A Cardiac Tetrodotoxin Binding Component: Biochemical Identification, Characterization, and Properties†

Alain Lombet, Jean-François Renaud, Robert Chicheportiche, and Michel Lazdunski*

ABSTRACT: Saturable, high-affinity binding of tritiated ethylenediamine–tetrodotoxin ([^{3}H]en-TTX) was identified in rat heart membranes. Specific [^{3}H]en-TTX binding was linear with tissue concentrations up to 1.5 mg of protein per mL. The saturation isotherms showed a single population of saturable high-affinity tetrodotoxin receptors in cardiac homogenates with an equilibrium dissociation constant (K_D) from 0.5 nM to 1.0 nM and a maximal binding capacity of 185 ± 25 fmol/mg of protein. A microsomal fraction prepared from rat heart homogenate was enriched 3.4-fold in maximal binding capacity for [^{3}H]en-TTX. Rat and guinea pig heart homogenates had a maximal binding capacity of about 175 fmol/mg of protein while the comparable values for chick embryo and rabbit hearts homogenates were 60–70 fmol/mg of protein. Innervated chick embryonic hearts and noninnervated chick embryonic heart cells in culture have very similar properties of association with [^{3}H]en-TTX (K_D and maximal binding capacity values). The density of the tetrodotoxin binding component was 2–4.5-fold higher in atrial tissue than in ventricular tissue. The rate constant of dissociation of the complex toxin receptor was 8.4×10^{-2} min^{-1} at 4 °C ($t_{1/2}$ = 8.25 min) with an activation energy of the dissociation process of 23 kcal mol^{-1}. Half-inhibition of [^{3}H]-en-TTX binding was obtained at pH 6.2. Tetrodotoxin and saxitoxin specifically displaced [^{3}H]en-TTX binding, with K_D values of 2.0 nM and 3.4 nM, respectively. Antiarrhythmic agents, local anesthetics and amiloride at concentrations up to 1 mM were without effect on the binding of this TTX derivative. Monovalent cations selectively displaced [^{3}H]en-TTX binding according to the sequence $Tl^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. The displacement of [^{3}H]en-TTX binding by monovalent ions was cooperative ($1.6 < n_H < 2.3$). Although divalent cations also displaced [^{3}H]en-TTX binding in the sequence $Mg^{2+} > Ca^{2+}$, they did not show any cooperativity in this displacement.

The action potential generated by excitable membranes requires rapid changes of sodium, potassium, and eventually calcium conductances. Among the voltage-dependent Na^+, K^+, and Ca^{2+} channels, only the Na^+ channel is accessible up till now to a biochemical approach. The reason is that this channel has a particularly rich pharmacology. Tetrodotoxin and saxitoxin (Narahashi, 1974; Ritchie & Rogart, 1977) block Na^+ entry through the transiently open form of the Na^+ channel, and many other toxic compounds such as batrachotoxin, veratridine, aconitine, grayanotoxin, pyrethroids, and polypeptide toxins extracted from scorpion venom and sea anemone (Albuquerque & Daly, 1977; Ulbricht, 1969; Herzog et al., 1964; Seyama & Narahashi, 1973; Jacques et al., 1980b; Koppenhöffer & Schmidt, 1968; Romey et al., 1976) modify the permeability characteristics to Na^+ ions by acting at the level of the gating system, i.e., by changing the kinetics of the voltage-dependent opening and closing process.

Tetrodotoxin and saxitoxin are the most widely used toxic compounds to study Na^+ channels in cell-excitable membranes including cardiac tissue (Hagiwara & Nakajima, 1966; Sperelakis, 1980). Preparations of tritiated saxitoxin and of tritiated tetrodotoxin derivatives at a high specific radioactivity have been recently described (Ritchie et al., 1976; Chicheportiche et al., 1980). These molecules have proven to be useful tools to study the molecular properties of the Na^+ channel in nerve and muscle (Ritchie & Rogart, 1977; Chicheportiche et al., 1980).

Although numerous electrophysiological studies have been carried out to analyze the properties of the Na^+ channel in cardiac cells [see, for reviews: Narahashi (1974), Sperelakis (1980), and Bernard (1975)], no biochemical work has been published up till now to describe the properties of this ionic channel in cardiac membranes. This paper describes the biochemical properties of interaction of a stable and highly radioactive tetrodotoxin derivative, [^{3}H]en-TTX, synthesized in this laboratory, to the Na^+ channel of cardiac cells of different animal origins. The very high specific radioactivity of this TTX derivative permits an analysis of the properties of the TTX receptor in excitable tissues which, like the cardiac cell, have a low density of Na^+ channels.

Materials and Methods

Tissue Preparation. Male Sprague-Dawley rats (150–300 g), male guinea pigs (300–500 g), and female F1 Blanc de Bouska X New Zealand rabbits (1.9–2.5 kg) were killed and their hearts were quickly taken out. They were washed in ice-cold oxygenated saline solution containing 115 mM Na^+, 5.4 mM K^+, 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, 5 mM glucose, and 25 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonate) adjusted to pH 7.4 with NaOH, until all the blood was removed. Connective tissue, large vessels, and fat were trimmed and removed. Dissection of the rat heart for regional distribution was performed in the saline medium continuously oxygenated and maintained at 4 °C. The right and left atria including floor were removes separately. The free wall of the right ventricle was incised and removed, leaving the intraventricular septum and the left ventricle intact. Finally the intraventricular septum was dissected from the wall of the left ventricle. In all other experiments, only the ventricles containing the intraventricular septum were used to obtain homogenates and microsomal fractions.

Hearts were minced with scissors, rinsed once with an ice-cold 20 mM Tris buffer containing 0.25 M sucrose and 1 mM EDTA at pH 7.5 (TSE buffer), mixed with 10 volumes

† From the Centre de Biochimie du C.N.R.S., Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France. *Received July 18, 1980.* This work was supported by the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique (ACC No. 76.7.0732), the Fondation pour la Recherche Médicale, and the Commissariat à l'Energie Atomique.

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of TSE buffer, and homogenized at setting 5 with a polytron apparatus (Brinkman Instruments) by using three 5-s bursts separated by 30-s pauses. The heart homogenates were filtered through four layers of cheesecloth and utilized directly for [^{3}H]en-TTX[1] binding assays. Heart homogenates were also used for the preparation of cardiac microsomal fractions (P_3) according to Paris et al. (1977). The preparation used in this study was obtained from 70 rat hearts which yielded 260 mg of microsomal protein. P_3 pellets were diluted to 32 mL with a 20 mM Tris buffer at pH 7.5 containing 0.25 M sucrose without EDTA. This preparation was divided into 1-mL fractions which were frozen and stored in liquid nitrogen until they were used.

Intact hearts from 11-day-old chick embryo were placed in the previously described saline solution. The solution containing the hearts was continuously oxygenated and maintained at 4 °C until the ventricles were minced and homogenized as described for rat hearts. Then the homogenized fraction was used directly for [^{3}H]en-TTX binding.

Heart cells from chick embryos were cultured in the form of monolayers according to Renaud (1980). Monolayers were used 2–3 days after plating, rinsed with the ice-cold TSE buffer, scraped, homogenized in the same buffer, and assayed for binding with [^{3}H]en-TTX.

Protein content was determined by the method of Hartree (1972) using bovine serum albumin as standard.

K^+-PNPase activities were measured according to Gache et al. (1976).

Binding Assays. Binding assays are carried out as follows: 0.2–1.0 mg of protein of cardiac homogenates are equilibrated in 1 mL of the standard incubation medium (20 mM Tris buffer containing 50 mM choline chloride at pH 7.5) in the absence (total binding) or in the presence (nonspecific binding) of 5 μM TTX. After 20 min at 4 °C, each incubation is stopped by filtering (less than 10 s) in duplicate two aliquots of 0.4 mL through prewetted GF/B glass fiber filters (Whatman) positioned over a vacuum Millipore filter flask apparatus. Filters are rinsed twice with 5 mL of an ice-cold 20 mM Tris buffer containing 200 mM choline chloride at pH 7.5. Aliquots of 0.1 mL are taken in parallel to determine the total radioligand concentration. [^{3}H]en-TTX which remained bound to cardiac membranes on the filter is extracted by vigourous shaking with 8 mL of Picofluor 30 (Packard) as scintillator. Free ligand concentration was obtained by subtracting the amount of [^{3}H]en-TTX bound to cardiac membranes from the total radioligand concentration. Specific [^{3}H]en-TTX binding was determined from the difference between the radioactivity determined in the absence and in the presence of 5 μM native TTX as described above. In experiments with the microsomal pellet P_3, the incubation medium (1 mL) was 20 mM Tris buffer containing 200 mM choline chloride at pH 7.5.

Binding experiments in physiological conditions were carried out in a Ringer solution at pH 7.4 containing 20 mM Tris-Cl, 140 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM glucose.

In all competition experiments, the molecule or ion to be tested as a possible competitor of [^{3}H]en-TTX was present in the incubation medium before the addition of the labeled ligand. When working with increasing concentrations of cations the ionic strength was maintained constant by adjusting the choline chloride concentration. Experiments with Tl^+, Li^+, Na^+, NH_4^+, guanidinium, Mg^{2+}, and Ca^{2+} were made at μ = 220 mM; experiments with K^+, Rb^+, and Cs^+ reached higher ionic strength (μ = 620 mM) to obtain correct displacement curves with these cations.

K_D values were determined in each experimental conditions of ionic strength and used to calculate appropriate inhibition constants. The buffer used to rinse the filters in that case was 20 mM Tris and 600 mM choline chloride at pH 7.5.

Dissociation of the Complex [^{3}H]en-TTX–Receptor. The dissociation was started by addition of 5 μL of 1 mM TTX per mL of incubation medium. For measurement of kinetic properties, dissociation was stopped at different times by quick filtration of 0.4 mL of the medium under the conditions previously described for equilibrium binding measurements. Rate constants of dissociation, k_{-1}, were calculated for various temperatures as follows:

$$k_{-1} = \frac{1}{t} \ln \frac{[\text{R-[}^3\text{H]en-TTX]}_e}{[\text{R-[}^3\text{H]en-TTX]}_t} \quad (1)$$

where R represents the tetrodotoxin binding component, $[\text{R-[}^3\text{H]en-TTX]}_e$ the concentration of complex formed at equilibrium before addition of unlabeled TTX (5 μM), and $[\text{R-[}^3\text{H]en-TTX]}_t$ the concentration of the toxin–receptor complex at time t during the dissociation. Half-lives were determined from eq 2.

$$t_{1/2} = \frac{\ln 2}{k_{-1}} \quad (2)$$

Analysis of Data. [^{3}H]en-TTX binding data (direct binding and competition experiments) were treated according to Atkins (1973), using a Wang 2200 calculator, to fit experimental points with theoretical curves. This fitting procedure gave Hill coefficients, maximal binding capacities, and K_D and IC_{50} values. The ability of a molecule or a cation to inhibit specific [^{3}H]en-TTX binding to its receptor was characterized by a IC_{50} value, i.e., the concentration of the compound which inhibits binding by 50% under the particular experimental conditions. The overall apparent dissociation constant $K_{0.5}$ of the inhibition for [^{3}H]en-TTX binding was calculated from IC_{50} (eq 3, where n_H is the Hill coefficient, $[\text{[}^3\text{H]en-TTX]}_f$

$$(K_{0.5})^{n_H} = \frac{(IC_{50})^{n_H}}{1 + \dfrac{[\text{[}^3\text{H]en-TTX]}_f}{K_D}} \quad (3)$$

the free concentration of radioligand at half-dissociation, and K_D the equilibrium dissociation constant for [^{3}H]en-TTX binding determined in Tris buffer (20 mM) at pH 7.5 and choline chloride at the appropriate concentration under the particular experimental conditions previously described).

Chemicals and Drugs. [^{3}H]en-TTX was synthesized according to Chicheportiche et al. (1980). Its specific radioactivity was determined by using the different methods previously described (Chicheportiche et al., 1980). This preparation of [^{3}H]en-TTX had a specific radioactivity of 27 Ci/mmol and a radiochemical purity of 90%.

Tetrodotoxin was obtained citrate-free from Sankyo Chemical Co. (Tokyo). Saxitoxin was obtained from the Food and Drug Administration (Washington D.C.). The chloride salts of all cations were of the highest purity commercially available. Experiments using Tl^+ were carried out with nitrate instead of chloride as anion.

Tetracaine and lidocaine were purchased from Sigma, procaine from Serva, D_{600} and verapamil from Knoll Pharmaceuticals, and nifedipine (Bay A 1040) from Bayer; ami-

[1] Abbreviations used: [^{3}H]en-TTX, tritiated ethylenediamine–tetrodotoxin; TTX, tetrodotoxin; STX, saxitoxin; [^{3}H]TTX, tritiated tetrodotoxin; [^{3}H]STX, tritiated saxitoxin.

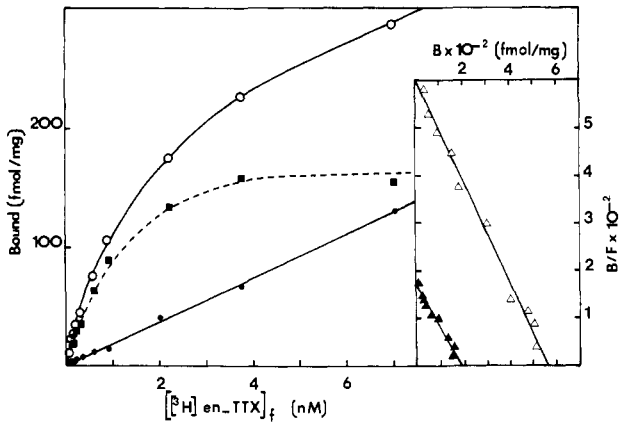

FIGURE 1: Binding of [^{3}H]en-TTX to rat heart homogenates. Protein concentration was 1.0 mg/mL. Total [^{3}H]en-TTX binding (○) and nonspecific binding (●) were made in parallel in the absence and presence of 5 μM TTX. Specific binding (■) is reported by dotted lines, calculated from total and nonspecific binding. In this typical experiment K_D and B_{max} values of 0.8 nM and 203 fmol/mg were obtained respectively. The inset shows a Scatchard plot from the specific [^{3}H]en-TTX binding data. (▲) K_D = 1.1 nM and B_{max} = 190 fmol/mg of protein. The same representation was obtained with microsomal fractions (P_3) of rate heart (△) with K_D = 0.93 nM and B_{max} = 560 fmol/mg of protein. The ordinate is bound over free ligand (mL/g of protein) and the abscissa is bound ligand (fmol/mg of protein).

loride was a gift from Professor Motais (Villefranche/sur/mer, France).

Results and Discussion

[^{3}H]en-TTX–Receptor Complex: Equilibrium Data. The specific binding of [^{3}H]en-TTX to its receptor is proportional to the concentration of cardiac homogenate from a concentration of 0.1–1.5 mg of protein/mL. This proprotionality was found by using different [^{3}H]en-TTX concentrations between 0.1 and 10 nM.

The saturability of [^{3}H]en-TTX binding to cardiac homogenates and microsomal fractions with increasing concentrations of the tritiated ligand is demonstrated in choline chloride medium (Figure 1). Scatchard plots (Scatchard, 1949) of the data gave single straight lines indicating a single population of high-affinity tetrodotoxin receptors. Eight different experiments on different preparations of cardiac homogenates from rat heart gave linear Scatchard plots (correlation coefficient ranging between 0.92 and 0.99) with values of K_D, the dissociation constant of the [^{3}H]en-TTX–receptor complex, between 0.5 and 1.0 nM, and a maximal binding capacity of 185 ± 25 fmol/mg of protein. For microsomal fractions, values obtained with the fitting procedure are K_D = 0.60 ± 0.15 nM at μ = 220 mM and a higher maximal binding capacity of 550 ± 50 fmol/mg of protein. A Scatchard plot of a typical experiment with the microsomal fraction gives a K_D value of 0.93 nM and a B_{max} value of 560 fmol/mg of protein (inset, Figure 1). Table I shows the enrichment factor of [^{3}H]en-TTX binding activity at different stages of preparation of a typical microsomal fraction. This purification follows closely that of the (Na^+,K^+)ATPase activity.

We have looked for the possible presence of low-affinity binding sites for tetrodotoxin using [^{3}H]en-TTX at concentrations ranging from 10^{-8} to 10^{-6} M. Such sites were not found to be present in this preparation.

Tetrodotoxin and saxitoxin displace [^{3}H]en-TTX binding (Figure 2). Dissociation constants were calculated as reported under Materials and Methods by using eq 3. They are 2.0 and 3.4 nM for TTX and STX, respectively. These values are very similar to those reported for nerve and skeletal muscle

Table I: Comparison of [^{3}H]en-TTX Binding with Plasma Membrane Marker during Purification of Heart Membranes[a]

fraction	[^{3}H]en-TTX specific binding (fmol/mg)	ouabain-sensitive (Na^+, K^+)ATPase (nmol min^{-1} mg^{-1})
homogenate H_0	185 (1)	13.7 (1)
1200*g* supernatant S_1	220 (1.2)	16.6 (1.2)
12000*g* supernatant S_2	175 (0.95)	12.6 (0.9)
100000*g* pellet P_3	635 (3.4)	42.5 (3.1)

[a] Purification and K^+PNPase activities were loaded according to Paris et al. (1977). Specific [^{3}H]en-TTX binding was measured as described under Materials and Methods. Purification factors are given in parentheses.

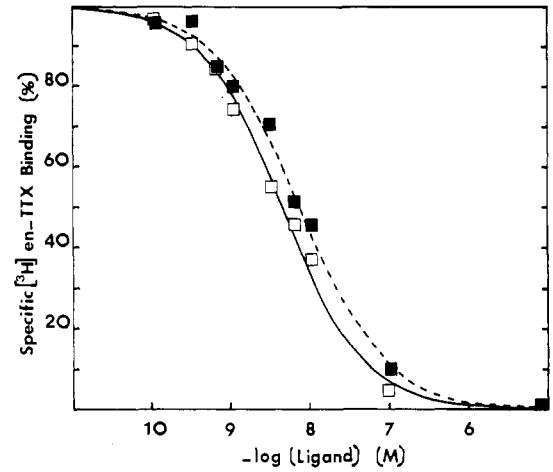

FIGURE 2: Displacement of specific [^{3}H]en-TTX binding in heart homogenates by TTX and STX at 4 °C. Half-displacement was obtained with TTX (□) and STX (■) at 4.4 and 7.3 nM, respectively. Experimental conditions were [[^{3}H]en-TTX] = 1.04 nM and K_D = 0.9 nM determined by direct binding in parallel on the same homogenate. Each point was made in triplicate by filtration of 0.4 mL of incubation medium containing 0.5 mg of protein/mL of homogenate on GF/B filters.

preparations [for reviews, see Narahashi (1974), Ritchie & Rogart (1977), and Barchi & Weigele (1979)].

Properties of Dissociation of [^{3}H]en-TTX from Its Receptor. The displacement of [^{3}H]en-TTX from its receptor by unlabeled tetrodotoxin follows first-order kinetics. This is another indication of the fact that there is a single set of binding sites for the toxin. Pseudo-first-order representations of the kinetic data are presented in Figure 3A for different temperatures between 0 and 20 °C. At 4 °C, for example, the rate constant of dissociation, k_{-1}, is 0.084 min^{-1}, corresponding to the half-life of 8.25 min. A similar value, k_{-1} = 0.1 min^{-1}, was found under the same conditions of pH and temperature by using microsomal membranes. The Arrhenius plot showing the temperature dependence of k_{-1} is linear (Figure 3B). The activation energy calculated from this representation is 23 kcal mol^{-1}. A similar activation energy was previously reported for the dissociation kinetics of the [^{3}H]TTX–receptor complex formed with crab axonal membranes (Balerna et al., 1975).

pH Dependence of [^{3}H]en-TTX Binding. Binding of [^{3}H]en-TTX to its cardiac receptor is pH dependent, being maximum at pH value higher than 7.0 (Figure 4) and completely prevented at pH values lower than 5.5. Half-maximal binding is observed at pH 6.2. Assuming that H^+ and TTX compete for the same ionizable site as was shown previously for other excitable membranes (Narahashi, 1974; Ritchie & Rogart, 1977), one can calculate an inhibition constant of 0.3 μM for H^+ using eq 3, corresponding to pH 6.2. The same

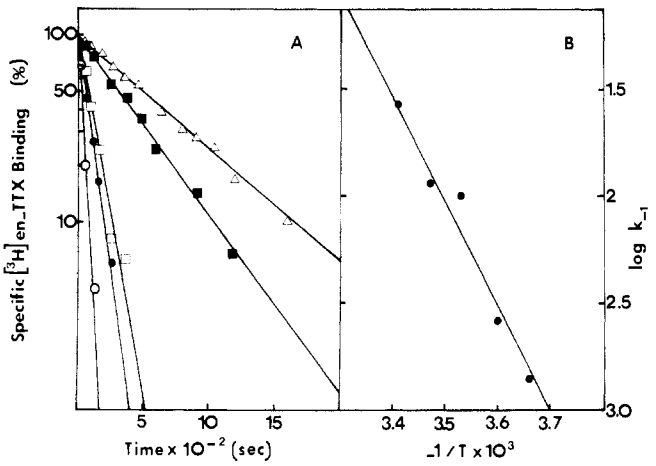

FIGURE 3: Kinetics of [^{3}H]en-TTX–receptor complex dissociation on rat heart homogenates. (A) Rates of dissociation of the specific [^{3}H]en-TTX binding as a function of time. Data are plotted semilogarithmically vs. time. Specific binding was determined as described under Materials and Methods. Rates of dissociation were measured at 0 °C (△), 5 °C (■), 10 °C (□), 15 °C (●), and 20 °C (○). The assay contained 0.5 mg of protein/mL with a [^{3}H]en-TTX concentration of 1.0 nM. (B) Arrhenius representation of the temperature dependence of the rate constant of dissociation k_{-1} of the [^{3}H]en-TTX–cardiac receptor complex. Temperatures varied from 0 to 20 °C at pH 7.5.

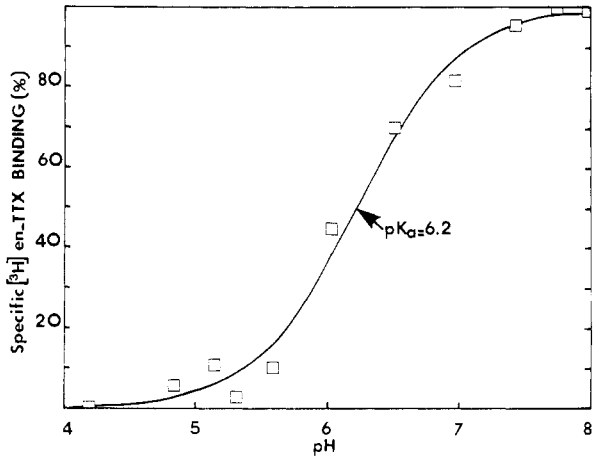

FIGURE 4: pH dependence of [^{3}H]en-TTX binding to rat heart homogenates at 4 °C. Specific binding was measured at 1.0 nM [^{3}H]en-TTX as described in binding assay. Half-maximal binding was obtained at pH 6.2.

type of pH dependence has previously been reported for TTX or STX binding to nerve, skeletal muscle, and electric organ membranes (Colquhoun et al., 1972; Weigele & Barchi, 1978a; Barchi & Weigele, 1979; Reed & Raftery, 1976; Agnew et al., 1978). pK_a values found with these systems range between 5.2 and 6.0.

Blockers of the Calcium Channel and Local Anesthetics Do Not Interfere with TTX Binding to Its Receptor. Antiarrhythmic drugs like verapamil, D_{600}, and nifedipine block the slow Ca^{2+} channel of cardiac cells at concentrations ranging between 0.001 and 0.1 mM (Sperelakis, 1980; Fleckenstein, 1977; De Barry et al., 1977). None of these molecules alter [^{3}H]en-TTX binding to cardiac membranes, even at concentrations as high as 0.1 mM.

Tetracaine, procaine, and lidocaine are local anesthetics which block action potentials in nerve, skeletal muscle, and heart muscle (Seeman, 1972; Kohlhardt et al., 1972) by inhibiting the functioning of ionic channels. Their blocking action is maximum at concentration of 1.5 to 30 μM (De Barry et al., 1977). None of these compounds inhibits the formation

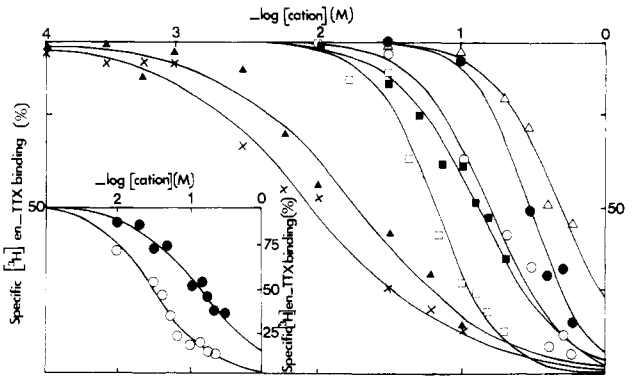

FIGURE 5: Displacement of specific [^{3}H]en-TTX binding on heart microsomal fraction by cations at constant ionic strength. Displacements were obtained with Mg^{2+} (×), Ca^{2+} (▲), Li^+ (□), Na^+ (■), K^+ (○), Rb^+ (●), and Cs^+ (△). Experimental conditions are $[[^3H]en\text{-}TTX]_f$ = 3.0 nM, μ = 220 mM for Mg^{2+} and Ca^{2+}; $[[^3H]en\text{-}TTX]_f$ = 1.4 nM, μ = 220 mM for Li^+ and Na^+; $[[^3H]en\text{-}TTX]_f$ = 2.3 nM, μ = 620 mM for K^+, Rb^+, and Cs^+. Inset: Displacement by guanidinium (○) and NH_4^+ (●); $[[^3H]en\text{-}TTX]_f$ = 2.3 nM, ionic strength = 220 mM, K_D = 0.6 nM.

Table II: Cation Interaction at the TTX and STX Binding Site of the Sodium Channel

cation	rat heart microsomes		rat synaptosomes,[a] (Weigele & Barchi, 1978b)	rat sarcolemma[a] (Barchi & Weigele, 1979)
	$K_{0.5}$ (mM)[b]	n_H	K_I (mM)	K_I (mM)
Tl^+	19.8	2.3	7.2	7.9
Li^+	35.2	2.0	22.6	16.7
Na^+	64.3	1.6	34.3	37.4
K^+	87.3	1.8	53.6	65.0
Rb^+	185.0	2.2	88.8	99.0
Cs^+	277.0	1.8	147.3	132.0
NH_4^+	24.4	1.0	20.2	
guanidinium	7.0	1.3	6.2	
Mg^{2+}	1.3	0.90	1.8	1.9
Ca^{2+}	3.2	0.92	3.6	4.1

[a] Data obtained from the indicated reference with [^{3}H]STX.
[b] Data calculated by using experimental n_H.

of the [^{3}H]en-TTX–receptor complex even at concentrations as high as 0.1 mM.

Amiloride, a specific blocker of Na^+ transport in epithelial cells at concentrations lower than 0.1 mM (Cuthbert, 1976), was also without effect on [^{3}H]en-TTX binding at concentrations as high as 1 mM.

Competition between [^{3}H]en-TTX Binding and Monovalent and Divalent Cations. Binding of [^{3}H]en-TTX to cardiac membrane under physiologic ionic conditions and in a choline chloride medium of similar ionic strength (μ = 220 mM) gave different results. The maximum binding capacities are the same (560 ± 50 fmol/mg of protein), but K_D values are different. In the Ringer solution which contains 20 mM Tris-Cl, 140 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM glucose at pH 7.4, the K_D value for the [^{3}H]en-TTX–receptor interaction is 3.2 nM instead of 0.6 nM in choline chloride. This increase of K_D by a factor of 6 is due to the presence in the Ringer solution of monovalent and divalent cations which compete with [^{3}H]en-TTX for binding to the Na^+ channel structure. Figure 5 shows how increasing concentrations of monovalent and divalent cations prevent the association of [^{3}H]en-TTX to its receptor at a constant ionic strength maintained with choline chloride. Results are summarized in Table II and compared with other results, using

Table III: Comparison between Densities of Tetrodotoxin Binding Component and Muscarinic Cholinergic Receptors in Hearts from Different Species[a]

	[^{3}H]en-TTX specific binding		muscarinic receptor binding (fmol/mg of protein)
	K_D (nM)	fmol/mg of protein	
rat	1.0 ± 0.3	185 ± 25 (8)	144 ± 12[b]
guinea pig	1.15 ± 0.28	164 ± 15 (4)	178 ± 13[b]
rabbit	1.02 ± 0.22	71 ± 18 (3)	57.2 ± 4.3[b]
11-day-old chick embryo	1.6 ± 0.2	62 ± 12 (3)	190 ± 13[c]

[a] Number of experiments is given in parentheses. [b] Fields et al. (1978). [c] Renaud et al. (1980).

either synaptosomes (Weigele & Barchi, 1978b) or skeletal muscle preparations (Barchi & Weigele, 1979) and [^{3}H]STX. Monovalent alkali cations bind to the TTX receptor in cardiac membranes in the sequence $Tl^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. The same sequences were also obtained on intact and solubilized garfish olfactory nerves (Henderson et al., 1974).

A very interesting result is that the binding of monovalent cations to the TTX receptor is cooperative with Hill coefficients ranging between 1.6 and 2.3 for the different monovalent cations. This observation indicates that there are multiple binding sites for Na^+ (or other substitute monovalent cations) at the mouth of the Na^+ channel. A Hill coefficient of 2 indicates a minimum number of two binding sites with homotropic interactions between the sites to explain the positive cooperativity. A completely different approach involving $^{22}Na^+$ flux measurements through the Na^+ channel of neuroblastoma cells or chick embryonic cardiac cells in culture (Jacques et al., 1978, 1980a) also strongly suggests that there are multiple and coupled binding sites at the mouth of the Na^+ channel. Cooperative modifications of STX binding by Na^+ have also been recently reported for an axolemna-enriched preparation obtained from myelinated axons (Rhoden & Goldin, 1979).

Organic cations like guanidinium or ammonium which are also known to be permeant cations for the Na^+ channel are among the monovalent cations with the highest affinity for the tetrodotoxin receptor (Table II). In contrast with inorganic monovalent cations, they inhibit [^{3}H]en-TTX binding, with Hill coefficients near 1. This observation would tend to indicate that there is no or very little positive cooperativity for the association of these cations to the TTX receptor moiety of the Na^+ channel.

Mg^{2+} and Ca^{2+} also prevent [^{3}H]en-TTX binding (Table II). These divalent cations associate with their receptor site more tightly than any of the monovalent cations. However, in contrast to monovalent ions, Mg^{2+} and Ca^{2+} binding is definitely noncooperative.

Species and Regional Distribution of Tetrodotoxin Receptors. The biochemical characteristics of the tetrodotoxin receptor of cardiac homogenates prepared from rat, guinea pig, rabbit, and chick embryo heart are given in Table III. The number of receptor sites per milligram of protein is higher in rat and guinea pig heart than it is in rabbit and chick embryonic heart. It is interesting to observe that the number of Na^+ channels as measured by [^{3}H]en-TTX binding in preparations of mammalian hearts is similar to the number of muscarinic receptors measured by binding assays using the antagonist quinuclidinylbenzilate (Fields et al., 1978). The situation is different for chick embryonic heart. Dissociation constants, K_D, for the [^{3}H]en-TTX–receptor complex are essentially the same for the four preparations.

Table IV: Regional Distribution of Tetrodotoxin Binding Component in Rat Heart

region	receptor density (fmol/mg of protein)
left ventricle	206 ± 15
right ventricle	145 ± 16
interventricular septum	119 ± 12
left atrium	529 ± 31
right atrium	275 ± 21

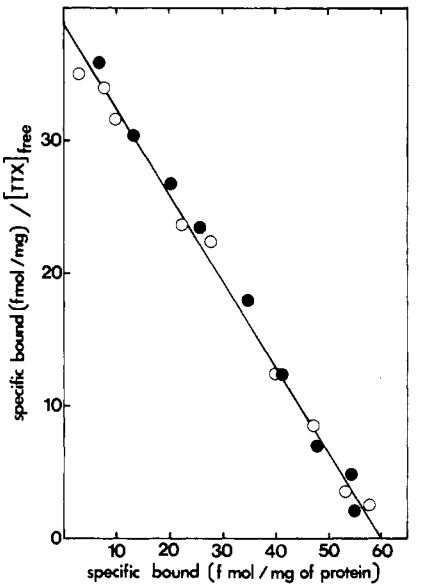

FIGURE 6: Scatchard plots of the [^{3}H]en-TTX binding to innervated and noninnervated chick embryonic heart cells at 4 °C, pH 7.5. (○) Ventricles from 11-day-old chick embryonic hearts; (●) cultured monolayers of 11-day-old chick embryonic hearts.

Regional distribution of ligand binding was carried out from saturation isotherms and Scatchard analysis. Receptor densities are given in Table IV. Atrial tissue contained 2 to 4.5 times more tetrodotoxin receptor per milligram of protein than did ventricular tissue. The greatest concentration of receptor was in the left atrium. This distribution of tetrodotoxin receptors again is similar to that found for the muscarinic cholinergic receptor (Fields et al., 1978). K_D values for the different region of the heart were essentially identical; they were all found to be between 0.23 and 0.39 nM.

Comparison of the Interaction of [^{3}H]en-TTX with Its Receptor in Innervated and Noninnervated Cardiac Cells. This comparison was made by using chick embryo cardiac cells. The [^{3}H]en-TTX binding was measured in parallel on 11-day-old chick embryo ventricles which are known to have both a parasympathic and a sympathic innervation (Pappano, 1977) and on ventricular cells in culture prepared from the same series of 11-day-old embryonic heart. These cell cultures have of course lost their innervation. Binding results with [^{3}H]en-TTX are presented in Figure 6. Both the maximal binding capacities (60 ± 9 fmol/mg of protein) and K_D values (1.55 ± 0.15 nM) are identical in innervated and noninnervated cardiac cells. This result is a strong indication that the TTX–receptor which is studied in this work belongs to the cardiac cell membrane and is not of a presynaptic origin.

Native TTX competes as usual with [^{3}H]en-TTX for the toxin receptor. The K_D for TTX found from competition studies, similar to those described in Figure 2, is 5 nM.

Conclusion

This paper describes the first biochemical characterization of the TTX binding component in cardiac cell membranes.

This characterization has been made possible by the use of a new and highly radioactive TTX derivative which we have recently synthesized (Chicheportiche et al., 1980).

A comparison between biochemical and electrophysiological results can be made for rat cardiac cells and for chick embryonic heart cells. K_D values found for the TTX–receptor complex are 2 and 5 nM for rat and chick cells, respectively. ED_{50} values found by measuring the influence of TTX on the maximal rate of rise $\dot{V}_{max}$ of the action potential of rat and chick cardiac cells are 100 and 20 nM, respectively (Athias et al., 1980, Iijima & Pappano, 1979). There are two reasons for the difference between biochemical and electrophysiological results. (i) K_D values were determined in a choline medium, in the absence of monovalent and divalent cations which are normally present in the physiological medium. Because cations like Na^+, K^+, or Ca^{2+} compete with TTX for association to the TTX receptor, K_D values measured in a Ringer solution are much higher than in choline medium. For rat cardiac cells an increase of K_D by a factor of 6 was found in physiological conditions. Of course ED_{50} are measured in physiological conditions, and for that reason they should be expected to be higher than K_D value measured in choline medium. (ii) The maximum rate of rise of an action potential is not linearly related to the Na^+ conductance value (Noble, 1975), i.e., to the number of functional Na^+ channels. True "physiological" dissociation constants for the TTX–receptor complex can be obtained by using a voltage-clamp analysis. Measurements of $\dot{V}_{max}$ at different TTX concentrations always give an ED_{50} value which is higher than that measured by voltage-clamp analysis. For example, the ED_{50} value for the interaction of TTX with the Na^+ channel in the Ranvier node is 3.6 nM when it is measured by the voltage-clamp technique and 11 nM when it is evaluated from $\dot{V}_{max}$ measurements (Schwarz et al., 1973).

One interesting observation made in this paper concerns the cooperative association of inorganic monovalent cations to the TTX part of the Na^+ channel that may suggest the existence of multiple interactive Na^+ binding sites. Differences in cooperativity between monovalent inorganic and organic cations on one hand and between monovalent and divalent inorganic cations on the other hand are not yet explained and deserve further studies.

The highly radioactive tetrodotoxin used in this study provides a convenient tool for further characterization of the Na^+ channel during the course of the cardiac ontogenesis and Na^+ channel differentiation. TTX-sensitive fast Na^+ channels are either absent or not functional at the very early stage of cardiac development (Sperelakis & Shigenobu, 1972). With our method, it seems now possible to differentiate between these alternatives, namely whether such channels exist in cardiac myocytes in a nonfunctional form or the fast Na^+ channels are synthesized later during the course of the embryonic development.

Acknowledgments

We are very grateful to N. Alenda and Dr. M. C. Lenoir for expert assistance, to Professor R. Motais for the generous gift of amiloride, to Dr. S. Paris for fruitful discussion, to Dr. G. De Vries for a careful reading of the manuscript, and to C. Bonifacino for skillful technical help.

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Dissociation Kinetics of the Estrogen Receptor Immobilized by Hydroxylapatite†

Willem de Boer‡ and Angelo C. Notides*

ABSTRACT: The estrogen receptor from the calf uterine cytosol was adsorbed to hydroxylapatite to provide a simpler and more controlled model for investigating in vitro the [^{3}H]estradiol dissociation kinetics of the estrogen receptor–nuclear complex. The dissociation of [^{3}H]estradiol at 29 °C from the estrogen receptor immobilized by hydroxylapatite showed biphasic kinetics with fast and slow components characteristic of the nonactivated and activated states of the receptor, respectively. The dissociation rate constant of the fast component (k_{-1} = 0.059 ± 0.004 min^{-1}), measured by exchange with estradiol, was one-third slower than that for the receptor free in the cytosol (k_{-1} = 0.162 ± 0.011 min^{-1}). The magnitude of the fast component was 17% when the receptor was bound to hydroxylapatite and 26% when in cytosol. The decrease in the rate of [^{3}H]estradiol dissociation and the reduction in the magnitude of the fast component suggest that receptor binding to hydroxylapatite facilitates activation of the estrogen receptor. The dissociation rate constant of the slower component, k_{-2}, at 29 °C was not significantly different whether the receptor was bound to hydroxylapatite (k_{-2} = 6.62 ± 0.26 × 10^{-3} min^{-1}), free in cytosol (k_{-2} = 7.25 ± 0.46 × 10^{-3} min^{-1}), or preactivated before being bound to the hydroxylapatite (k_{-2} = 6.93 ± 0.52 × 10^{-3} min^{-1}). The [^{3}H]estradiol dissociation rate from the activated estrogen receptor bound to hydroxylapatite, when measured by exchange, was not influenced by a 150- to 1500-fold excess of estradiol (0.5–5 μM estradiol), although massive quantities (10 or 200 μM) of estradiol increased the dissociation rate (k = 12.2 × 10^{-3} min^{-1} and 0.088 min^{-1}, respectively). Combinations of 1 μM estradiol plus 200 μM progesterone increased the dissociation rate (k = 0.067 min^{-1}), while 200 μM progesterone alone did not displace the bound [^{3}H]estradiol or affect receptor stability. High concentrations of steroids exerted a nonspecific detergent action on the receptor. Triton X-100 (0.01%) markedly increased [^{3}H]estradiol dissociation (k = 0.11 min^{-1}). The use of Triton X-100 to isolate nuclei containing receptors affects the [^{3}H]estradiol dissociation kinetics of the receptor and may influence the receptor's interactions with the nucleus. The hydroxylapatite-immobilized estrogen receptor is useful in investigating the kinetics and mechanism of estrogen receptor activation and serves as a tool for understanding the estrogen receptor–nuclear interactions.

The existence of a specific estrogen-binding protein or receptor and the receptor's relationship to the tissue responses initiated by the estrogens have been reviewed (Gorski et al., 1968; Jensen & De Sombre, 1973; Gorski & Gannon, 1976). The complex formed between estradiol and the receptor results in activation (transformation) and the translocation of the receptor from the cytoplasm to the nucleus (Jensen et al., 1968; Shyamala & Goski, 1969). We have described the molecular properties and mechanism of the transformation of the cytoplasmic 4S form of the receptor, a monomer with a molecular weight of (7–8) × 10^4, into the activated or nuclear 5S receptor, a dimer with a molecular weight of (13–14) × 10^4 (Notides & Nielsen, 1974, 1975; Notides et al., 1975). We have recently found that the receptor exists in two estrogen-binding affinity states and that estrogen binding shifts the equilibrium between the monomer and dimer toward the higher affinity state, the activated 5S form of the receptor (Weichman & Notides, 1977). The two affinity states of the receptor are readily measured by the unique, biphasic [^{3}H]-estradiol dissociation kinetics of the receptor. The fast [^{3}H]estradiol dissociating phase is generated by the nonactive 4S receptor, while the second, slower dissociating component is a property of the activated 5S receptor (Weichman & Notides, 1979).

In the light of these findings, it is preferable to use the [^{3}H]estradiol dissociation kinetics which are sensitive and specific as a probe of the receptor interactions with the nucleus. To establish the validity of this approach, we report here the properties and kinetics of the [^{3}H]estradiol dissociation from the receptor that has been immobilized to hydroxylapatite. These findings serve as an important prerequisite and model for understanding the interactions of the estrogen receptor with the nucleus as described in the accompanying paper (de Boer and Notides, 1981).

Experimental Procedures

Materials. The 17β-[2,4,6,7-^{3}H]estradiol (108 Ci/mmol), 17β-[2,4,6,7,16,17-^{3}H]estradiol (160 Ci/mmol), and Triton X-100 (scintillation grade) were obtained from Amersham/Searle. The radiochemical purity of the estrogens was verified by thin-layer chromatography in a chloroform–ethyl acetate (3:1) solvent system. Unlabeled 17β-estradiol and progesterone

† From the Department of Radiation Biology and Biophysics, University of Rochester, Rochester, New York 14642. *Received August 20, 1980.* This work was supported by Research Grant HD 06707 from the National Institutes of Health and the NIEHS Center Grant ES 01247.
‡ W. de Boer was partially supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). Present address: Biochemical Laboratory, Nyenborgh 16, 9747AG Groningen, The Netherlands.

0006-2960/81/0420-1285$01.25/0