Covalent Labeling of the Tetrodotoxin Receptor in Excitable Membranes

Abstract. *A photoaffinity labeling technique is described by which a tetrodotoxin analog is covalently bound to receptor sites associated with the sodium pores of excitable membranes. The biological activity of the toxin analog is retained after the covalent binding reaction.*

Interest in the interaction of tetrodotoxin with excitable membranes resides principally in the toxin's selective action on transient sodium conductance (*1, 2*). This specificity of action, expressed at nanomolar concentrations, has raised the possibility of using tetrodotoxin in the identification and characterization of membrane sodium channels (*3*). Specific tetrodotoxin binding components have, in fact, been isolated and partially characterized from garfish olfactory nerves with the aid of tritiated tetrodotoxin (*4, 5*). Further attempts have been made to solubilize these components with sodium cholate and Triton X-100 (*5, 6*). The binding of tetrodotoxin to these "solubilized" preparations occurs rapidly and reversibly to components with a molecular weight of about 500,000 (*6*). The partial inhibition of tetrodotoxin binding by treatment with trypsin, α-chymotrypsin, and pronase has been considered suggestive evidence of a tetrodotoxin-protein interaction. Enhancement of this inhibition of binding by prior treatment with phospholipase suggests that the binding sites are embedded in a phospholipid environment (*4*).

While it is reasonable to assume from the binding data that the solubilized component responsible for tetrodotoxin binding is closely associated with the sodium channels of the excitable membrane, the reversibility of the tetrodotoxin-receptor interaction raises the possibility of secondary nonspecific interactions. This would be especially true during further attempts to resolve the isolated binding component (or components) with sodium cholate or Triton X-100 treatment, where substantial mixing of the membrane components would be expected. Work with artificial lipid membranes has suggested an appreciable interaction of tetrodotoxin with cholesterol (*7*). While the full significance of this observation must await further investigation, it has been suggested that an interaction with cholesterol may represent one component responsible for the nonspecific binding of tetrodotoxin known to occur in intact nerve (*8*).

In view of the reversibility of tetrodotoxin binding and possible secondary interactions with other membrane components during attempts at resolution, further indications of the actual constituents at the tetrodotoxin receptor site can be gained only by indirect means (*9*) or by a change in experimental methodology. A powerful tool for specific labeling of the receptor sites for a particular ligand (such as tetrodotoxin) is that of affinity labeling (*10*). In the general method of affinity labeling, it is necessary to obtain the highest possible specificity of reaction with the desired binding site. The most effective ligand might therefore be expected to be the natural substance into which has been incorporated a highly reactive group; this group is then responsible for formation of a covalent bond to the receptor site after the initial, normal, ligand-receptor interaction.

In contrast to the necessary specificity of the binding reaction, the subsequent covalent bonding reaction should be nonselective, reacting with the receptor site whatever its chemical constitution. However, most of the potential chemical modifications of ligands do not ensure that the added group will be sufficiently active to ensure reactivity following binding but not so active that bonding occurs before receptor-ligand formation. Many affinity analogs have had the disadvantage of either requiring a long period of time for the insertion reaction or bringing about a relatively high percentage of nonspecific labeling (*10*). What is required is assurance that receptor site binding will occur and that, after binding, the reagent will be able to covalently attach itself onto the receptor.

The specialized technique of photoaffinity labeling can readily satisfy these requirements. Westheimer and co-workers (*11*) first showed the feasibility of rapid photochemical generation of carbenes at the active site of enzymes. The rapid generation of a reactive reagent from a stable precursor already situated at the active site of an enzyme was shown to result in covalent labeling of that site. In addition, photoactive nitrene probes are effective means of achieving such covalent insertions (*12*). Carbenes can be generated photochemically from diazo compounds and nitrenes from azides. These are the only known common chemical species capable of inserting directly into C-H bonds. The chemistry of the arylazides makes them particularly effective. Arylazido analogs are potentially effective photoaffinity labels because photolysis results in the generation of a highly reactive arylnitrene, which bonds covalently and rather indiscriminately to amino acid side chains (*13*). The application of carbodiimidazole-catalyzed esterification reactions by Jeng and Guillory (*14*), using arylazidocarboxylic acid derivatives, resulted in the synthesis of arylazido adenine and pyridine nucleotide analogs. These analogs act as substrates for a number of enzymes (*14, 15*). They are competitive inhibitors for these enzymes in the presence of the normal substrate and act as irreversible inhibitors on photolysis. The irreversible inhibition is associated with specific and stoichiometric covalent labeling.

In view of the known chemical structure of tetrodotoxin (*2*) and the successful application of the carbodiimidazole-catalyzed esterification of the ribose hydroxyls of nucleotides (*14*), we attempted the chemical synthesis of arylazido-β-alanine (*16*) analogs of tetrodotoxin. We report here that this method has resulted in the synthesis of biologically active arylazido analogs of tetrodotoxin. Experiments are described which indicate that these analogs act as active site directed reagents for covalent insertion at the tetrodotoxin binding site of excitable membranes. [Hucho *et al.* (*17*) recently developed a different photoaffinity reagent for the investigation of the tetraethylammonium receptors of nerve.]

Arylazido-β-alanine was prepared as described by Jeng and Guillory (*14*) and was coupled to tetrodotoxin (Calbiochem) by the method described for its coupling to adenosine triphosphate, except that the procedure was scaled down 1/100. The aqueous solution containing the reaction products (450 μl) was streaked in a thin band (8 by 0.2 cm) on a thin-layer precoated plastic sheet measuring 20 by 10 cm (Cellulose 254, 0.1 mm thick) and developed in the dark for 4 hours with *n*-butanol, H_2O, and acetic acid (5:3:2). In this solvent, the unreactive tetrodotoxin remained near the origin and the unreactive arylazido-β-alanine moved with the solvent front (R_F 0.94). In addition to the reactants, two reaction products were observed by virtue of the yellow to orange color of the arylazido-β-alanine adjunct: a weak yellow band at R_F 0.61 and a strong orange band at R_F 0.75.

The materials chromatogramming at R_F 0.61 and 0.75 were scraped from the plate and extracted from the solid sup-

port cellulose with a total of 1 cm^3 of H_2O. These aqueous extracts were then separated from the solid cellulose support by filtration over a small Whatman 2.4-cm glass paper filter. We will designate the R_F 0.75 product as tetrodotoxin-arylazido-β-alanine$_1$ (TTX-AβA$_1$), and that with R_F 0.61 as TTX-AβA$_2$. Both products had an ultraviolet absorption band characteristic of arylazido-β-alanine alone. The concentration of each substance was estimated by using the molar extinction coefficient 27.2×10^3 at 260 nm (*14*).

The biological activity of TTX may be assayed in a wide variety of electrically excitable membrane systems (*2*). We chose to utilize an amphibian skeletal muscle preparation since the density and accessibility of the saturable TTX binding sites (*18*) and the electrical properties of this membrane (*19*) have recently been extensively studied. Earlier observations showed that exposure to Ca-free media can produce a readily reversible Na-dependent, TTX-sensitive depolarization in both crayfish (*20*) and amphibian (*21*) muscle. The biological activity of the TTX analogs was therefore assessed by their ability to prevent this TTX-sensitive depolarization after exposure to Ca-free media.

Figure 1A shows a typical control experiment utilizing the iliofibularis muscle of the toad *Bufo marinus*. The muscle was immersed initially in Ringer solution (*22*). The membrane potentials of ten individual fast skeletal muscle fibers were sampled for every data point shown, by using standard electrophysiological techniques. The Ringer solution was then replaced at intervals by a Ca-free Ringer solution (*23*) containing different TTX concentrations (3×10^{-7}, 7×10^{-7}, and $1 \times 10^{-6} M$). In comparison with the substantial depolarization that occurs when the muscle is placed in Ca-free Ringer in the absence of TTX (Fig. 1A, panel b), only a small depolarization occurs in the presence of TTX (Fig. 1A, panels d, f, and h). Since this component was not affected by a threefold increase in TTX concentration, and occurred at concentrations substantially greater than those found to be sufficient to saturate the specific TTX receptors (*24*), we conclude that a small, readily reversible, TTX-insensitive depolarization occurs under these conditions.

Figure 1B shows a comparable experiment with TTX-AβA$_1$. This analog, at an estimated final concentration of $8.5 \times 10^{-7} M$, produces an inhibition of depolarization similar to that observed with unmodified TTX (compare Fig. 1A, panel b and Fig. 1B, panel b). In comparison with control experiments involving a similar duration of exposure with unmodified TTX, washout of TTX-AβA$_1$ was more complete (Fig. 1B, panel d), suggesting some minor steric hindrance of receptor-ligand binding associated with the presence on the TTX molecule of the photoaffinity label. This effect is also noticeable when comparing Fig. 1A, panel d and Fig. 1B, panel b; in this case TTX-AβA$_1$ was slightly less effective than unmodified TTX at one-third the concentration. The analog was then reintroduced (Fig. 1B, panel f) and the muscle preparation photoirradiated (*25*) at a sufficient intensity (*14*) to generate the highly reactive arylnitrene from the reactive azido group. Subsequent exposure to a Ca-free medium (Fig. 1B, panel h) resulted in no detectable loss of TTX activity (compare Fig. 1B, panel b) despite repeated washings in the Ca-free medium. It should be noted that in both the nonirradiated and the irradiated sequence, the TTX analog was in contact with the membrane for the same period of time (25 minutes). Control experiments, with and without photoirradiation, showed that arylazido-β-alanine alone was without TTX-like activity (*26*).

We conclude that photoirradiation results in an apparently irreversible binding of TTX-AβA$_1$ to biologically active membrane receptors. The chemistry of the arylazido group (*13*) can explain the irreversibility of the TTX-like effect as being due to covalent binding of the TTX analog to these receptor sites.

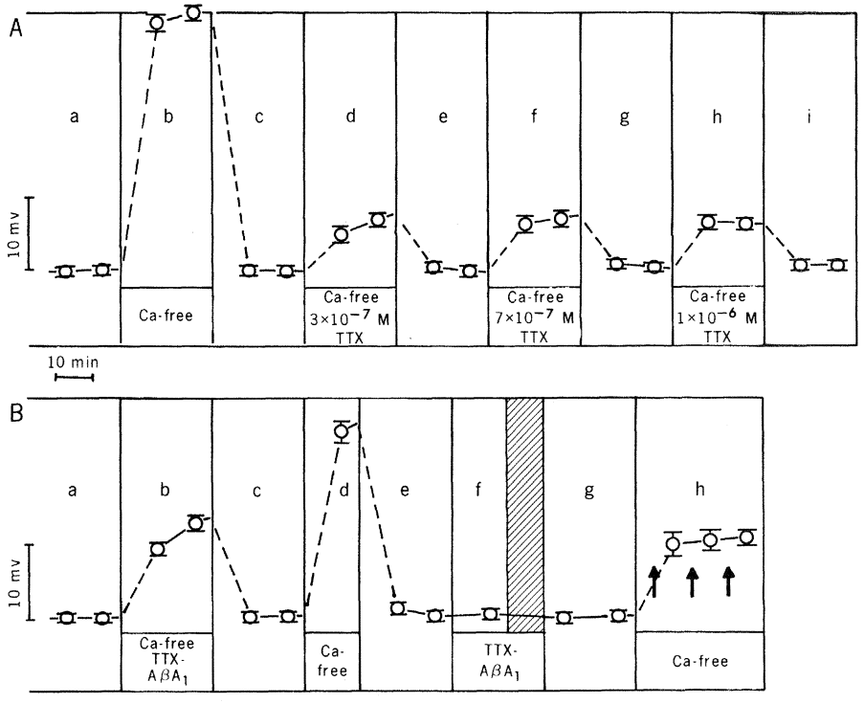

Fig. 1. (A) Mean membrane potential (± standard error of the mean, $N = 10$) from muscle fibers of a *Bufo marinus* iliofibularis muscle. The muscle was immersed in a normal Ringer solution during the periods represented by panels a, c, e, g, and i; it was exposed to Ca-free medium without TTX in panel b and with TTX (at the concentrations shown) in panels d, f, and h. The initial resting membrane potential was 88.9 ± 0.41 mv in this muscle. (B) A similar experiment with the photoaffinity-labeled tetrodotoxin analog TTX-AβA$_1$. The muscle was immersed in control Ringer solution in panels a, c, e, and g, and in the Ca-free medium in panels d and h. The TTX-AβA$_1$ was added in panels b and f. The muscle preparation was photoirradiated (*24*) in panel f (shaded region). Three complete solution changes were made at each of the arrows in panel h. The initial resting membrane potential was 91.1 ± 0.55 mv in this muscle.

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16. This is 3-[*N*-(4-azido-2-nitrophenyl)] aminopropionic acid.
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22. The composition of the Ringer solution was 120 m*M* NaCl, 2.5 m*M* $KHCO_3$, and 2 m*M* $CaCl_2$, adjusted to *p*H 7.6.
23. The composition of the Ca-free Ringer solution was as given in (*22*) except that 1 m*M* EDTA was used in place of the $CaCl_2$. The muscle bath was flushed three times at each solution change.
24. It is necessary to show not only that the initial concentration of TTX is sufficient to saturate these receptor sites, but also that uptake of TTX by these high-affinity receptors does not significantly alter the initial TTX concentration. The total binding (specific plus nonspecific) reported by Almers and Levinson (*18*) indicates that a maximum 5 percent concentration change would occur under the experimental conditions of this study.
25. The preparation was photoirradiated for five 1-minute periods, at 1-minute intervals, to avoid heating the muscle tissue. Two tungsten halogen projector lamps (650 watts, DVY, 3400°K) were situated 10 cm to either side of the preparation bath but slightly above the fluid level to ensure direct illumination of the submerged muscle. Cooling was supplied as necessary to maintain the bathing medium at room temperature (22°C).
26. Although TTX-AβA$_2$ also showed TTX activity, the activity was not maintained when the preparation was washed after photoirradiation. It seems reasonable to suppose that in this analog the stereochemical positioning of the arylazido photoactive ligand on the TTX molecule prevented its covalent insertion during photoirradiation.
27. We thank S. J. Jeng for her helpful assistance in the preparation of the arylazido-β-alanine tetrodotoxin analogs and T. Kosaki for his assistance in the measurements of muscle membrane potential. Supported in part by grants from the American Heart Association (R.H.G.) and by NSF grants PCM76-02974 (R.J.G.) and BNS76-02647 (J.S.D.).

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Allelopathic Influence on Blue-Green Bloom Sequence in a Eutrophic Lake

Abstract. *The bloom sequence in a eutrophic lake, Linsley Pond, over a period of 3 years is correlated to the effects of cell-free filtrates of dominant blue-green algae on both their successors and their predecessors. There is unbroken correspondence between the effects of heat-labile probiotic and antibiotic filtrates and the rise and fall of bloom populations in situ. All organisms in vitro were axenic or unialgal (bacterized) isolates from Linsley Pond.*

When for fiscal or logistic reasons excess nutrients cannot be eliminated from freshwater lakes, certain species of phytoplankton grow to excess. Such species might be better controlled if we could obtain a clearer understanding of bloom sequence; that is, of which phytoplankter will grow to excess, and when.

Bloom sequence in eutrophic freshwater lakes is the overt expression of multiple, interacting factors, and any phytoplankter which produces a bloom population is one which is momentarily most favored by the summation of the effects of these factors. Such effects function as either coarse or fine adjustors of the natural bloom sequence. Coarse adjustors, such as light (*1*) and macronutrients (*2*), determine the production capacity of the system and, to a degree, the major categories of dominant organisms; for example, diatom blooms reflect silicon availability (*3*). In contrast, fine adjustors, such as micronutrients (*4*) and variations in temperature patterns (*5*), determine the specific organisms of dominance. No fine adjustor, however, has been identified which accounts for bloom sequence. Rather, the best examples presented account for single instances of dominance (*6*).

Since the early 1900's (*7*), the effects of extracellular metabolites (allelopathy, probiosis, and antibiosis) have been suggested as playing a major role in sequence determination, and decades of speculation and investigation have followed (*8*). There are many reports of interactions occurring between growing organisms in vitro (*9*). Such interactions are frequently attributed to the effects of extracellular metabolites. Although many of the reported effects can be assigned to direct competition for nutrients or to other nonmetabolite influences, several authors have presented evidence that algal metabolites are both suitable in quality and sufficient in quantity to account for reported interactions (*6, 10*). Yet, extrapolation of such data from experiments in vitro to explanations of dominance in situ is universally challenged. The most severe criticisms concern either the differences in the dilutions of active metabolites in vitro and in situ, or the relationships of study organisms to each other or to any natural community. The advisability of extrapolating quantitative information is specifically questioned because population densities in vitro have far exceeded those in situ. It has been suggested that in a lake such factors as *p*H or nutrients could totally mask an allelopathic response observed in vitro only because of the increased concentration of the toxic plant product under laboratory conditions (*11*). Qualitative extrapolation is similarly challenged because (i) species are used which do not occur together in nature (organisms in tests are isolated from different locales, often from a convenient culture collection), and (ii) bacterial activity is not taken into account.

In consideration of these criticisms certain procedures were established for this study. All organisms were isolated from the natural community of a single, eutrophied lake—Linsley Pond, North Branford, Connecticut. Population densities in metabolite-producing (producer) cultures (*12*) were monitored and were found similar to those of epilimnetic blooms in Linsley. This similarity reflects both the high population densities in Linsley and the composition of growth media. The basic growth medium in producer cultures consisted of equal volumes of separately aged and charcoal-treated water samples obtained from Linsley before and after the fall turnover (*13*). Macronutrient additions (*14*), treatment of the cultures (*15*), and inocula for producer and test cultures were identical, and all *p*H values were within a range of 0.1 unit for any given set of tests (*16*) and control (*17*) cultures. The only difference between treatments of test and control cultures was the autoclaving of control cultures prior to enrichment, *p*H check, and inoculation with bioassay organisms.

Although examples of heat-stable allelopathic effects were noted, only heat-labile effects are included in this report (*18*). For *Anabaena holsaticum* the heat-labile substance was removed from filtrates by ultrafiltration, by dialysis, and by ether extraction. In each instance the substance was active when returned to bioassay cultures at its natural concentration, or at two or five times the natural concentrations. Cultures diluted to 0.5 times also retained activity.

Table 1 shows a summary of the probiotic and antibiotic effects of axenic, cell-free filtrates of each bloom-dominant phytoplankter on organisms which bloomed before or after it. Filtrates of each dominant species produced only negative, or neutral, effects on its imme-