Threshold and Receptor Reserve in the Action of Neurohypophyseal Peptides

A study of synergists and antagonists of the hydroosmotic response of the toad urinary bladder

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ABSTRACT The interrelationship of several physiological receptors which influence the hydroosmotic response of the toad urinary bladder was studied employing neurohypophyseal peptides, prostaglandin E_1, theophylline, and cyclic nucleotides. The binding property of agonists (pD_2), synergists (pS_2), competitive antagonists (pA_2), and noncompetitive antagonists (pD_2') was determined after a suitable methodology had been developed. A series of neurohypophyseal peptides was examined in detail for their catalytic activity. It was found that the replacement of the hydroxy radical of the tyrosine residue in oxytocin by a methoxy and then by an ethoxy radical led to a progressive decline in the catalytic activity of the hormone—corresponding to a change from agonist to partial agonist to competitive antagonist. [4-Leucine]-mesotocin behaved as a competitive antagonist of oxytocin. Prostaglandin E_1 (PGE_1) was found to be a noncompetitive inhibitor of neurohypophyseal peptides and theophylline; whereas the maximal hydroosmotic response of the bladder to [2-O-methyltyrosine]-oxytocin and theophylline was greatly depressed by PGE_1, the response to saturating concentrations of oxytocin was only slightly diminished— a finding which reveals a "receptor reserve" for oxytocin. Saturating concentrations of [2-O-ethyltyrosine]-oxytocin, inactive per se, potentiate theophylline—disclosing a "threshold phenomenon" for the mediation of neurohypophyseal hormone action. It is concluded that neurohypophyseal peptides are capable of producing graded effects on adenyl cyclase both below and above the range of enzyme activity which evokes graded changes in membrane permeability.

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INTRODUCTION

Sutherland and associates (35, 43) have shown that a number of hormones with diverse physiological functions operate at their target cells through the adenyl cyclase-3′,5′-AMP system. We have been interested in the hydro-osmotic response of the toad bladder to neurohypophyseal hormones where the primary hormone-receptor interaction appears to be translated into an alteration in the rate of formation of cyclic AMP from ATP (33), and where the cyclic nucleotide in turn triggers through one or more intermediate steps the final effector machinery of the cell. In this study we have investigated neurohypophyseal peptides, theophylline, and prostaglandin E_1 (PGE₁), as modulators of the intracellular level of cyclic AMP in order to gain insight into the question of whether each increment of adenyl cyclase activity can be translated into an increment of hydroosmotic activity, or whether steps subsequent to adenyl cyclase activation are limiting in the over-all response of the tissue to hormone. In order to characterize the interaction of these compounds with their receptors, we have drawn on the approach which has been applied to drug-receptor interactions (3, 20, 45).

MATERIALS AND METHODS

Symbols

$[A]$, concentration of agonist, i.e. a compound capable of evoking a hydroosmotic response in the toad urinary bladder

$[B]$, concentration of a competitive antagonist of A

$[B']$, concentration of a noncompetitive antagonist or a synergist of A

E_m, maximal hydroosmotic response capacity of the target tissue

E_{50}, half-maximal hydroosmotic response

E_A, hydroosmotic response to a given concentration of A

E_{AB}, hydroosmotic response to A in the presence of B

E_{A_m}, maximal hydroosmotic response reached with saturating concentrations of A

$E_{AB'_m}$, hydroosmotic response to a given concentration of A in the presence of saturating concentrations of a synergist B'

h_m, $(E_{AB'_m} - E_A)/E_m$, maximal synergistic effect of a synergist, B', on the response to A expressed as a fraction of the maximal response of which the target tissue is capable

$E_{A_m B'_m}$, hydroosmotic response to saturating concentrations of A in the presence of saturating concentrations of noncompetitive antagonist B'

h_m', $(E_{A_m} - E_{A_m B'_m})/E_m$, maximal antagonistic effect of a noncompetitive inhibitor, B', on the response to A expressed as a fraction of the maximal response of which the target tissue is capable

$[A]_{50}$, concentration of A which will evoke an E_{50} response

$[A]_{50B}$, concentration of A which will evoke an E_{50} response in the presence of a given concentration of B

$[B]_{50}$, the concentration of B which establishes the ratio $[A]_{50B}/[A]_{50} = 2$

$[B']_{50}$, the concentration of B' which elicits a half-maximal synergistic or noncompetitive inhibitory effect

K_A, K_B, dissociation constants for the complexes of A or B and their receptors

pD_2, $-\log [A]_{50}$, where A is an agonist

pA_2, $-\log [B]_{50}$, where B is a competitive antagonist

pS_2, $-\log [B']_{50}$, where B' is a synergist

pD_2', $-\log [B']_{50}$, where B' is a noncompetitive antagonist

Reagents and Procedures for Measuring Water Permeability of the Toad Bladder

Toads, *Bufo marinus* L. (National Reagents Inc., Bridgeport, Conn.), weighing from 150–250 g were kept on moist peat moss. Specimens of both sexes were pithed and
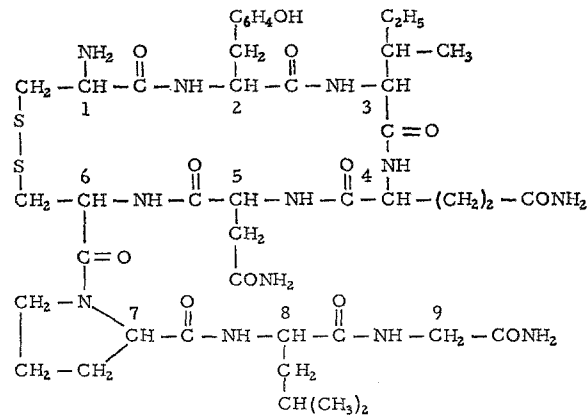

FIGURE 1.   Structure of oxytocin. Numbers indicate the position of the individual amino acid residues.

their urinary bladders placed in Ringer fluid of the following composition (mM): NaCl, 110; KCl, 3; $CaCl_2$, 1; $MgCl_2$, 1; $NaHCO_3$, 3; dextrose, 5.5; tris(hydroxymethyl)aminomethane hydrochloride (Trizma),® 10; pH = 8.4; tonicity, 245 ± 3 milliosmols per liter.

The following compounds were employed in this study: [8-arginine]-vasopressin (15), oxytocin (16, 17) (Fig. 1), crystalline [1-β-mercaptopropionic acid]-oxytocin (deamino-oxytocin) (19), [5-ornithine]-oxytocin (22), [2-O-methyltyrosine]-oxytocin (25, 27), [2-O-ethyltyrosine]-oxytocin (46), [4-leucine]-mesotocin, [4-leucine-8-isoleucine]-oxytocin (36), prostaglandin E_1 (PGE₁) (Upjohn Co., Kalamazoo, Mich.), cyclic 3′,5′-adenosine monophosphate (cyclic AMP), N^6-2′-O-dibutyryl cyclic 3′,5′-adenosine monophosphate (Schwarz Bio Research, Inc., Orangeburg, N.Y.), and theophylline. The neurohypophyseal hormones and synthetic analogues were checked for their physical properties (specific optical rotation), purity (thin-layer chroma-

tography), and biological activity (fowl vasodepressor assay [29]) just prior to use; the data obtained agreed with those reported for these compounds in the references cited above. For estimating the permeability to water of the toad urinary bladder the sac preparation of Bentley (9) was employed with minor modifications. In brief, hemibladders of comparable sizes are tied to the end of glass tubes so that the mucosa forms the inside of a sac which is filled with 7 ml of dilute Ringer fluid (50 milliosmols per liter). Four or more bladders are suspended in a 100 ml bath of full strength Ringer fluid stirred with an air jet. After a 2 hr period of incubation in this solution, bladders are transferred to the challenge bath. The rate at which water escapes from the mucosal fluid through the bladder wall is monitored by removing the sac preparation from the serosal bath and weighing it at intervals of 30 min. After each weighing, bladders are emptied and filled with fresh dilute Ringer fluid, reweighed immediately, and placed into a fresh bath.

General Protocol for Determination of the E_{50}

The half-maximal hydroosmotic response of a hemibladder to a given agonist (or an agonist in combination with an inhibitor or synergist) is determined in the following manner: the hemibladder is exposed for the first 30 min interval to a low concentration of agonist (a concentration which will evoke a 10–40% response), then for a second 30 min interval to twice that concentration, and finally for a third 30 min interval to a concentration above that known to be needed for a maximal effect. The rate of weight loss of the bladder following the two submaximal challenges is expressed as a fraction of the maximal response; and the concentration of agonist corresponding to the half-maximal response, $[A]_{50}$ or $[A]_{50B}$, is determined by interpolation on a logarithmic scale between the two submaximal response values as illustrated in Fig. 2. In several experiments the response of the hemibladder was tested at more than three concentrations of agonist. Bladders in which the initial low concentration of agonist evoked a response in excess of 50% were discarded.

Potencies of Agonists (pD_2)

The interaction of a hormone (A) with its receptor (R) can be represented by models based on the mass action law or the Langmuir adsorption isotherm which describe the probability of a given number of molecules of A interacting with a given number of molecules of R:

$$[A] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [AR]; \qquad \frac{k_2}{k_1} = K_A$$
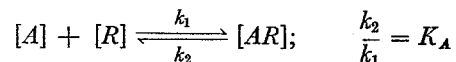
The interaction of A with R is assumed to result in a stimulus and, according to current pharmacological concepts concerning occupancy of receptor sites (3, 20, 45), the stimulus, S_A, generated as a consequence of an agonist, A, interacting with its specific receptor can be quantitated as follows:

$$S_A/S_m = \frac{\alpha}{1 + K_A/A} \qquad (44)$$

Stimulation expressed as a fraction of the maximal possible stimulus (S_A/S_m) is dependent upon the concentration of compound A, the dissociation constant of the

agonist receptor complex (K_A), and the proportionality constant (α), relating the degree of receptor occupation by A to the degree of stimulus formation. The constant, α, is referred to as intrinsic activity of A (2). The equation given above assumes that the agonist, A, reacts with a single type of receptor or a set of receptors
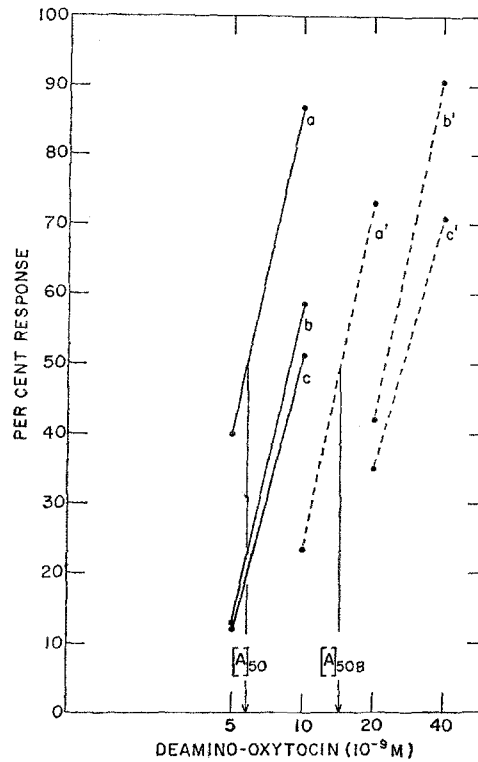

FIGURE 2. Method for determining $[A]_{50}$ and $[A]_{50B}$ values. The hydroosmotic responses of three sets of contralateral hemibladders to a given concentration of de-amino-oxytocin alone (●——●) and a deamino-oxytocin–[2-O-ethyltyrosine]–oxytocin mixture (●——●). Deamino-oxytocin alone is employed at two levels of concentration, the lower concentration selected to evoke less than the half-maximal response (E_{50}) of the bladder and the higher concentration being twice that of the lower. The response is plotted against the logarithm of the deamino-oxytocin concentration. The concentration of deamino-oxytocin which evokes a half-maximal response of one hemibladder (bladder a, b, or c) is read off the abscissa as the $[A]_{50}$ value. The concentration of deamino-oxytocin which, in the presence of inhibitor, evokes a half-maximal response in the contralateral hemibladder (bladder a', b', or c', respectively) is read off the abscissa as the $[A]_{50B}$ value.

with similar K_A values. The applicability of this assumption to the receptors associated with the hydroosmotic response of the toad bladder is supported by a detailed analysis of the dose-response relationships of several neurohypophyseal hormones and synthetic analogues reported previously (18).

When the relationship between stimulus and final effect is linear, the concentra-

tration of agonist which will evoke a half-maximal response ($[A]_{50}$) equals K_A and the binding constant (affinity) of A for its receptor is given by $1/K_A$. The negative logarithm of the concentration of agonist that will evoke a half-maximal effect is referred to as its pD_2 value (5, 28). With these considerations in mind we derived pD_2 values for a number of neurohypophyseal peptides on the toad bladder. However, it will be shown in this paper that the bladder exhibits threshold and receptor-reserve phenomena, so that, strictly speaking, a 50 % hydroosmotic response usually does not imply 50 % receptor occupation by the nonapeptide. Only when the number of receptor sites below threshold equals the receptor sites that constitute the receptor-reserve will $[A]_{50}$ equal K_A—an assumption which cannot be made a priori for the interaction of a given agonist with the toad bladder. Therefore, when the receptor-reserve exceeds the subthreshold, the K_A of an agonist will be underestimated, although the resultant error in the pD_2 value is minimized by the fact that the slopes of dose-response curves are steep; i.e., a large change in response (ordinate) is associated with a relatively small change in the concentration of agonist (abscissa).

Since bladders from different toads vary considerably in their sensitivity to a given challenge, it is advisable to compare the concentration of an unknown agonist which will evoke a half-maximal hydroosmotic response, i.e. its $[A]_{50}$ value, in one hemibladder to the concentration of a standard which will evoke the same response in the contralateral hemibladder of the same toad. We have employed crystalline deamino-oxytocin as a reference standard (18) and have found the $[A]_{50}$ value for this standard to be 5.53×10^{-9} M $\pm$ 0.34 (sem).[1] The $[A]_{50}$ value for the unknown substance is then normalized in the following manner:

$$\text{Normalized } [A]_{50} \text{ unknown} = 5.53 \times 10^{-9} \text{ M} \times \frac{[A]_{50} \text{ unknown}}{[A]_{50} \text{ deamino-oxytocin}}$$

The pD_2 value of the unknown agonist is then equal to the negative logarithm of the normalized $[A]_{50}$.

Potencies of Competitive Antagonists (pA_2)

When an antagonist B competes with an agonist A for the same receptor system, the dose-response curve for A will be shifted in a parallel manner progressively to the right along the log-dose axis as the concentration of B is increased. Since the antagonism is surmountable, increasing concentrations of agonist will result in a response. The magnitude (7) by which the dose-response curve is shifted at E_{50} is given by:

$$\frac{[A]_{50B}}{[A]_{50}} = 1 + \frac{[B]}{K_B}$$

The fraction $[A]_{50B}/[A]_{50}$ denotes the extent by which the competitive antagonist B displaces the midpoint of the concentration-response curve of agonist A to the right (see Fig. 3). The concentration of competitive inhibitor which produces a twofold shift of the dose-response curve of the agonist to higher concentrations is related to the dissociation constant of the antagonist-receptor complex ($[B] = [B]_{50} = K_B$). The

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[1] This value was obtained from dose-response curves on 86 toads, both sexes, including studies carried out in all seasons.

negative logarithm of $[B]_{50}$ has been referred to as pA_2 by Schild (40). The parallel shift of the dose-response curve is a consequence of a parallel shift in the dose-stimulus curve; therefore, threshold or reserve phenomena do not influence this displacement (4).

TABLE I

DETERMINATION OF THE pA_2 VALUE
FOR [2-O-ETHYLTYROSINE]-OXYTOCIN

Agonist: Deamino-oxytocin, A
Antagonist: [2-O-Ethyltyrosine]-oxytocin, B

Toad No.	$[A]_{50}$* × 10⁻⁹M	$[A]_{50B}$* × 10⁻⁹M	$\dfrac{[A]_{50B}}{[A]_{50}}$
		when $[B] = 1.0 \times 10^{-7}$M	
1	7.7	10.5	1.36
2	8.2	11.2	1.36
3	6.4	10.2	1.59
4	7.2	9.2	1.27
5	6.8	12.2	1.79
6	7.6	12.2	1.60
7	6.8	12.8	1.88
8	6.8	11.0	1.61
		Average	1.56 ± 0.08
		when $[B] = 2.5 \times 10^{-7}$M	
9	8.9	22.3	2.51
10	6.6	15.6	2.36
11	6.6	16.5	2.50
12	7.6	18.5	2.43
13	8.0	19.6	2.45
14	5.8	14.5	2.50
15	5.6	13.6	2.43
16	9.8	26.8	2.73
		Average	2.49 ± 0.04

$[A]_{50} = 7.27 \times 10^{-9}$M ± 0.27. $[B]_{50} = 1.55 \times 10^{-7}$ M ± 0.10‡. $pA_2 = 6.81 ± 0.03$.§
* Values of $[A]_{50}$ and $[A]_{50B}$ determined graphically as illustrated in Fig. 2.

‡ When $\dfrac{[A]_{50B}}{[A]_{50}} = 2.00$; $[B] = [B]_{50} = 1.55 \times 10^{-7}$ M ± 0.10 (SEM measured graphically as described in text).

§ $pA_2 = -\log 1.55 \times 10^{-7}$ M $= 6.81 ± 0.03$.

With respect to the hydroosmotic activity in the toad bladder the affinity constant of a competitive inhibitor is best evaluated by determining the $[A]_{50}$ value of the reference agonist in one hemibladder and an $[A]_{50B}$ value for any given concentration of antagonist B in the contralateral hemibladder (see Fig. 2). This procedure is repeated on fresh hemibladders with a different concentration of B. The concentration of B is varied in such a manner that at least one value for the ratio, $[A]_{50B}/$

$[A]_{50}$, is obtained which is greater and one value which is less than 2. The concentration of B which produces a twofold shift of the dose-response curve, $[B]_{50}$, is determined by plotting the ratio of $[A]_{50B}/[A]_{50}$ on the ordinate vs. the log of $[B]$ on the abscissa and reading off the value of $[B]$ corresponding to $[A]_{50B}/[A]_{50} = 2$. The standard error for the mean $[B]_{50}$ value was measured graphically by (a) drawing a line parallel to the abscissa through the point on the ordinate corresponding to $[A]_{50B}/[A]_{50} = 2$ and (b) connecting upper and lower limits respectively of the SEM values for fractions $[A]_{50B}/[A]_{50}$. The limits of the SEM for $[B]_{50}$ are then given by the points of intersection of curves (a) and (b), values which are read off on the abscissa.

The procedure for measuring pA_2 values is illustrated in Table I with [2-*O*-ethyltyrosine]-oxytocin, which competitively inhibits the hydroosmotic action of deamino-oxytocin. Unlike the pD_2 values for different agonists, the pA_2 values for different antagonists have not been normalized, since both hemibladders of the toad are required for determining the pA_2 value of a given antagonist. However, $[A]_{50B}$ values for different concentrations of B have been normalized with respect to the $[A]_{50}$ value of a given agonist (see Fig. 3) as follows:[2]

$$\text{Normalized } [A]_{50B} = \text{average } [A]_{50} \times \frac{[A]_{50B}}{[A]_{50}} .$$

Potencies of Synergists (pS_2) *and Noncompetitive Antagonists* (pD_2')

A stimulus generated as a consequence of an agonist interacting with its primary receptor may be amplified or dampened by the action of substances influencing any step other than the initial agonist-receptor interaction. It is useful to define the potency of such substances, synergist or noncompetitive antagonist, by the concentration which will evoke a half-maximal synergistic or antagonistic effect, and then to express that concentration in terms of the negative logarithm.

The following protocol was employed for the evaluation of synergists: a control set of hemibladders is challenged at 30 min intervals with three or four successively increasing concentrations of agonist alone. The experimental set of hemibladders from the same toads is also challenged with increasing concentrations of agonist, but in the presence of a fixed concentration of synergist. For each concentration of agonist to be tested fresh mucosal and serosal baths are used; for each level of synergist to be tested, fresh hemibladders are employed for both the control and the experimental sets. The concentration of synergist is raised in separate experiments until a further increase in concentration will not evoke a further increase in response. The maximal synergistic effect, (h_m), is given by the vertical[3] distance between the steep portion of

[2] For example with the agonist deamino-oxytocin ($[A]_{50} = 7.27 \times 10^{-9}$ M $\pm$ 0.27) and the antagonist [2-*O*-ethyltyrosine]-oxytocin with toad 1 in Table I:

$$\text{Normalized } [A]_{50B} = 7.27 \times 10^{-9} \text{ M} \times \frac{10.5 \times 10^{-9} \text{ M}}{7.7 \times 10^{-9} \text{ M}} = 9.89 \times 10^{-9} \text{ M}.$$

[3] We have chosen in this study to evaluate the synergistic effect by measuring the vertical rather than the horizontal distance between concentration-response curves, because we have interpreted the interaction between adenyl cyclase activators and phosphodiesterase inhibitors in terms of a virtual increase in intrinsic activity rather than in affinity.

the log-dose response curve of the reference agonist A alone and that of the agonist in the presence of saturating concentrations of synergist evaluated in matched hemibladders. The h_m value depicted in Fig. 4 represents the average of several h_m values each derived from individual pairs of matched hemibladders. The concentration of synergist that will yield a half-maximal synergistic effect, $h_m/2$, is determined by interpolation between response values just above and below $h_m/2$. We have referred to the negative logarithm of the molar concentration of the synergist which will elicit a half-maximal effect as its pS_2 value; i.e., $pS_2 = -\log[B']_{50}$.

The concentration of a noncompetitive antagonist which will produce half-maximal inhibition is determined as follows: The protocol for obtaining families of dose-response curves is identical to that employed for the evaluation of synergists. The maximal effect of the antagonist is most accurately measured in combination with a partial agonist, i.e. an agonist which is incapable of eliciting the maximal hydroosmotic response of which the bladder is capable ($E_{A_m}/E_m < 1.0$); the maximal antagonistic effect, h_m', is given by the difference in maximal responses to saturating concentrations of partial agonist alone and in the presence of saturating concentrations of antagonist as well (see Fig. 5 A and B). The negative logarithm of the concentration of the noncompetitive antagonist for half-maximal inhibition has been termed the pD_2' value; i.e., $pD_2' = -\log[B']_{50}$ (5).

RESULTS

Competitive Antagonism with [2-O-Ethyltyrosine]-oxytocin and [4-Leucine]-mesotocin

[2-O-Ethyltyrosine]-oxytocin and [4-leucine]-mesotocin failed to increase the osmotic flow of water across the toad bladder, when added to the serosal medium to give concentration levels as high as 10^{-5} M. However, both these agents inhibited the hydroosmotic action of active neurohypophyseal principles. The log dose-response curves of deamino-oxytocin, [8-arginine]-vasopressin, [2-O-methyltyrosine]-oxytocin, and [5-ornithine]-oxytocin were shifted to the right progressively and in a parallel manner by increasing concentrations of [2-O-ethyltyrosine]-oxytocin. This effect of [2-O-ethyltyrosine]-oxytocin is illustrated in Fig. 3, where deamino-oxytocin served as the reference agonist. As can be seen in Table II, we selected neurohypophyseal peptide agonists with a wide spectrum of affinities, ranging in their pD_2 values from 4.92 ± 0.04 for [5-ornithine]-oxytocin to 9.40 ± 0.07 for [8-arginine]-vasopressin. However, the affinity of the antagonist [2-O-ethyltyrosine]-oxytocin was largely independent of the potency of the agonists as manifested by the finding that the pA_2 value for (2-O-ethyltyrosine]-oxytocin varied only between 6.60 ± 0.04 and 7.22 ± 0.04 (Table II). Because of the small amount of [4-leucine]-mesotocin available, its pA_2 value was only studied with respect to one reference agonist, oxytocin, and found to be 5.94 ± 0.05. The inhibitory action of both antagonists on the hydroosmotic response of the bladder was reversible and specific for the neurohypophyseal
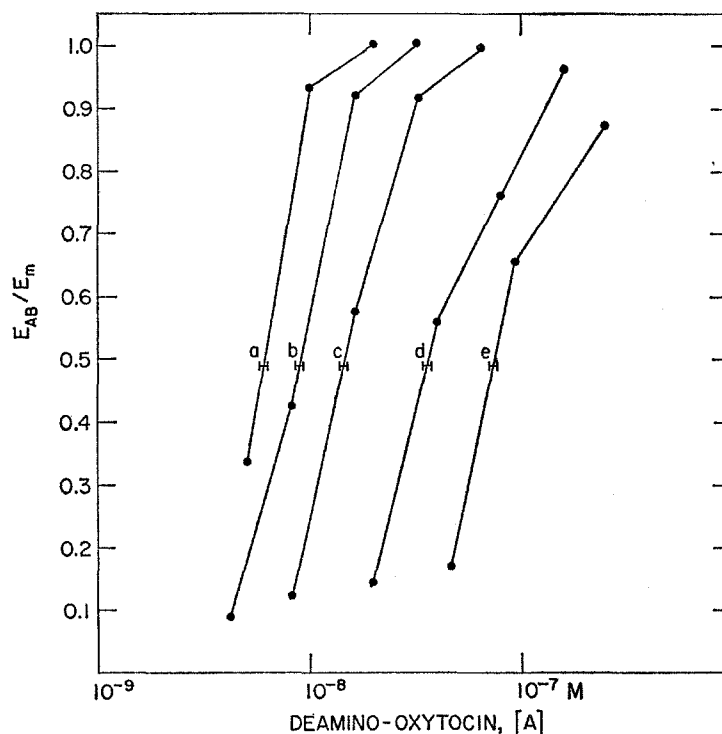
FIGURE 3. Inhibition of the hydroosmotic response of the toad bladder to deamino-oxytocin caused by increasing concentrations of [2-O-ethyltyrosine]-oxytocin [B]. Curve a represents the response of the bladder in relation to the logarithm of the concentration of deamino-oxytocin [A] in the absence of inhibitor; curves b, c, d, and e represent the response of the bladder to deamino-oxytocin in the presence of 1.0, 2.5, 5.0, and 20.0 × 10^{-7} M [2-O-ethyltyrosine]-oxytocin, respectively. Curves b, c, d, and e are normalized with respect to the midpoint of curve a and the midpoint of curves b, c, d, and e is shifted to the right by a factor of: 1.56 ± 0.08 (SEM), 2.49 ± 0.04, 6.33 ± 0.10, and 10.90 ± 0.11, respectively. Each curve represents the average response of eight fresh hemi-bladders.

TABLE II

COMPETITIVE INHIBITION OF SOME
NEUROHYPOPHYSEAL PEPTIDES BY [2-O-ETHYLTYROSINE]-OXYTOCIN
AND [4-LEUCINE]-MESOTOCIN

Reference agonist	pD_2 of reference agonist (±SEM)	pA_2 of [2-O-Ethyltyrosine)-oxytocin (±SEM)	pA_2 of [4-Leucine]-mesotocin (±SEM)
[8-Arginine]-vasopressin*	9.40 ± 0.07	6.66 ± 0.05	—
Oxytocin*	8.63 ± 0.02	6.85 ± 0.03	5.94 ± 0.05
Deamino-oxytocin	8.25 ± 0.03	6.81 ± 0.03	—
[2-O-Methyltyrosine]-oxytocin‡	7.09 ± 0.05	7.22 ± 0.04	—
[5-Ornithine]-oxytocin§	4.92 ± 0.04	6.60 ± 0.04	—
	Average	6.83 ± 0.11	

* pD, values calculated on the basis of data reported by Eggena et al. (18).

‡ The pD_2 value of 7.09 corresponds to an $[A]_{50}$ value of 1.23 × 10^{-7} M. The $[A]_{50}$ which we reported earlier (18) is superceded.

§ Data reported by Havran et al. (22).

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hormonal receptor because they did not diminish the hydroosmotic action of cyclic AMP and theophylline; indeed the effect of theophylline was potentiated (see below).

Synergism of [2-O-Ethyltyrosine]-oxytocin and Theophylline

While [2-O-ethyltyrosine]-oxytocin competitively antagonized the action of neurohypophyseal hormones, this analogue was found to facilitate the perme-
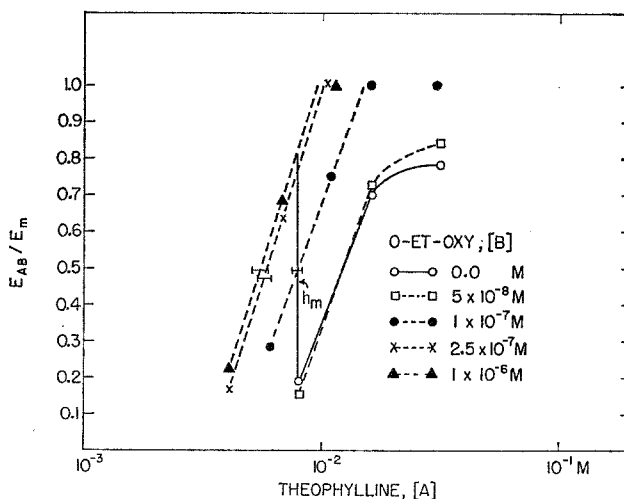

FIGURE 4. Synergism of theophylline and [2-O-ethyltyrosine]-oxytocin. The log dose-response curve for theophylline alone (o——o) is compared with the log dose-response curve for theophylline in the presence of 5 × 10⁻⁸ M (□----□), 1 × 10⁻⁷ M (●----●), 2.5 × 10⁻⁷ M (×----×), and 1 × 10⁻⁶ M (▲----▲) [2-O-ethyltyrosine]-oxytocin (O-Et-Oxy), respectively. The synergistic effect of maximal concentrations of [2-O-ethyltyrosine]-oxytocin is given by h_m. Each curve represents an average of six or more experiments on fresh hemibladders. All curves have been normalized with respect to the control curve (o——o). Standard errors (SEM) are indicated for mean concentrations of theophylline which will evoke E_{50} responses; see text for SEM of h_m.

ability response of the bladder to theophylline. In the presence of increasing concentrations of [2-O-ethyltyrosine]-oxytocin (5.0 × 10⁻⁸ to 2.5 × 10⁻⁷ M) the midpoint of the dose-response curve for theophylline was shifted to the left along the log-dose axis and there was a 22% increase in maximum response (Fig. 4). A further increase in the concentration of [2-O-ethyltyrosine]-oxytocin to 1.0 × 10⁻⁶ M was without significant effect. The maximal synergistic effect, h_m, amounts to 0.60 ± 0.04 (SEM), and the concentration of [2-O-ethyltyrosine]-oxytocin which elicits a half-maximal synergistic effect (i.e. $h_m/2$ = 0.30) was determined to be 1.0 × 10⁻⁷ M; consequently, its pS_2 value amounts to 7.00 ± 0.05 (SEM).

Noncompetitive Antagonist: Prostaglandin E_1

In confirmation of the findings of others (34) PGE_1 per se did not increase the bladder permeability to water when tested in the serosal bath at concentrations as high as 2.86 $\times$ 10^{-6} M. PGE_1 also proved to be a potent inhibitor of the hydroosmotic action of neurohypophyseal peptides and theophylline, but only minimally influenced the action of dibutyryl cyclic AMP (as shown in Fig. 5 D, a maximal response to dibutyryl cyclic AMP of 0.80 $\pm$ 0.04 (SEM)[4] was depressed to 0.72 $\pm$ 0.05 by 2.9 $\times$ 10^{-7} M PGE_1). The dose-response curves of both theophylline and of [2-O-methyltyrosine]-oxytocin are influenced similarly by PGE_1.

As shown in panel A of Fig. 5, the maximal response to the partial agonist [2-O-methyltyrosine]-oxytocin, unlike the response to oxytocin, is progressively depressed from 0.88 $\pm$ 0.03 (SEM) to a minimal value of 0.10 $\pm$ 0.01 (SEM) as the concentration of PGE_1 is increased. The $[B']_{50}$ of PGE_1 in the case of [2-O-methyltyrosine]-oxytocin amounted to 5.1 $\times$ 10^{-10} M which corresponds to a pD_2' value of 9.29 $\pm$ 0.05 (SEM).

Similarly, the inhibition by PGE_1 of the theophylline-induced hydroosmotic response is characterized by a marked reduction of the maximal response from 0.78 $\pm$ 0.04 (SEM) to 0.10 $\pm$ 0.01 (SEM) (Fig. 5 B). In this case the $[B']_{50}$ of PGE_1 was found to be 5.4 $\times$ 10^{-10} M which corresponds to a pD_2' value of 9.27 $\pm$ 0.07 (SEM).

The dose-response curves of theophylline and of [2-O-methyltyrosine]-oxytocin are clearly influenced in a similar fashion by PGE_1; however, the response to oxytocin appears at first glance to be influenced differently by PGE_1. The results of a representative experiment are illustrated in Fig. 5 C. When PGE_1 was studied in the presence of oxytocin, an agonist that can evoke the maximum response of which the bladder is capable, the dose-response curve of the hormone was shifted to the right along the log-dose axis ultimately with a diminution in the maximal response averaging 0.77 $\pm$ 0.10. Both the shift of the dose-response curves and the PGE_1-induced diminution of the maximal response to oxytocin exhibited considerable variation. Finally, we compared the effect of PGE_1 on the synergistic response of the combination of cyclic AMP and theophylline as well as on the synergistic response of the combination of oxytocin and theophylline. PGE_1 was employed at a concentration of 2.9 $\times$ 10^{-7} M which is sufficiently high to inhibit completely the responses of the bladder to a low concentration of either oxytocin or theophylline. As noted above, PGE_1 has little effect on the action of exogenous dibutyryl cyclic AMP on the bladder. Whereas the near maximal hydroosmotic

[4] It should be noted that maximal concentrations of exogenous dibutyryl cyclic AMP gave a maximal hydroosmotic response somewhat lower than that obtained with maximal concentrations of deamino-oxytocin.

response evoked by the oxytocin-theophylline combination was reduced by PGE_1 to almost basal levels, PGE_1 reduced the response to the cyclic AMP-theophylline combination only minimally (Fig. 6).
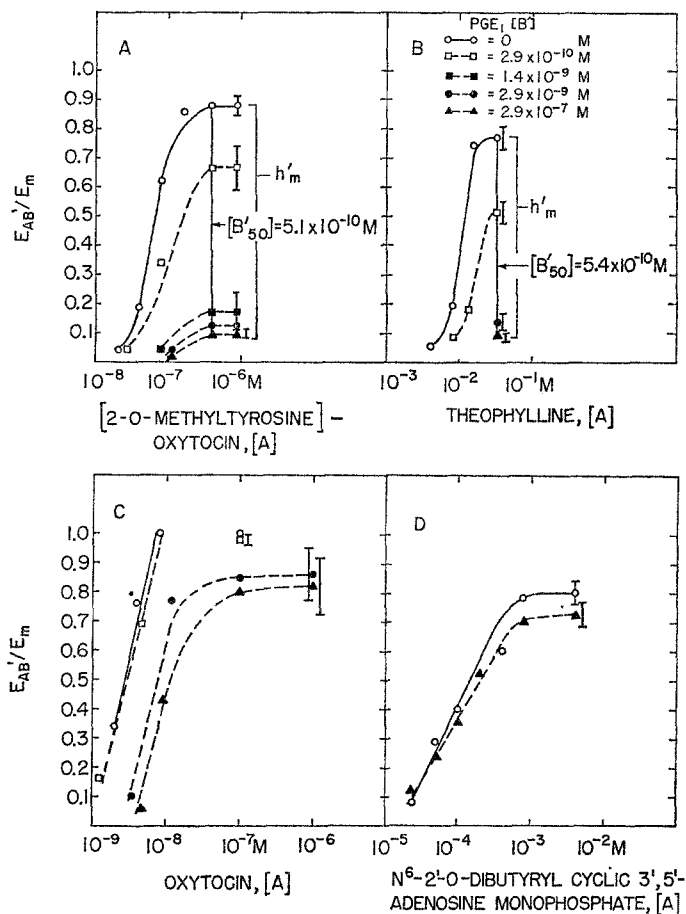

FIGURE 5.   Inhibitory action of prostaglandin (PGE_1) on the hydroosmotic response of the toad bladder to the following agonists: (A) [2-O-methyltyrosine]-oxytocin, (B) theophylline, (C) oxytocin, (D) N^6-$2'$-O-dibutyryl cyclic $3',5'$-adenosine monophosphate. Log dose-response of agonist A alone (o⎯⎯o) and in the presence of increasing concentrations of PGE_1, B' (2.9 × 10^{-10} M, ⎕-----⎕; 1.4 × 10^{-9} M, ■----■; 2.9 × 10^{-9} M, ●----●; 2.9 × 10^{-7} M, ▲----▲). The maximal antagonism by PGE_1 is given by h_m' and the concentration of PGE_1 associated with half-maximal antagonism is given by the $[B']_{50}$ value. Each point represents the average of six or more experiments. The dose-response curves of the agonists in the presence of PGE_1 have been normalized with respect to the response curve for the agonist alone. The per cent maximal hydroosmotic response reached with saturating concentrations of these agonists, E_{Am}/E_m, has been determined in separate experiments by direct comparison with deamino-oxytocin in contralateral hemibladders. Standard errors (SEM) are given for the responses obtained with the highest concentrations of agonists tested.
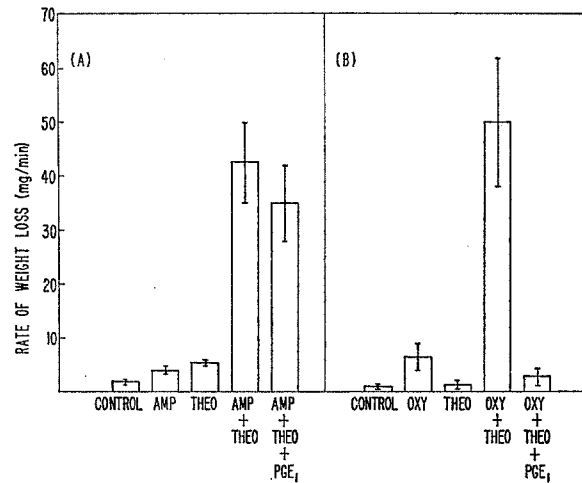
FIGURE 6. Effect of prostaglandin (PGE$_1$) on (A) the response of the bladder to a theophylline-cyclic AMP combination (THEO-AMP) and on (B) the response of the bladder to a theophylline-oxytocin combination (THEO-OXY). (A) Initially two sets of hemibladders were kept for 30 min in plain Ringer fluid (control); during the following 30 min period one set of hemibladders was exposed to 2.5 × 10^{-3} M AMP and the contralateral set of hemibladders was exposed to 4 × 10^{-3} M THEO. For the last 30 min interval one of the two sets of hemibladders was exposed to the combination of 2.5 × 10^{-3} M AMP and 4 × 10^{-3} M THEO, while the contralateral set was exposed to a medium containing 2.5 × 10^{-3} M AMP, 4 × 10^{-3} M THEO, and 2.9 × 10^{-7} M PGE$_1$. (B) The experimental procedure was identical to that used in (A); however, in these experiments 1 × 10^{-9} M OXY was substituted for AMP. The graph depicts the mean and standard error of the mean (SEM) of values obtained in eight experiments.

DISCUSSION

The water permeability response of the toad urinary bladder is rather specific in that only three types of compounds have to date been found capable of eliciting this effect reversibly: neurohypophyseal hormones (9, 23, 41), methylxanthines, and cyclic AMP (33). There is considerable evidence that cyclic AMP is involved in the mediation of the action of neurohypophyseal hormones as well as of theophylline (32). Both the hormones and theophylline raise the intracellular level of cyclic AMP; the former by stimulating the adenyl cyclase system, the latter by inhibiting cyclic nucleotide phosphodiesterase, the enzyme which inactivates cyclic AMP by hydrolysis to give 5'-AMP (13, 21).

In the present study we are exploring the interplay of neurohypophyseal hormonal peptides, theophylline, and prostaglandin E$_1$ (PGE$_1$)—all of which affect the intracellular level of cyclic AMP. Specifically, we are investigating whether each increment of adenyl cyclase activity can be translated into an increment of hydroosmotic activity, or whether steps subsequent to adenyl

cyclase activation are limiting in the over-all response of the tissue to hormone.

In this context we studied a series of neurohypophyseal peptides (oxytocin, [2-O-methyltyrosine]-oxytocin, and [2-O-ethyltyrosine]-oxytocin) possessing different intrinsic ("catalytic") activities with respect to their ability to evoke the hydroosmotic response. The two latter compounds had already been shown to exhibit inhibitory properties or decreased maximal effects in several other hormone-sensitive tissues (37); moreover, in an earlier investigation we had observed a diminution in the intrinsic hydroosmotic activity of [2-O-methyltyrosine]-oxytocin on the toad bladder (18).

We noted (18) that the replacement of the hydroxy radical in tyrosine by a methoxy moiety results in a decline in the maximal hydroosmotic response from 100% to 88%. The replacement of the hydroxy moiety by the ethoxy radical abolished the response completely, a finding which could be due on the one hand to the inability of this analogue to bind to the receptor at the concentrations used, or, on the other hand, to the inability of the hormone-receptor complex to catalyze a permeability response. In the latter case [2-O-ethyltyrosine]-oxytocin should competitively inhibit the bladder response to oxytocin and other active analogues. We therefore studied the effect of [2-O-ethyltyrosine]-oxytocin on the dose-response relationship of oxytocin, dea-mino-oxytocin, [8-arginine]-vasopressin, [5-ornithine]-oxytocin, and [2-O-methyltyrosine]-oxytocin, a group of agonists which vary greatly in their affinity constants for the receptor. It was established that [2-O-ethyltyrosine]-oxytocin was an equipotent competitive inhibitor of all these agonists as indicated by the minimal variation in the pA_2 value (see Table II).

The fact that the binding constant of [2-O-ethyltyrosine]-oxytocin (indicated by a pA_2 = 6.83 ± 0.11 (sem)) is the same within the limits of experimental error as that of [2-O-methyltyrosine]-oxytocin (indicated by a pD_2 = 7.09 ± 0.05 (sem))—although the maximal hydroosmotic activity of these two analogues was found to be 0 and 88%, respectively—shows that the structural alteration of the tyrosine residue resulted in a selective change in the catalytic function of this hormonal principle. Thus the intrinsic activities of oxytocin, [2-O-methyltyrosine]-oxytocin, and [2-O-ethyltyrosine]-oxytocin decline progressively resulting in a functional mutation of the hormone from agonist to partial agonist to competitive antagonist.

In this context it is of interest that the graded properties of partial agonism shown by [2-O-methyltyrosine]-oxytocin and [2-O-ethyltyrosine]-oxytocin in the toad bladder system have an exact parallel in the action of these peptides as well as in the action of the corresponding analogues of [8-lysine]-vasopressin, on the isolated rat uterus and on the isolated rattail artery (38).

In addition to the above studies, we found that [4-leucine]-mesotocin (36)—an analogue of mesotocin (1, 39) in which the glutamine residue in position 4 is replaced by a leucine residue—did not evoke a hydroosmotic response,

but inhibited competitively the action of oxytocin. In the light of this finding, it would be of interest to investigate whether the analogue with an analogous replacement in the oxytocin series, namely [4-leucine]-oxytocin (14), has comparable antagonistic properties in the hydroosmotic assay.[5]

Thus, [2-*O*-ethyltyrosine]-oxytocin and [4-leucine]-mesotocin appear to bind to the hormonal receptor but do not evoke the hydroosmotic effect. However, the absence of a final effect need not imply that the interaction of the peptide with its receptor had not produced an increase in the activity of adenyl cyclase because cyclic AMP production may be increased above the basal rate and yet be insufficient to assure the minimum intracellular level (threshold) needed to trigger the event(s) leading to a hydroosmotic response. In order to determine whether the toad bladder exhibits such a threshold phenomenon (8, 6, 26 44) we studied the effect of [2-*O*-ethyltyrosine]-oxytocin on the bladder by using theophylline to retard the destruction of cyclic AMP and thereby to amplify any subthreshold effects which the analogue may have had on the adenyl cyclase system. The fact that the relationship of hydroosmotic activity to theophylline dosage is shifted to the left (Fig. 4) by the influence of [2-*O*-ethyltyrosine]-oxytocin shows that the latter compound can act as a synergist of theophylline. It seems likely that this synergism is a consequence of adenyl cyclase activation by [2-*O*-ethyltyrosine]-oxytocin rather than a peptide-dependent increase of the effect of theophylline on phosphodiesterase; in the latter instance one would not expect the observed increase in the *maximal* response of the bladder to theophylline following addition of the peptide (Fig. 4).

In order to substantiate the idea that this synergism is a direct consequence of the interaction of [2-*O*-ethyltyrosine]-oxytocin with the same receptor responsible for adenyl cyclase activation by oxytocin, it was necessary to show for both the antagonistic and the synergistic actions of [2-*O*-ethyltyrosine]-oxytocin identity of binding constants, a criterion indicative of receptor identity. For this purpose we determined the affinity constant of the synergist and referred to the logarithm of this constant as pS_2. As an antagonist [2-*O*-ethyltyrosine]-oxytocin proved to have an average pA_2 of 6.83 $\pm$ 0.11 (SEM), a value which closely approximates the pS_2 value of 7.00 $\pm$ 0.05 (SEM) found for its synergistic action. These results support the contention that [2-*O*-ethyltyrosine]-oxytocin—although incapable by itself at concentrations as high as 10^{-5} M of eliciting a hydroosmotic response—stimulates the adenyl cyclase enzyme system above its basal rate of activity. Moreover, this experiment suggests that low levels of adenyl cyclase stimulation are not translated into permeability changes and, accordingly, that the effector system, i.e. the

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[5] *Note Added in Proof* It has indeed recently been reported that this analogue competitively inhibits the hydroosmotic action of [8-arginine]-vasopressin and [8-arginine]-vasotocin in the toad bladder (Chiu, P.J.S. and W.H. Sawyer. 1970). *Amer. J. Physiol.* 218:838.

hydroosmotic permeability barrier of the toad bladder epithelium, exhibits a threshold with respect to cyclic AMP.

The activities of adenyl cyclase and phosphodiesterase in unstimulated target tissues are balanced so that the level of cyclic AMP is maintained below threshold; this may be physiologically significant in preventing fortuitous hydroosmotic responses to small fluctuations in enzyme activity and, thereby, serve to keep the target tissue under the control of its primary hormonal regulator.

It has recently become apparent that prostaglandins participate in a critical way in modulating the action of a number of hormones in different tissues (11, 24). Butcher and Baird (12) have shown that prostaglandin E_1 diminishes the lipolytic effect of several hormones by lowering hormone-dependent cyclic AMP levels in isolated fat cells of the rat, while in other tissues (hemidiaphragm, spleen, kidney, and testis) PGE_1 alone raises cyclic AMP levels. Others (10, 34) have postulated that prostaglandin may function as a feedback regulator on the cyclic AMP mechanism either by influencing the activity of adenyl cyclase or of phosphodiesterase. We therefore reexamined the inhibitory properties of PGE_1 on the hydroosmotic action of neurophyophyseal peptides, theophylline, and cyclic AMP and its dibutyryl derivative. In confirmation of the observations of Orloff, Handler, and Bergström (34), we found PGE_1 to be a potent inhibitor of neurohypophyseal hormones but not of cyclic AMP or its more potent dibutyryl derivative (Fig. 5 D). Under the experimental conditions employed theophylline was likewise inhibited by PGE_1. Contrary to the suggestion (31) that PGE_1 competes with vasopressin for a common receptor site, our findings indicated that PGE_1 behaves as a noncompetitive inhibitor of neurohypophyseal hormones and theophylline. The noncompetitive nature of this inhibition can be most clearly illustrated with a partial agonist for which a receptor reserve is excluded. Hence a stepwise elimination of receptors will result in a corresponding decline of the dose-response curve. This is shown in Fig. 5 A in which the maximal response of the bladder to the partial agonist, [2-O-methyltyrosine]-oxytocin, is progressively depressed with increasing doses of PGE_1, a finding which indicates that the inhibitory action of PGE_1 is insurmountable. Moreover, the maximal PGE_1-induced depressions of the log dose-response curves were similar for [2-O-methyltyrosine]-oxytocin and for theophylline (Fig. 5 B), and the affinity constants of PGE_1 for the "inhibited" receptor(s) were the same within the limits of error.

The inhibitory effect of PGE_1 on oxytocin (Fig. 5 C) appears at first sight to be competitive because increasing concentrations of PGE_1 cause apparently parallel shifts to higher concentrations of the oxytocin dose-response curve— suggesting that in the case of oxytocin the inhibitory action of PGE_1 may be
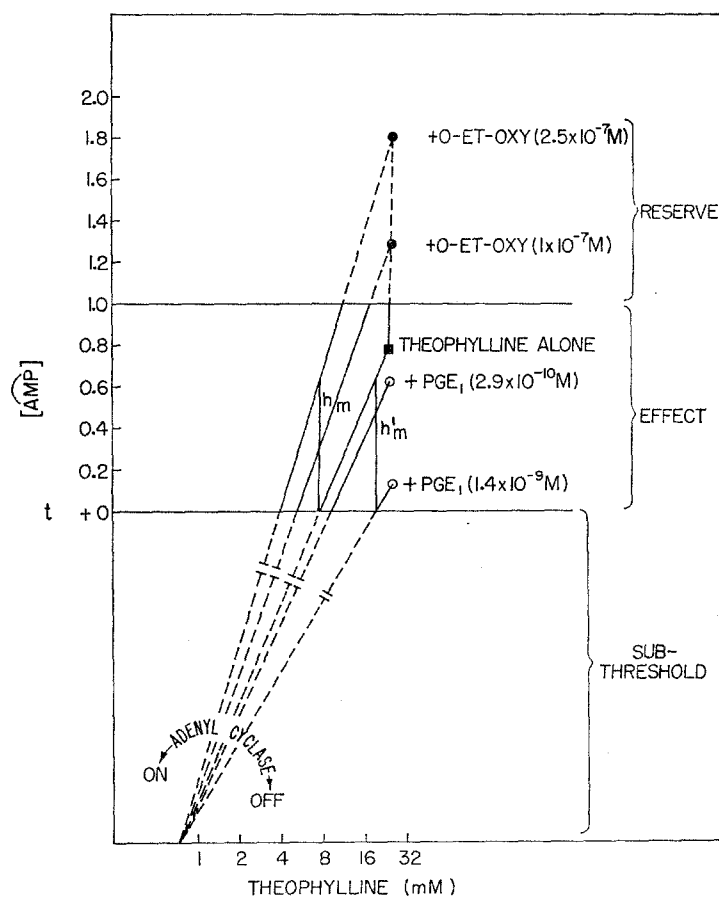
FIGURE 7. Interpretation of the relationship between the "second messenger" (AMP) and the final effector response (hydroosmosis) of the toad urinary bladder to neuro-hypophyseal peptides, theophylline, and prostaglandin E_1. The relation between the level of intracellular cyclic AMP (ordinate), and the theophylline-induced inhibition of cyclic nucleotide phosphodiesterase represented in terms of theophylline concentration (abscissa) suggests that this hormone-sensitive tissue exhibits a subthreshold and a reserve region with regard to the response to cyclic AMP. The coefficient of proportionality relating intracellular [AMP] and [Theophylline] is the adenyl cyclase activity. Thus the slope of the regression of [AMP] on .Theophylline] (■——■) will increase with adenyl cyclase stimulation by neurohypophyseal peptides (●——●) and decrease with adenyl cyclase inhibition by PGE_1 (o——o). The quantitative relationships shown in the subthreshold and reserve regions are derived by extrapolation from experimental data obtained in the effect region (Figs. 4 and 5B).

surmountable. But, unlike the action of a competitive inhibitor, the action of PGE_1 exhibits a saturation phenomenon (i.e. there is a limit to the degree to which PGE_1 can displace the oxytocin dose-response curve to the right [Fig. 5 C]). This observation, together with the evidence that oxytocin and

[2-O-methyltyrosine]-oxytocin act on the same receptors,[6] suggests that the inhibition of oxytocin by PGE_1 is also noncompetitive.

Assuming, therefore, that PGE_1 is indeed a noncompetitive inhibitor of oxytocin, the fact that maximal oxytocin concentrations in combination with maximal PGE_1 concentrations evoke an almost full hydroosmotic response can be explained if oxytocin at saturating concentrations generates a stimulus considerably in excess of that required for eliciting the maximal hydroosmotic response. These results are consistent with the view that oxytocin and certain other neurohypophyseal peptides are capable of evoking a maximal hydroosmotic response in the toad bladder with only partial occupation of the total number of receptors available—a phenomenon referred to as "receptor reserve" (6, 30, 42). Peptides which exhibit this phenomenon evidently can produce levels of intracellular cyclic AMP well above that required for a maximal response. Supramaximal levels of intracellular cyclic AMP can also be obtained with certain neurohypophyseal peptides which are themselves incapable of eliciting a hydroosmotic response provided that the degradation of the intracellular cyclic AMP formed is sufficiently retarded. Most probably this situation was encountered in the case of the synergism between [2-O-ethyltyrosine]-oxytocin and theophylline (Fig. 4). In the present study both processes, synergism and noncompetitive antagonism, appear to modulate the stimulus formation rather than the final effector process. The foregoing analysis and experimental findings are presented schematically in Fig. 7.

The last question investigated was whether PGE_1 acts directly on adenyl cyclase, phosphodiesterase, both, or neither. As can be seen from Fig. 5 A and B, PGE_1 possesses an identical binding constant for its inhibitory actions on the response to [2-O-methyltyrosine]-oxytocin and theophylline, findings which suggest that both inhibitory effects are probably exerted through an action of PGE_1 at the same receptor. This receptor must be functionally connected with adenyl cyclase because both exogenous cyclic AMP and its dibutyryl derivative are essentially unaffected by PGE_1. To obtain direct evidence on the question of whether or not PGE_1 also acts on the phosphodiesterase system, we compared the effect of PGE_1 on a theophylline-oxytocin combination vs. a theophylline–exogenous cyclic AMP combination (Fig. 6). The synergistic effect of the theophylline-cyclic AMP combination, which is independent of adenyl cyclase activity, but dependent upon 3′,5′-phosphodiesterase activity, was only minimally affected by the presence of PGE_1.

In conclusion, our studies of the graded catalytic activities of oxytocin and selected analogues have shown that the toad urinary bladder—with respect to its hydroosmotic reactivity—exhibits threshold and receptor reserve phenomena. Thus the stimulus-effect relationship in the mechanism of action of

[6] Equivalent pA_2 values for the competition of [2-O-ethyltyrosine]-oxytocin with oxytocin and with [2-O-methyltyrosine]-oxytocin (Table II) suggest that all three compounds act at the same receptor.

neurohypophyseal hormones is discontinuous inasmuch as an increasing stimulus in the subthreshold range evokes no response at all, and an increasing stimulus in the receptor-reserve range evokes no further increase in response. Therefore, the intrinsic activity of a hormonal peptide is better defined by its effect on the catalytic activity of adenyl cyclase, than by its final biological effect on membrane permeability.

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