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Photoaffinity Labeling of the Antidiuretic Hormone Receptor

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Summary. A study to determine the feasibility of photoaffinity labeling the antidiuretic hormone receptor in the toad urinary bladder has been carried out. Two photoactivated derivatives of oxytocin have been synthesized, purified, and characterized chemically and biologically. Photolysis of the toad bladder in the presence of one of these derivatives, 2-nitro-5-azidobenzoylglycyloxytocin, produces a permanent inhibition of the response to native oxytocin. This inhibition can be relieved by protecting the hormone receptor with excess oxytocin during the photolysis. These results suggest that the photolysis-dependent inhibition of the response to native hormone is due to covalent incorporation of the photoaffinity label into the hormone receptor.

The potential use of photoactivated ligand analogs to characterize complex biological receptors was proposed five years ago (Cooperman, 1976; Knowles, 1972). However, there have been few reports of the application of photoaffinity labeling to the identification of peptide hormone receptors. It has been shown that an aryl azido derivative of gastrin pentapeptide will photoincorporate into a hydrophobic binding site on bovine serum albumin (Galardy, *et al.*, 1974). The present study was undertaken to determine the feasibility of derivatizing the peptide hormone oxytocin with photoactivated reagents and investigating the interaction of these hormone analogs with the antidiuretic hormone receptor in the isolated toad urinary bladder. Oxytocin was chosen as the model peptide for this study because it was available in synthetic form and a large body of structure-activity data for oxytocin and its analogs in target tissues was available (Rasmussen *et al.*, 1963; Rudinger, Pliska & Krejci, 1972; Sawyer & Manning, 1973).

Two photolabile analogs of oxytocin have been synthesized and completely purified, an *a*-keto diazo derivative, N-EDMoxytocin and an aryl azide derivative NAB-gly-oxytocin (Fig. 1) (J.M. Stadel, D.B.P. Goodman and H. Rasmussen, *in preparation*). Both oxytocin analogs

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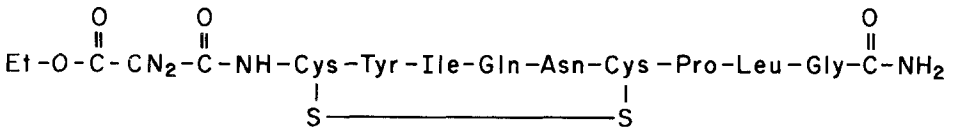
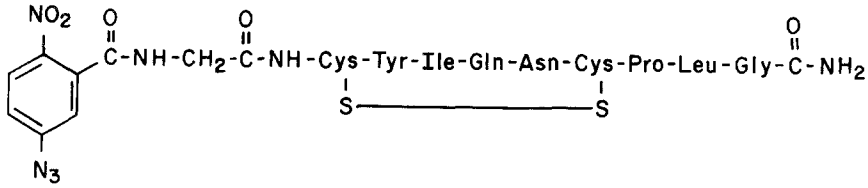


N-E DM oxytocin



NAB-gly-Oxytoxin

Fig. 1. The structure of the photoaffinity-labeled oxytocin derivatives: N-(ethyl-2-diazomalonyl)oxytocin (N-EDM-oxytocin) and 2-nitro-5-azidobenzoyl-glycyloxytocin (NAB-gly-oxytocin)

lose nitrogen upon photolysis to generate highly reactive electrophilic intermediates that react rapidly and nonselectively with their chemical environments, making covalent incorporation into the receptor possible. The two hormone analogs are the product of a single modification of oxytocin at the N-terminal amine, and the derivitizations did not interfere with the binding specificity of the hormone for the antidiuretic hormone receptor in the isolated toad urinary bladder.

N-EDMoxytocin is a competitive antagonist for native oxytocin. When hemibladders are mounted as sacs for the hydroosmotic assay (Bentley, 1958) and N-EDMoxytocin added, there is no alteration of bulk water permeability (Fig. 2). When native oxytocin is added to tissue in the presence of N-EDMoxytocin the response is inhibited. This inhibition is reversible since a full hydroosmotic response is induced by native oxytocin after the hormone analog is washed out (data not shown). At 10^{-5} M N-EDMoxytocin approximately a 50% inhibition of the response to 7.3×10^{-9} M native oxytocin is observed. This inhibition can be overcome by increasing concentrations of native oxytocin, indicating that N-EDM oxytocin is a competitive inhibitor of the antidiuretic hormone response. When transepithelial sodium transport is measured as short-circuit current (SSC), N-EDM-oxytocin again does not exhibit any

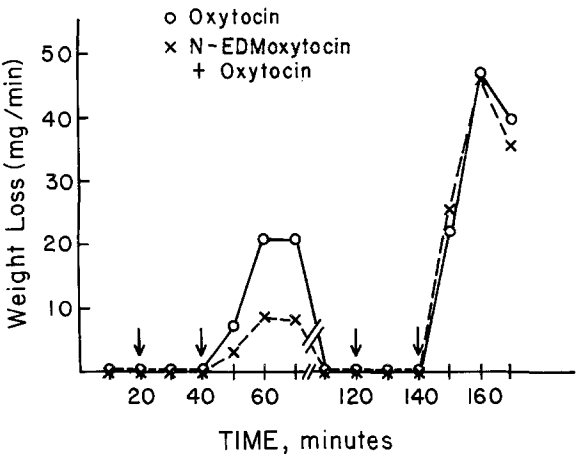


Fig. 2. The effect of N-EDMoxytocin on the hydroosmotic response. Paired hemibladders were mounted as sacs and equilibrated for 20 min. N-EDMoxytocin (10^{-5} M) was then added to one set of bladders. After an additional 20 min all hemibladders were challenged with native oxytocin (7.3×10^{-9} M). After recording the response the bladders were washed free of hormones and baseline water permeability reestablished. N-EDM-oxytocin (10^{-5} M) was then readded to one set of hemibladders, and 20 min later oxytocin (5×10^{-7} M) was added to all hemibladders

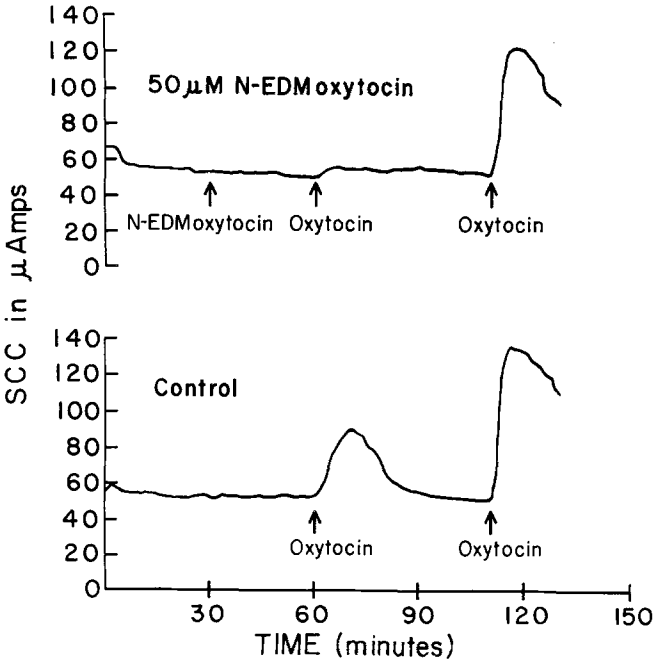


Fig. 3. The effect of N-EDMoxytocin on transepithelial sodium transport. Tissue was mounted in a double chamber Ussing apparatus and short-circuit current continuously recorded. Initially N-EDM-oxytocin (5×10^{-5} M) was added to one side of the tissue. Subsequently both sides of the tissue were treated first with 10^{-8} and then 5×10^{-7} M oxytocin

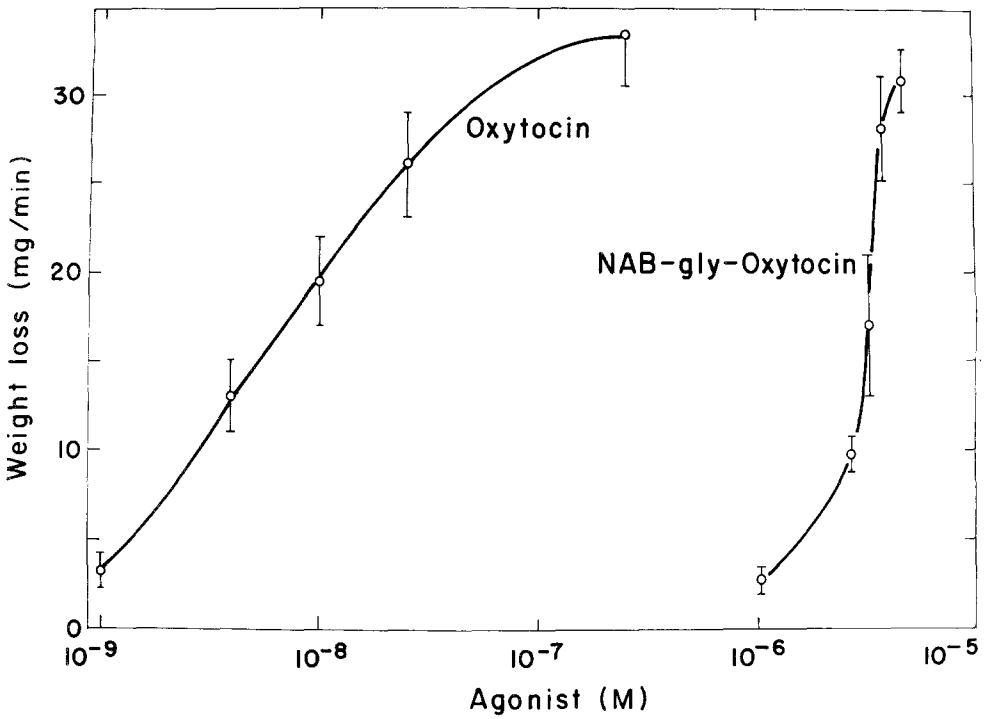


Fig. 4. The hydroosmotic response to native oxytocin and NAB-gly-oxytocin

antidiuretic hormone activity, i.e., SCC is not stimulated (Fig. 3). The hormone analog acts as an inhibitor, preventing the usually observed increase in SCC after the addition of native oxytocin.

NAB-gly-oxytocin, by contrast, retains antidiuretic hormone activity. In the hydroosmotic assay NAB-gly-oxytocin is less potent than native oxytocin (one-half maximal activity $= 3.2 \times 10^{-6}$ M), but it possesses full agonist activity (Fig. 4). When repurified the hormone analog maintained constant specific activity, indicating that the observed antidiuretic hormone activity was intrinsic to the hormone analog. Additionally, when antidiuretic hormone activity was assayed by measuring [^{14}C] urea movement, NAB-gly-oxytocin was a full agonist (Fig. 5).

When the serosal surface of the toad bladder was equilibrated with buffer alone, repeated photolysis ($\lambda \geqq 320$ nM) had no effect of oxytocin stimulated ^{14}C urea permeability. In the presence of NAB-gly-oxytocin (8×10^{-6} M) repetitive photolysis produced a time-dependent permanent inhibition of oxytocin stimulated [^{14}C] urea permeability (Table 1). This inhibition could be prevented by incubating the tissue with native oxyto-

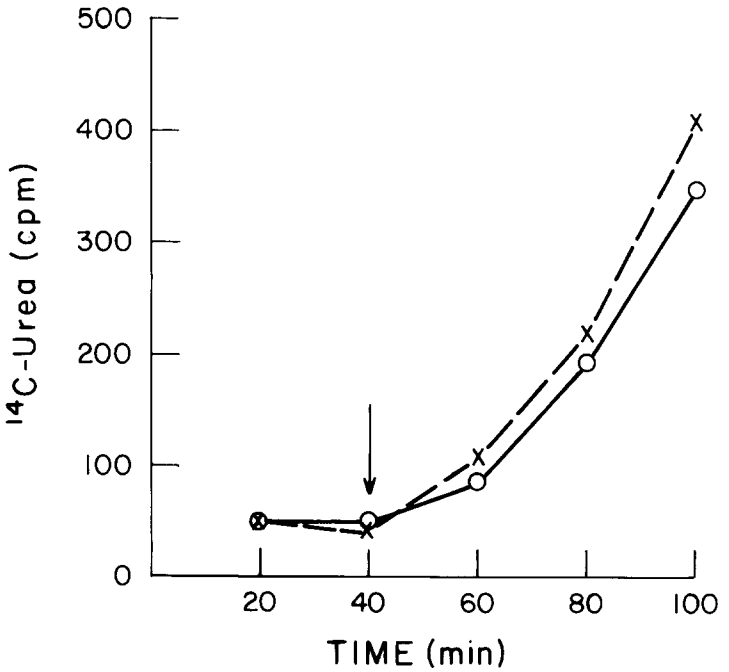


Fig. 5. The effect of native oxytocin and NAB-gly-oxytocin on the [^{14}C] urea permeability of the toad urinary bladder. Tissue was mounted as in Fig. 3. At the indicated time native oxytocin (1.67×10^{-7} M) (○—○) or NAB-gly-oxytocin (6.5×10^{-6} M) (×—×) was added to the appropriate serosal bathing solutions

Table 1. The response of the toad urinary bladder to native oxytocin after photolysis in the presence of NAB-gly-oxytocin and/or oxytocin[a]

NAB-gly-oxytocin	Oxytocin	Time of Photolysis	% Inhibition of Control	*n*
0	0	5×1 min	0	3
8×10^{-6}	0	3×1 min	29.9	2
8×10^{-6}	0	5×1 min	49.7	4
8×10^{-6}	10^{-6}	3×1 min	13.9	3

[a] Tissue [^{14}C] urea permeability was determined after incubation and photolysis as indicated. The response of photolyzed tissue is compared to control tissue shielded from photolysis by black plastic.

cin (10^{-6} M) during the repetitive photolysis experiments. These data thus strongly suggest that the permanent inhibition is due to covalent labeling of the receptor.

The present study demonstrates the feasibility of photoaffinity labeling a peptide hormone receptor. To verify the covalent incorporation of the photolabel into the receptor and to utilize the hormone analog

to characterize and identify the receptor it will be necessary to introduce a radioactive marker, either iodine or tritium, into the photoaffinity label. The specific radioactivity will have to be quite high (>30 Ci/mmole) because there are relatively few receptor sites (~1000/cell) in the plasma membrane of epithelial cells (Bockaert *et al.*, 1972) and because approximately 10% of the receptors are covalently labeled during each photolysis. We have attempted to introduce iodine into the tyrosine of oxytocin by enzymatic iodination (Morrison & Schonbaum, 1976; McIlhinney & Schulster, 1974), but the resulting monoidooxytocin was inactive in the hydroosmotic assay both as an agonist or antagonist. Methods have been developed to incorporate tritium (20–30 Ci/mmole) in the phenolic side chain of the tyrosine in position 2 of oxytocin by catalytic substitution of iodine with tritium (Morgat *et al.*, 1970). However, the product yield from this process is very low (<1%). Due to the shift in activity of the derivatized hormone in the bioassay systems milligram quantities of radioactive photolabel analog would be required.

It is also possible to introduce the radioactivity into the photoreagent. At present none of the precursors of ethyl-2-diazomalonyl chloride are available at sufficiently high specific radioactivity. By utilizing an amino acid to link the aryl azide to oxytocin it is possible to introduce a radioactive marker into NAB-gly-oxytocin. Unfortunately (^{3}H) glycine is available at only 5–15 Ci/mmole and the specific radioactivity is reduced during the synthesis of the photoactivated hormone analog. It may be possible to use other amino acids such as tyrosine which can be iodinated or leucine which is available at a high specific radioactivity (70–100 Ci/mmole). It is unknown what the effect of a substitute amino acid as the linker between the aryl azide and the hormone might have on the binding specificity of the receptor for the photolabel.

It has been pointed out previously that the interpretation and verification of the accuracy of photoaffinity labeling can be difficult (Cooperman, 1976). By derivatizing radioactive oxytocin with two photoreagents it would be possible to compare labeling results; common labeling patterns would suggest that the receptor had been identified.

The present study also indicates that the two photoreagents employed, ethyl-2-diazomalonyl chloride and the N-hydroxysucchinimide ester of 2-nitro-5-azidobenzylglycine, may have general application to the derivitization of other hormones and biologically important ligands for photoaffinity labeling studies. These two reagents react rapidly and nearly quantitatively with primary amines and could be used to derivatize these compounds.

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