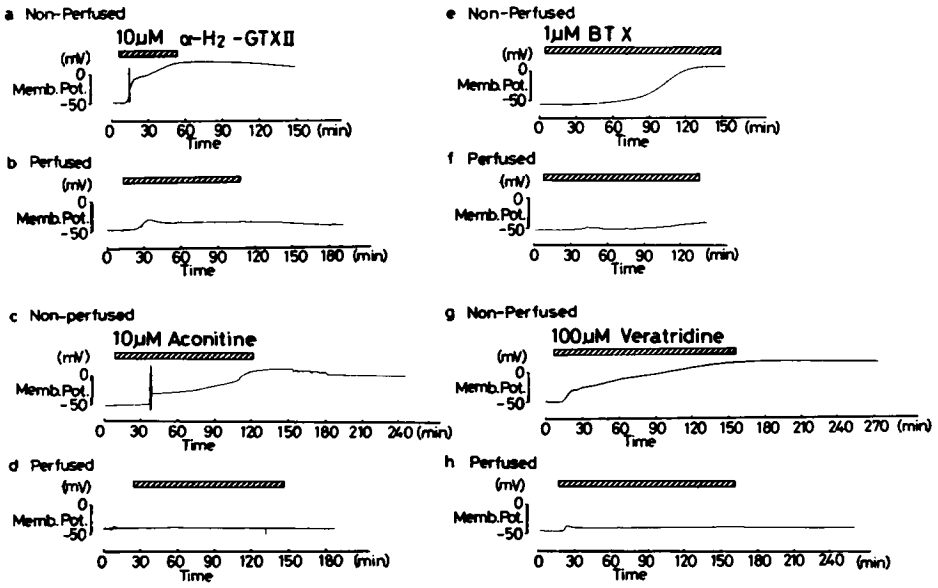

FIGURE 1 Effect of internal perfusion on the degree of depolarization of the axonal membrane caused by each of the four biological toxins. A pair of axons were isolated from *Loligo edulis*. One axon was perfused and the other served as a nonperfused control in each experiment. (*a, b*) α-H_2-GTX II; (*c, d*) aconitine; (*e, f*) BTX; (*g, h*) veratridine. (*a, c, e, g*) Nonperfused fibers. (*b, d, f, h*) Perfused fibers. Changes in membrane potential after application of toxins are depicted. The toxins were applied externally during the period indicated by the hatched area above each graph.

bound to its receptor, is slowly released. The possibility of a leak of [^{3}H]α-H_2-GTX II through the mechanical damage along the axon was discarded based on the following findings. When [^{14}C]Sucrose and [^{3}H]α-H_2-GTX II were simultaneously applied to the external medium, only counts of [^{3}H]α-H_2-GTX II significantly increased in the effluent and those of [^{14}C]Sucrose were in the background range regardless of the rate of internal perfusion (Fig. 2 *B*). Another evidence to support the notion that the site of action is in the internal phase is that 1×10^{-4} M desacyl asebotoxin VII (ATX VII) exerts depolarizing action only when applied internally, indicating that there is no receptor for grayanotoxin on the external surface. It has been reported that this analogue fails to produce a transient depolarization on the frog skeletal muscle despite having the groups essential for biological action (8). Since ATX VII is extremely hydrophilic, it may not be able to permeate through the cell membrane and cannot reach the site of action only accessible from the internal phase.

These data clearly show that α-H_2-GTX II can penetrate through the membrane. It was considered worthwhile to test whether or not α-H_2-GTX II directly passes through the pores of the Na channel. When applied externally, 540 μM [^{3}H]α-H_2-GTX II depolarized the membrane of the internally perfused axon from -56 mV to -21 mV. Whereas tetrodotoxin (TTX; 1×10^{-6}M) repolarized the membrane to the original resting potential (Fig. 3, *top*), counts in the effluent monotonically increased irrespective of the change in the membrane potential (Fig. 3, *bottom*). Since TTX specifically blocks the Na channel from the external surface (9), the route used by α-H_2-GTX II to pass through the membrane is not likely to be the pore of the Na channel. Another possible route of transporting

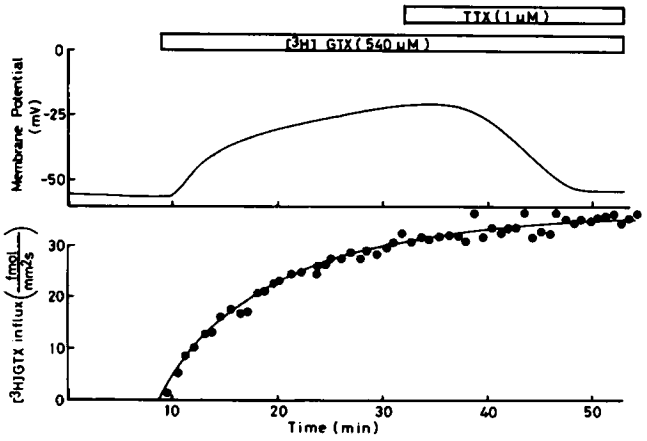

FIGURE 3 Effect of TTX on the penetration of [^{3}H]α-H_2-GTX II through the cell membrane. (*Top*) The transmembrane potential as a function of time in an internally perfused squid axon (*Sepioteuthis lessoniana*). (*Bottom*) The time course of [^{3}H]α-H_2-GTX II appearance in the internal perfusate. Each circle indicates the number of counts from two droplets of perfusate that were collected. Upper bar indicates the time of application of 1 μM TTX, and lower bar, the presence of 540 μM [^{3}H]α-H_2-GTX II in the external medium.

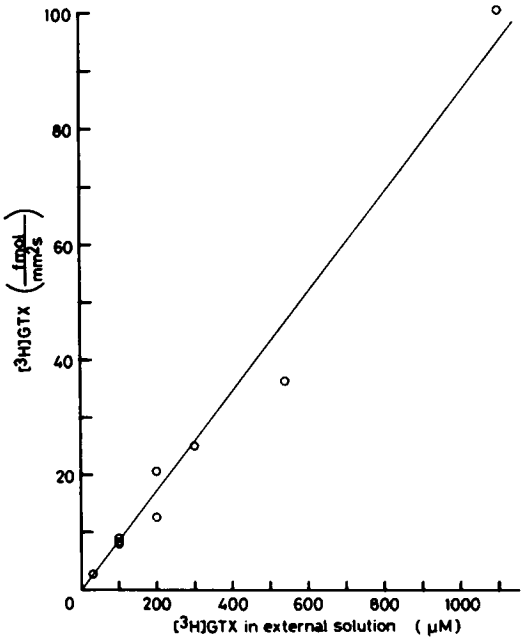

FIGURE 4 Linear relationship between the concentration of [^{3}H]α-H_2-GTX II in the external medium and [^{3}H]α-H_2-GTX II influx. The counts of effluent when transudation reached a steady-state were plotted against the concentration of [^{3}H]α-H_2-GTX II in the external medium. Solid line was obtained by the least squares method ($Y = 0.086X$). Ordinate is the [^{3}H]α-H_2-GTX II influx (fmol/mm^2) and abscissa, the concentration of [^{3}H]α-H_2-GTX II in the external medium (μM).

α-H_2-GTX II is via a carrier-mediated system. This possibility is also in doubt by virtue of the following three experiments. (*a*) When the uptake of α-H_2-GTX II reached a steady state in the presence of 100 μM [^{3}H]α-H_2-GTX II in the external medium, an excess of unlabeled α-H_2-GTX II (1 mM) was applied externally. Although the membrane depolarized further, uptake of tritium-labeled α-H_2-GTX II remained constant. If there was a specific carrier for α-H_2-GTX II, one should have observed a reduction in uptake of [^{3}H]α-H_2-GTX II (due to its displacement from a specific binding site, the *cis*-effect), instead of a constant uptake of [^{3}H]α-H_2-GTX II. (*b*) It was also determined whether labeled α-H_2-GTX II can be displaced from an internal site (*trans*-effect). To do this, the concentration gradient of α-H_2-GTX II was reversed, i.e., its concentration in the intracellular phase was made three times higher than that in the extracellular phase where [^{3}H]α-H_2-GTX II was applied. Counts of the perfusate in this case were about the same as those in the simple application of [^{3}H]α-H_2-GTX II of the same external concentration, indicating that a *trans*-effect is not observable in this condition. (*c*) Counts of [^{3}H]α-H_2-GTX II in the perfusate were linearly proportional to the concentration of [^{3}H]α-H_2-GTX II in the external medium in the range from 30 μM to 1.1 mM (Fig. 4). It is suggested that transport of α-H_2-GTX II through the membrane is nonsaturable and hence is consistent with simple diffusion. These observations do not completely preclude the possible

involvement of a carrier-mediated transport system but favor the notion that α-H_2-GTX II diffuses through the cell membrane. In considering the nonpolar nature of α-H_2-GTX II, it is reasonable to speculate that the route α-H_2-GTX II takes in permeating the cell membrane is via the lipid phase. This speculation is also supported by the fact that the hydrophilic grayanotoxin derivative, grayanotoxin III (GTX III), is much less effective in producing depolarization when externally applied than when internally applied (8, 11). Since GTX III in the external medium tends to stay in the aqueous phase, it is difficult for GTX III to accumulate in the intracellular phase. The observation that the more hydrophobic α-H_2-GTX II is equally potent regardless of the side of the drug application further reinforces this reasoning. In view of the qualitative similarly of the physiological actions of four biological toxins on the axonal membrane, it may be reasonable to extend the rationale of α-H_2-GTX II to other toxins.

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