A Neurohypophyseal Hormone Analog with Selective Oxytocin-Like Activities and Resistance to Enzymatic Inactivation: An Approach to the Design of Peptide Drugs*

(conformation/hormone action/smooth muscle contractility/induction of labor)

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ABSTRACT Stepwise, synthetic alteration of oxytocin, guided by conformational and enzymological considerations, has led to analogs with specifically modified activity profiles and resistance to enzymatic inactivation. General requirements for the design of peptides with therapeutic value are discussed.

Peptides thus far have not played a major commercial role as therapeutic agents, with certain exceptions such as insulin and adrenocorticotropic hormone (ACTH). However, this situation is rapidly changing as ever more naturally occurring or synthetic low molecular weight peptides with interesting biological activities are being discovered. Additional clinical value can be gained with the development of conceptual approaches for synthetic modification of the molecule in order (i) to endow the analog ideally with only one specific activity (i.e., to change the activity profile of the peptide so that only the desired biological property is expressed, whether agonistic or antagonistic) and (ii) to increase the resistance of the peptide to enzymatic inactivation and to alter tissue distribution, with the hope of prolonging the duration of hormonal action.† Chemical modifications of the hormonal peptide based on conformational considerations, rather than on an empirical or intuitive approach, should most readily lead to success in obtaining an analog with the required properties.

In the case of neurohypophyseal hormones their proposed preferred conformations (2, 3) suggest that substitutions of amino-acid residues in the corners of the β-turns of the hormones (positions 3, 4, 7, and 8, which are not primarily involved in the intramolecular stabilization of the peptide backbone and, therefore, are available for intermolecular interactions) would lead to analogs with a selectively modified activity profile. Thus, in these analogs certain activities will be enhanced while others will be diminished or even abolished (4, 5). Because of its compact conformation the 20-membered ring component of the neurohypophyseal hormones is already rather resistant to enzymatic cleavage [e.g., α-chymotrypsin fails to cleave the 20-membered ring of oxytocin and vasopressin despite the presence of aromatic residues (6, 7); pregnant primates have an arylamidase which slowly cleaves the Cys-Tyr bond (8), and the S-S bond is susceptible to reductive cleavage (9)]. In each tissue studied to date, we have found most rapid enzymatic inactivation to occur by hydrolytic cleavage of -CO-NH- linkages present in the acyclic portion of the neurohypophyseal hormones (10). These findings are in accord with our general considerations of the relationship between the conformation of neurohypophyseal hormones and their susceptibility to enzymatic inactivation (5).

With this as background we became interested in neurohypophyseal hormone analogs with amino acid residues other than Pro in position 7. First, the Pro residue occupies the one corner of the β-fold which, in contrast to positions 3, 4, and 8, has not been found to be substituted in the course of evolution of the neurohypophyseal hormone principles (11); second, Pro is located in the enzymatically vulnerable, acyclic part of the hormones and substitution of this residue may yield analogs which render the acyclic peptide portion more resistant to enzymatic attack. In the present, initial study biological activities and relative resistance to enzymatic degradation of [7-glycine]oxytocin (12, 13) and [1,6-aminosuberic acid, 7-glycine]oxytocin (14) are compared with those parameters of oxytocin (the structures of the compounds are given in Fig. 1). The results require analysis from a point of view of

Abbreviation: Asu, 2-aminosuberic acid.

† The most frequently applied approach to render a peptide resistant to enzymatic attack has been by replacement or modification of chemical functional groups, such as N- and C-terminal groups, disulfide bond, etc. The nature of these changes is usually such that the hydrophobicity of the peptide is enhanced. Thus the analogs may permeate into the cell instead of remaining in the circulatory system or being excreted as is the case with peptide hormones (1). If these agents cannot be broken down in vivo but rather accumulate intracellularly, their clinical application—particularly for routine usage over long periods of time—may have undesirable consequences in patients.

CH2—S—S—CH3
H2N-CH-CO-Tyr-Ile-Gln-Asn-HN-CH-CO-Pro-Leu-Gly-NH2 (I)
CH2—S—S—CH3
CH2—CH3—CH2—CH3
CH2—CO-Tyr-Ile-Gln-Asn-HN-CH-CO-Gly-Leu-Gly-NH2 (III)

Fig. 1. Structures of oxytocin (I), [7-glycine]oxytocin (II), and [1,6-aminosuberic acid, 7-glycine]oxytocin (III).
**Materials and Methods**

For all standard bioassays the four-point design was used in which the neurohypophyseal extract obtained from the U.S. Pharmacopeia was the reference standard; this extract possesses 2.5 units/ml of oxytocic activity and 2.1 units/ml of rat pressor activity. A minimum of four animals was used for the quantification of the oxytocin-like activities and no less than six animals for the determination of the vasopressin-like activities. Determinations of oxytocic activities and cumulative dose–response curves using isolated uterine horns were performed on rats in natural estrus according to Holton (15) as modified by Munsick (16), utilizing Mg++-free van Dyke-Hastings solution as bathing fluid. In vitro oxytocic assays were carried out as described by Chan and Kelley (17). Milk-ejecting activity was determined on urethane-anesthetized, lactating rabbits following the procedure of van Dyke et al. (18) as modified by Chan (19). Avian vasodepressor assays were performed on conscious chickens (20) according to the procedure of Coon (21). The pressor properties of the poly peptides were determined on atropinized, urethane-anesthetized male rats following the procedures of the U.S. Pharmacopeia (22). The ability of the poly peptides to enhance water transport (i.e., to induce diuresis) was examined in In...
From previous studies with rat uterine homogenate we suspect that the rapid inactivation of I is due to glycaminamide release (28), which does not take place with II and III (10, 29). Probably some degree of disulfide cleavage in II accounts for its slow loss of activity (30, but see also 31). Similar relative inactivation rates were obtained with I, II, and III using crude homogenates of rat kidney and median eminence, toad urinary bladder, and kidney of mouse, hamster, pig, pigeon, and human. Use has been made of the enzymatic stability of III§ to screen various biological preparations for either the presence of enzymes which cleave neurohypophyseal hormones at loci not encountered before or for a known kind of enzymic activity (e.g., activities releasing glycaminamide or the C-terminal dipeptide moiety§), which would have to be present in the given preparation to an unprecedentedly high degree (10).

Our enzymatic findings, in conjunction with the high specificity for smooth muscle receptors exhibited by II and III, suggested that these hormonal analogs would exhibit protracted action and might be useful in inducing uterine contractions leading to labor. This approach could also eventually yield analogs useful for therapeutic abortion§. However, in vitro responses of the rat uterus to single equimolar doses of II are of shorter duration than to I; although III does exhibit protracted responses as compared to II, they are still shorter than those induced by equimolar doses of I (Fig. 3). Experiments in intact rats showed a somewhat different pattern. While I induced rhythmic contractions for about 3 min, peptide II did so only for 2 min. However, analog III induced contractions for at least 8 min (Fig. 4). In view of their relative inactivation patterns, one could have expected an even more pronounced differentiation of duration of action between I on the one hand and II and III on the other. Differences in permeation of the analogs into the tissue or, in the case of the in vitro study, rapid excretion, or both, may account for part of the results. Studies of tissue distribution and investigations of excretion patterns of the individual analogs (preferably radioactively labelled) are warranted not only in the rat, but also in primates including human. Topological differences between hormonal peptides not only contribute, through changes in the hydrophilic-hydrophobic balance or changes in susceptibility to enzymatic attack, to the duration of hormone action, but also account for differences in "specific activity."

Considering "specific activity"—as opposed to duration of action—the high oxytocin-like activities of oxytocin are thought to be a result of its relatively compact conformation,

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§ Under such severe conditions as treatment with purified a-chymotrypsin for 24 hr at 37°C at a ratio of 1:1 w/w, the enzyme still was unable to hydrolyze the Tyr-Ile peptide bond, but it removed the glycaminamide from I (10). However, recently it was found that a-chymotrypsin inactivated 1,6-aminosuberic acid analogs of vasopressin more rapidly than the parent hormone (7) and that the enzyme hydrolyzes the Tyr-Ile bond in [1,6-aminosuberic acid]-oxytocin (Sakakibara and Yamanaka, unpublished). Thus, some degree of hydrolysis of the Tyr-Ile bond of III could occur under certain circumstances. However, preparations which release C-terminal dipeptides or glycaminamide from neurohypophyseal hormones fail to affect III (for summary of such enzymes see ref. 10).

§ Note in this context that this peptide (like 1,6-aminosuberic acid analogs in general) is expected to have an unlimited shelf life, while the activity of oxytocin can diminish upon prolonged storage, likely by polymerization involving disulfide bond interchange reactions. Moreover, the lack of charged groups should increase the rate of uptake of III, allowing sublingual administration.
Fig. 4. Typical response patterns of the rat uterus in vivo. In terms of oxytocic units about equal doses of each peptide were injected into the jugular vein. S indicates time of injection.

which contains a large hydrophobic surface (5). For example, the fact that deamino oxytocin (32) exhibits a higher oxytocic activity than oxytocin (as is generally observed with neurohypophyseal hormone analogs when the terminal amino group is replaced by a hydrogen) is probably associated with the tighter structure of the deamino analog rather than the elimination of the chemical functional group per se (5). While Gly is, from a conformational standpoint, a good replacement for Pro, it lacks the structural rigidity of Pro (13). Extrapolating from the proposed stabilizing effect Pro has on the 20-membered ring of oxytocin and vice versa (5), we expected that both the ring and the acyclic tripeptide sequence of II and III would exhibit a greater conformational freedom than I; a loss of compactness of structure is thought to be accompanied by a reduction of the specific uterotonic activity (determined as area measured under the peaks resulting from stimulation with the agonist, rather than height of first contraction). As a result, 1,6-aminobenzic acid oxytocin (30, 31) despite the possibility of its enzymatic inactivation in certain tissues, may actually exhibit a greater specific uterotonic activity than III.

The neurohypophyseal hormone analog most potent and selective in terms of controlling smooth muscle motility may be obtained by combining within a single molecule the properties of a compact conformation, stability to enzyme action and a certain degree of hydrophilicity. 1,6-Aminobenzic acid, 7-glycineoxytocin possesses some of these properties, and preliminary clinical trials in Japan have demonstrated its efficacy in facilitation of labor® with reduction of some of the side effects (pressor and antidiuretic) caused by oxytocin. More importantly, these investigations open new routes to the possible use of the contractile activity of neurohypophyseal hormones for fertility control in humans. A major problem is that oxytocin and its analogs facilitate myometrial contractility in vivo only in the pregnant uterus at term; during the early stages of pregnancy and in the non-pregnant uterus oxytocin has little effect (33, 34), although uterine sensitivity to oxytocin and especially to vasopressin varies during the menstrual cycle (35). However, the recent finding of a differential sensitivity of various animal smooth muscle systems to a given neurohypophyseal hormone or analog (36) may lead to the development of peptides acting selectively at sites other than the corpus of the human uterus which are involved in pregnancy, e.g., the Fallopian tube (37).

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