Affinity Labeling of the Digitalis Receptor with \( p \)-Nitrophenyltriazene-Ouabain, a Highly Specific Alkylating Agent*

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Bernard Rossi, Paul Vuilleumier, Christian Gache, Marco Balerna, and Michel Lazdunski
From the Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06934 Nice Cedex, France

Three derivatives of ouabain have been synthesized which alkylate the digitalis receptor. These derivatives were formed through reductive amination of \( p \)-nitrophenyltriazene (NPT) ethylenediamine to the periodate-oxidized rhamnose moiety of ouabain.

The non-covalent binding of the ouabain derivatives (NPT-ouabain, designated I, II, and III) was followed (i) by their ability to inhibit the activity of sodium- and potassium-activated ATPase ((Na\(^+\),K\(^+\))-ATPase) purified from the electric organ of *Electrophorus electricus* (6), (ii) by the binding of \([\text{H}]\text{NPT}-\text{ouabain}\) I to the enzyme, and (iii) by the inhibition of \([\text{H}]\text{ouabain}\) binding with unlabeled NPT-ouabain I.

Covalent modification of the digicalis site of \((\text{Na}^+, \text{K}^+)\)-ATPase occurs after long periods of time. At pH 7.5 (25°C) the best alkylating derivative, NPT-ouabain I, gives maximum covalent labeling after 6 h. Only the large polypeptide chain \((M_r = 93,000)\) of the purified enzyme is specifically labeled with \([\text{H}]\text{NPT-ouabain}\) I while the glycoprotein chain \((M_r = 47,000)\) is not significantly labeled. Labeling of a microsomal fraction of the electric organ with \([\text{H}]\text{NPT-ouabain}\) I gave the same type of gel pattern as that observed with the purified enzyme.

\([\text{H}]\text{NPT-ouabain}\) I was also used to label the digicalis receptor in highly purified axonal membranes and in cardiac membranes prepared from embryonic chick heart. Although the \((\text{Na}^+, \text{K}^+)\)-ATPase in both types of membranes has a low affinity for ouabain, \([\text{H}]\text{NPT-ouabain}\) I proved to be a very efficient affinity label for the digitalis receptor. In the complex mixture of polypeptides found in these membrane preparations, only a single polypeptide chain having a \(M_r = 93,000\) is specifically labeled by \([\text{H}]\text{NPT-ouabain}\) I.

Digitalis preparations have proven to have great value clinically in the treatment of chronic congestive failure and other disorders associated with the cardiovascular system. In addition to its clinical importance, this family of molecules has attracted the interest of biochemists, physiologists, and biophysicists because of its rather unique action on the transmembranal transport of sodium and potassium. It is well known that the sodium pump in the plasma membrane of eukaryotic animal cells is inhibited by low concentrations of digitalis glycosides (1–3). Studies on the nature of the inter-
sariat à l'Energie Atomique. \(^{1}H\)Ouabain (19 Ci/mmol) was obtained from New England Nuclear.

Methods—Unless indicated otherwise, thin layer chromatography was on precoated Silica Gel 60 plates; development was in chloroform: methanol (4:1, by volume). \(R_f\) values were as follows: oxidized ouabain, 0.27; NPT-ouabain I, 0.40; NPT-ouabain II, 0.49; NPT-ouabain III, 0.55. Cardiac steroids were assayed according to Kedde (17) and primary amines according to Inman and Dintzis (18) as described by Forbush et al. (9). Products were detected by long wave UV absorption (\(p\)-nitrophenyltriazene ethylenediamine and ouabain derivatives), iodoine vapor (\(p\)-nitrophenyltriazene ethylenediamine, oxidized ouabain, and ouabain derivatives), and 3,5-dinitrobenzoic acid or \(Cl\)COOH/hypochloride sprays or \(^3\)H counting (cardiac steroids).

Synthesis of \(p\)-Nitrophenyltriazene Ethylenediamine—\(p\)-Nitrophenyltriazene ethylenediamine (1-nitrophenyl-3-(2-aminoethyl)-triazene) was prepared using a modification of the procedure described by Sinnott and Smith (19). Typically, 14.65 mg of ethylenediamine (0.244 mmol) were dissolved in 3 ml of ice-cold water and the pH adjusted to 7.0 with HCl. \(p\)-Nitrophenylazidomethane (tetrafluoroborate (28.9 mg, 0.122 nmol) was suspended in 2 ml of ice-cold water and then added to the ethylenediamine solution. The suspension was maintained at 0°C and stirred constantly. Throughout the reaction the pH was at pH 7.0 (Radiometer Titrator II, equipped with a SBR2c recorder). After 5 min, the solid phase of the reaction medium was separated by filtration and washed with 2 ml of ice-cold water. The filtrate was extracted with 5 ml ice-cold diethyl ether which had been previously saturated with water. The aqueous phase was then extracted 4 times with 5 ml of ice-cold water. The combined butanol extracts were evaporated under vacuum at room temperature (15.8 mg, 62% yield). After quantitative determinations of primary amines (18), we used a molecular absorption coefficient of \(e_{250} = 22,000 \text{ M}^{-1} \times \text{cm}^{-1}\), a value in accordance with that reported by Sinnott and Smith (19) for \(p\)-nitrophenyltriazene. The product was either immediately used or stored at -60°C and repurified before use on a Sephadex LH-20 column (elution with methanol).

Synthesis of \(p\)-Nitrophenyltriazene Ethylenediamine Ouabain Derivatives (NPT-Ouabain)—The synthesis of NPT-ouabain is outlined in Fig. 1. Schiff base formation between \(p\)-nitrophenyltriazene and the aldehyde groups of oxidized ouabain was followed by a reduction with NaCNBH\(_4\). The synthesis yielded three NPT derivatives of ouabain.

Ouabain oxidation was performed according to Rogers and Lazdunski (10) except that iodate was removed on a QAE-Sephadex column (Pharmacia) instead of precipitation with lead (II) acetate.

Oxidized ouabain (10 mM) was incubated with 20 mM NPT ethylenediamine in absolute methanol at room temperature. The reaction mixture was adjusted to an apparent pH between 5 and 6 (20) by adding acetic acid or triethylamine. NaCNBH\(_4\) was then added to a final concentration of 20 mM. The reaction was followed by analytical thin layer chromatography. Three spots appeared with \(R_f\) values of 0.40 (NPT-ouabain I), 0.49 (NPT-ouabain II), and 0.55 (NPT-ouabain III). After 4 h, the reaction products were separated by preparative thin layer chromatography (2 mm). The three products, each representing approximately 5% of the starting material, were eluted with methanol. The three compounds had identical UV spectra, each consisting of two peaks (Fig. 2). The first peak (\(\lambda_{max} = 362 \text{ nm}\)) represents the triazenyl group. A shift of \(\lambda_{max}\) occurs during the coupling of NPT ethylenediamine (\(\lambda_{max} = 352 \text{ nm}\)) to ouabain. The second peak represents the lactone ring of ouabain (\(\epsilon_{222} = 14,500 \text{ M}^{-1} \times \text{cm}^{-1}\) and to some extent the secondary peak of \(p\)-nitrophenyltriazene ethylenediamine (\(\epsilon_{272} = 8,500 \text{ M}^{-1} \times \text{cm}^{-1}\), inset of Fig. 2).

Radiolaabeled \(p\)-nitrophenyltriazene ouabain was synthesized by two methods, the usual procedure being similar to that for the synthesis of unlabeled NPT-ouabain. (i) \(^{1}H\)NPT ethylenediamine (25.8 mmol; specific radioactivity, 30 Ci/mmol) and 200 nmol of oxidized ouabain (10) were incubated in 100 ml of absolute methanol at pH 6.0 to 7.0. After 15 min, NaCNBH\(_4\) was added to a final concentration of 10 mM; reaction time was 5 h. Analysis by UV absorption indicates a total product yield relative to ouabain of 23% (approximately 8% for each of the three derivatives). Specific radioactivity of the product is about 60% relative to the specific radioactivity of \(^{1}H\)ethylenediamine. (ii) Oxidized \(^{1}H\)ouabain (2 pmol; specific activity, 6 Ci/mmol) and NPT ethylenediamine (4 mmol) were incubated in 620 ml of absolute ethanol at pH 6.0 to 7.0. After 15 min, NaCNBH\(_4\) (final concentration, 10 mM) was added and the reaction continued for 4 h. By this method, total product yield was again approximately 25%. The specific radioactivity of oxidized ouabain was approximately 60% of that of the initial sample of \(^{1}H\)ouabain.

Enzyme and Membrane Preparations—Purified (Na\(^{+}\),K\(^{+}\))-ATPase from the electric organ of Electrophorus electricus was purified according to Dixon and Hokin (21). A sarcosomal fraction of the electric organ was prepared as described by Agnew et al. (22). Crab axonal membranes and cardiac plasma membranes from 14-day-old chick embryonic hearts were prepared according to Balerna et al. (23) and Paris et al. (24), respectively.

Enzyme Assays—(Na\(^{+}\),K\(^{+}\))-ATPase activity was measured spectrophotometrically at 340 nm with a pyruvate kinase-lactate dehydrogenase-linked system using conditions previously described by

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**Fig. 1.** Reaction scheme and proposed structures for the coupling products.

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**Fig. 2.** Absorbance spectrum of NPT-ouabain derivatives I, II, or III. UV visible spectrum of 40 \(\mu\)M NPT-ouabain in methanol. Inset, UV visible spectrum of NPT ethylenediamine at 40 \(\mu\)M.
**Table I**

Chemical methods used to identify and quantify the ethylenediamine group associated with p-nitrophenyltriazene in NPT ethylenediamine

| Experiment | A. Determination of p-nitrophenyltriazene moiety | B. Determination of ethylenediamine moiety | A/B molar ratio |
|------------|-----------------------------------------------|----------------------------------------|----------------|
| 1          | UV absorption, ε\textsubscript{22,000} = 22,000 | Titrimetric method\textsuperscript{a} | 1.1            |
| 2          | UV absorption, ε\textsubscript{22,000} = 22,000 | Primary amine determination (Inman and Dintzis \textsuperscript{18}) | 0.85           |
| 3          | UV absorption, ε\textsubscript{22,000} = 22,000 | \[\text{H}]\text{Ethylene diamine counting} | 0.95           |
| 4          | NMR analyses of aromatic protons \[\text{H}]\text{Ethylene diamine counting} | NMR analyses of ethylenediamine protons | 1.0            |
|            | \(88.3 (d, 2, J = 9 \text{ Hz})\) | \(83.9 (m, 2, -\text{CH}_2\text{NH}_2)\) |                |
|            | \(87.4 (d, 2, J = 9 \text{ Hz})\) | \(81.16 (t, 2, -\text{NHCH}_3)\) |                |

\textsuperscript{a}Molecular absorption coefficient taken from Sinnott and Smith \textsuperscript{19}.

\textsuperscript{b}Titration of amino group was performed with a pH-stat Radiometer Titrator II equipped with a SBR 2C recorder.

The proton NMR analyses were performed on a Bruker 90 MHz with Fourier transform in dimethyl-d₆ sulfoxide.

**Table II**

Chemical methods used to identify and quantify the p-nitrophenyltriazene ethylenediamine group associated with ouabain after the coupling step

| Experiment | A. Determination of p-nitrophenyltriazene ethylenediamine moiety | B. Determination of ouabain moiety |
|------------|-------------------------------------------------------------|----------------------------------|
| 1          | UV absorption, ε\textsubscript{22,000} = 22,000 | UV absorption, ε\textsubscript{22,000} = 14,500 | 0.95 |
| 2          | UV absorption, ε\textsubscript{22,000} = 22,000 | Cardiac steroid determination (Kedde \textsuperscript{17}) | 1.0 |
| 3          | UV absorption, ε\textsubscript{22,000} = 22,000 | \[\text{H}]\text{Ouabain counting} | 1.0 |
| 4\textsuperscript{c} | NMR analyses of aromatic protons \(88 (d, 2, J = 9 \text{ Hz})\) | NMR analyses of C₂ vinylic proton \(86 (s, 1)\) | 1.0 |
|            | \(\delta 6.7 (d, 2, J = 9 \text{ Hz})\) | | |

\textsuperscript{c}The proton NMR analyses were performed on a Bruker 90 MHz with Fourier transform in dimethyl-d₆ sulfoxide.

**RESULTS**

**Characterization of PNT-Ouabain Derivatives—Evidence for the synthesis of p-nitrophenyltriazene ethylenediamine appears in Table I. All the results indicate a 1:1 ratio of the two moieties in the final structure. Mass spectral analysis performed on a AEI MS 902 instrument was in agreement with the theoretical molecular weight of this product. The same type of strategy was used to estimate the molar ratio of ouabain on the one hand and p-nitrophenyltriazene ethylenediamine on the other hand in NPT-ouabain derivatives. Different methods of analysis gave a 1:1 stoichiometry of each of the two moieties in the final NPT-ouabain derivatives (Table II).**

**Reversible Binding of PNT-Ouabain to Its Receptor Site—**

The time course of the inhibition of activity of (Na\textsuperscript{+,K\textsuperscript{-}})-ATPase purified from the electric organ of *E. electricus* is nearly the same for ouabain and NPT-ouabain I (data not shown). The inhibitory potencies of the different ouabain derivatives were compared to the inhibitory potency of ouabain under a variety of experimental conditions (Table III). In the presence of ATP, Mg\textsuperscript{2⁺}, and Na\textsuperscript{+}, the three NPT derivatives are nearly equipotent to ouabain itself. In the presence of Na\textsuperscript{+} alone, none of the compounds has any inhibitory activity. Differences which are seen between NPT derivatives and ouabain in the presence of ATP, Mg\textsuperscript{2⁺}, and K\textsuperscript{+} probably reflect a lower rate of association of the NPT-ouabain derivatives with (Na\textsuperscript{+,K\textsuperscript{-}})-ATPase.

The data in Fig. 3 indicate that in the presence of ligands which favor inhibition by ouabain and NPT-ouabain derivat-
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tives (i.e. ATP, Na\textsuperscript{+}, and Mg\textsuperscript{2+}). [\textsuperscript{3}H]NPT-ouabain I forms a complex with (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase which has a dissociation constant of 110 nM. Under the same conditions, a dissociation constant of 30 nm was obtained for an ouabain-(Na\textsuperscript{+}, K\textsuperscript{+})-ATPase complex. Therefore, the introduction of the NPT substituent in the ouabain molecule decreases its affinity for the enzyme only by a factor of about 4. The number of binding sites for both [\textsuperscript{3}H]NPT-ouabain I and [\textsuperscript{3}H]ouabain is 3 nmol/mg of protein.

Direct evidence that NPT-ouabain I and ouabain interact with the same receptor site in the (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase molecule came from observations that unlabeled NPT-ouabain I displaces the [\textsuperscript{3}H]ouabain bound to the enzyme (Fig. 4). The concentration of NPT-ouabain that is able to induce 50% displacement of labeled ouabain bound to its receptor is 0.65 \mu M. The following equation was used to calculate $K_d$, the true dissociation constant of the complex:

$$K_d = \left[ \frac{K_i \times (RL)}{(L_0) - (RL)} \right] \times \left[ \frac{(C_0) - (R_0) + (RL) \left(1 + \frac{K_i}{(L_0) - (RL)} \right)}{(R_0) - (RL) \left(1 + \frac{K_i}{(L_0) - (RL)} \right)} \right]$$

where $K_i$, dissociation constant for the [\textsuperscript{3}H]ouabain-receptor complex; $K_d$, dissociation constant for the NPT-ouabain-receptor complex.

**TABLE III**

Conditions of interaction of ouabain and ouabain derivatives NTP I, II, and III with (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase

| Conditions | Percentage of (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase inhibition produced by NPT-ouabain |
|------------|-----------------------------------------------------|
| Na\textsuperscript{+} | 1 0 0 0 |
| ATP, Mg\textsuperscript{2+}, Na\textsuperscript{+} | 99 90 88 88 |
| Mg\textsuperscript{2+}, K\textsuperscript{+} | 97 96 96 90 |
| ATP, Mg\textsuperscript{2+}, K\textsuperscript{+} | 49 48 5 2 |

**Fig. 3** (left). Specific binding of [\textsuperscript{3}H]ouabain (a) and [\textsuperscript{3}H]NPT-ouabain I (c) to (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase. The enzyme is a purified solubilized preparation from the electric organ of *E. electricus* (21). Measurements were carried out at 25°C for 30 min in 50 mM triethanolamine-HCl buffer at pH 7.4 in the presence of 100 mM Na\textsuperscript{+}, 2 mM Mg\textsuperscript{2+}, 2 mM ATP, 1 mM dithiothreitol, and increasing concentrations of labeled ligands. Bound ligand was separated from unbound ligand by filtration. Unspecific binding was estimated from measurements in the presence of 1 mM unlabeled ouabain.

**Fig. 4** (center). Competition between NPT-ouabain I and [\textsuperscript{3}H]ouabain for the binding to (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase. Conditions are the same as for Fig. 3 except that the binding at a constant concentration of 1 nm [\textsuperscript{3}H]ouabain (L_0) was measured in the presence of increasing concentrations of unlabeled NPT-ouabain (C_0). The experimental points are well described by a theoretical curve calculated for the competitive binding of two ligands to a single class of binding sites, with a concentration of sites of $R_0 = 0.165 \mu M$, $K_d$ ([\textsuperscript{3}H]ouabain) = 30 nm and $K_d$ (NPT-ouabain I) = 88 nm (for equation see "Methods").

**Fig. 5** (right). Time course of the covalent labeling of the (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase by NPT-ouabain I, II, and III. Association of the (Na\textsuperscript{+}, K\textsuperscript{+})-ATPase from *E. electricus* with the three ouabain derivatives was done in the same medium as in Figs. 3 and 4. Protein concentration was 75 \mu g/ml. Covalent labeling at different times was measured after acid denaturation of the enzyme and separation from free and covalently bound ligand by filtration (for details see "Experimental Procedures"). •, 1 \mu M NPT-ouabain I; ○, 1 \mu M NPT-ouabain II; ■, 0.62 \mu M NPT-ouabain III.
that protein concentration was about 0.5 mg/ml. ['HINPT-ouabain was then diluted with an equal volume of 4% sodium dodecyl sulfate as described in the legend of Fig. 5 and under "Methods," except each membrane preparation was incubated with 1 mM unlabeled ouabain. A, purified (Na',K')-ATPase of E. electricus; B, crude microsomal preparation of E. electricus; C, crab axonal membranes from Cancer pagurus; D, cardiac plasma membranes from chick embryo. Arrows designated as a, b, c, indicate positions corresponding to $M_r = 95,000, 45,000, and migration front, respectively. Ligand that has not reacted with proteins migrates ahead of the tracking dye.

"Experimental Procedures." In all cases, films were exposed for 3 days before developing. Each panel was organized as follows: left lane, Coomassie-stained gel of the incubate; middle lane, gel autoradiography; right lane, gel autoradiography of the same incubate previously exposed to 1 mM unlabeled ouabain. A, purified (Na',K')-ATPase of E. electricus. A nearly complete covalent labeling is probably due to the fact that the reactive intermediate formed from NPT-ouabain I has a low affinity for ouabain, inhibition constant for the ouabain-ATPase complex is 100 $\mu$M (25). Because of these two properties (i.e. low (Na',K')-ATPase content and low affinity for ouabain), the number of ouabain binding sites could not be determined by titration with [1H]NPT-ouabain. Nevertheless, [1H]NPT-ouabain I was an excellent label for the membrane preparation. Radioactivity was specifically incorporated into only one of the membrane polypeptides, the large chain of (Na',K')-ATPase which is also labeled by $[^35]$P]ATP (25).

The plasma membrane of chick embryo cardiac cells also contains a (Na',K')-ATPase with a low affinity for ouabain ($K_a = 44 \mu$M, Ref. 31). This membrane preparation, which contains a large number of polypeptide chains, was labeled by [1H]NPT-ouabain I on only two chains. However, only the large of these ($M_r = 95,000$) is completely protected against covalent labeling by [1H]NPT-ouabain I in the presence of an excess ouabain. Previous labeling of cardiac membranes with photoaffinity derivatives of ouabain or strophanthidin gave much more complex patterns.

Covalent labeling of polypeptides in the low molecular
weight region \( M_r = 12,000 \) is not consistent with the labeling pattern expected for an integral component of the \((Na^+,K^+)\)-ATPase. First, radioactivity incorporation in this band is not the ratio between band c \( (M_r = 12,000) \) and band a \( (M_r = 93,000) \) varies largely with the nature of the preparation used, decreasing with the degree of purity in \((Na^+,K^+)\)-ATPase of the preparation. This implies that the labeling in the band c area does not occur exclusively on a polypeptide chain belonging to \((Na^+,K^+)\)-ATPase.

At present the situation with respect to the small proteolipid \( (M_r = 12,000) \) is as follows: (i) it is labeled by radioactive photoaffinity derivatives of ouabain which have a photoactivatable group on the steroid portion of the molecule; (ii) it is labeled by tritiated NPT-ouabain I, but this labeling is not prevented by an excess ouabain.

The significance of these results is difficult to evaluate at the present time; therefore, it cannot be concluded that the \( M_r = 12,000 \) chain is a constituent of the \((Na^+,K^+)\)-ATPase. Furthermore Goldin (30) seems to rule out a functional role for this peptide since it is not required to reconstitute ATPase transport function.

In summary, this report describes the synthesis and properties of alkylating derivatives of ouabain which can be prepared with very high specific radioactivities. One of these derivatives, NPT-ouabain I, appears to be better than any of the previously described affinity labels derived from cardiac glycosides. This derivative is extremely useful for identifying a polypeptide chain which bears an ouabain binding site, even if this site is present in small quantities and has a low affinity for its specific ligand. NPT-ouabain I is also potentially useful for a variety of other purposes, including the mapping of the ouabain site in the polypeptide chain of the \( M_r = 100,000 \) subunit of \((Na^+,K^+)\)-ATPase once the sequence of this polypeptide chain is known.

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