# OXYTOCIN RECEPTOR IN FROG BLADDER EPITHELIAL CELLS
## Relationship of [$^3$H]oxytocin binding to adenylate cyclase activation

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## 1. Introduction

The presence on the isolated frog skin epithelium of two categories of [$^3$H]oxytocin binding sites with different affinities and maximum capacities has already been demonstrated [1]. It was shown that the high affinity binding sites (apparent $K_m = 3.0 \times 10^{-9}$ M) are probably involved in the initiation of the biological response (increase in sodium transport and water permeability). The low affinity binding sites (apparent $K_m = 5.0 \times 10^{-8}$ M) have a capacity 12 times higher but their eventual physiological role has still to be determined.

It seems very likely (e.g. see [2–4]) that both the biological effects of neurohypophysial peptides on amphibian skin and bladder epithelial cells are mediated through an increase in cyclic adenosine 3'-5' monophosphate (cyclic AMP) cell content.

The active hormonal concentrations on amphibian skin and bladder epithelial cells are very similar. Oxytocin stimulates the adenylate cyclase activity present in particulate fractions of frog or toad bladder epithelial cell homogenates [5–7]. However, the dose of oxytocin giving half maximum hormonal stimulation of adenylate cyclase ($1.5 \times 10^{-7}$ M) is sixty times higher than the concentration eliciting a half-maximum response in the intact structure ($3.0 \times 10^{-9}$ M) [1].

In order to decide if the receptors involved in adenylate cyclase activation correspond or not to the high affinity receptor sites detected on the intact structure, the binding of [$^3$H]oxytocin to the broken cell preparation was studied. It was found that both high and low affinity binding sites are present, but only the high affinity sites are involved in adenylate cyclase activation.

It is suggested that these two categories of sites correspond to those detected on the intact structure and that tissue homogenization and purification are responsible for the loss in affinity observed.

In addition the effects of calcium ions on the receptor–cyclase system were reexamined. Previous experiments [5] have shown that very low calcium concentrations ($10^{-6}$ M to $10^{-5}$ M) are required for hormonal stimulation, and that higher concentrations, from $10^{-5}$ to $10^{-3}$ M, reduce the maximum activation without changing the apparent affinity for the hormone. Two alternative explanations were given: either $Ca^{2+}$ ions could act by reducing the total number of accessible receptor sites, or it might reduce the effectiveness of the receptor–cyclase coupling. It is shown in this paper that calcium acts at the coupling step.

## 2. Materials and methods

### 2.1. *Materials*

Oxytocin (Oxy.) chlorhydrate (374 IU/mg) and lysine vasopressin (L.V.P.) chlorhydrate (285 IU/mg) were kindly donated by Dr. Boissonnas of Sandoz, [$\alpha$-$^{32}$P]ATP (sodium salt, 880 to 1027 mCi per mmole) stored at −25° in 50% ethanol was purchased from the Commissariat à l'Energie Atomique (Saclay France). The [$^3$H]oxytocin used was labelled by Pradelles et al. as previously described [8]. Its specific radioactivity was 24 Ci/mmole and its radiochemical and biological purity was 100% [1].

### 2.2. *Methods*

Particulate fractions of isolated frog bladder epithelial cells were prepared using the technique previously described [5]. Enzyme I designates enzyme prepared in 5 mM ethylenediamine tetraacetic acid (EDTA) and 25 mM Tris-HCl, pH 8.

Enzyme II is enzyme I treated with 20 mM ethylene glycol bis (beta-aminoethyl ether)-*N*-*N*′ tetraacetic acid (EGTA) and extensively washed in 25 mM Tris-HCl, pH 8. Adenylate cyclase activity was assayed using the method the authors reported earlier [5]. The cyclic AMP was separated using the technique described by Ramachandran et al. [9].

Binding reaction was carried out in an incubation medium identical to that used for adenylate cyclase assay. Under standard conditions the incubation medium contained 100 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 0.25 mM ATP, 1 mM cyclic AMP, and 50 to 120 $\mu$g of enzyme in a total volume of 100 $\mu$l. (Identical results were obtained with or without an ATP regenerating system.) Two techniques were used to separate bound [$^3$H]oxytocin from free labelled hormone.

#### 2.2.1. Separation by centrifugation

75 $\mu$l aliquotes of the incubation medium were carefully layered on 350 $\mu$l of a solution containing 36% sucrose (w/v), 5% bovine serum albumin (w/v), 100 mM Tris-HCl pH 8, and 10 mM $MgCl_2$, contained in plastic microtest tubes at 0°. The tubes were immediately centrifugated at 22,000 *g* in a Wifug centrifuge for 5 min at 4°. The tubes were then frozen in liquid nitrogen, the bottom of each tube, containing the particulate fraction, was cut, solubilized in soluene (0.5 ml) at 60° for 20 min and counted in 8 ml of Bray's scintillation medium [10]. Control samples without enzyme were treated in exactly the same way. The radioactivity present in these controls was the same whatever the [$^3$H]oxytocin concentration used and represented 0.038% of the total radioactivity contained in the tube when [$^3$H]oxytocin was used at a concentration of $10^{-7}$ M.

#### 2.2.2. Separation by filtration through Millipore filter EAWP 0.45 $\mu$m

Two ml of a cold solution containing 0.25 mM ATP, 1 mM cyclic AMP, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 8 (solution A) were added to each sample. The

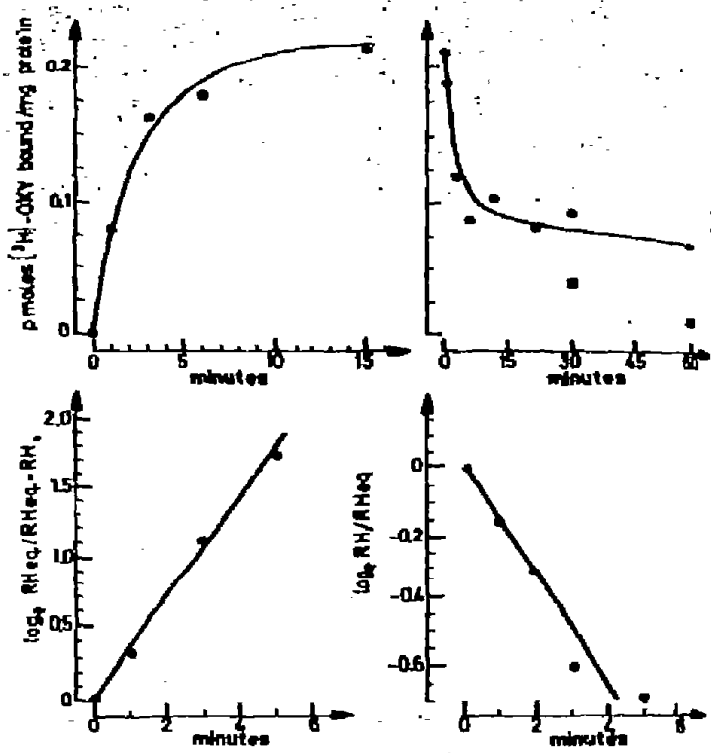


Fig. 1. Time-course of the association of [$^3$H]oxytocin with its high affinity receptors; reversibility of binding. Enzyme I (92 $\mu$g protein) was incubated in a final volume of 100 $\mu$l in the presence of [$^3$H]oxytocin ($2.5 \times 10^{-7}$ M). Oxytocin binding was measured as a function of time using the Millipore technique as indicated under Methods (see association curve, left part). To test binding reversibility the enzyme was first incubated for 15 min at 30° in the presence of [$^3$H]oxytocin ($2.5 \times 10^{-7}$ M). The hormonal concentration in the incubation medium was then lowered to $1.25 \times 10^{-8}$ M by dilution with an incubation medium containing no hormone (sol. A, see Methods). Residual binding was measured as a function of time after dilution (see dissociation curve, right part). In a different series of samples (■), the diluting medium contained dithiothreitol ($10^{-2}$ M). The $k_1$ and $k_2$ association and dissociation constants were determined from the semilogarithmic plots shown in the lower part of the figure assuming i) that the binding process corresponds to the equilibrium: $H + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RH$, in which H, R, and RH are, respectively, free hormone, free receptor, and the hormone receptor complex; ii) that the free hormone concentration is constant (the amount of bound hormone at equilibrium is negligible in relation to the total amount present in the incubation medium). The association and dissociation time-courses are rendered by equations 1 and 2 respectively: (1) $\log_e [RHeq]/([RHeq]-[RH]) = (k_1[H] + k_2)t$, (2) $\log_e [RH]/[RHeq] = -k_2 t$ with [RHeq] = concentration of hormone receptor complex at equilibrium. For the semilogarithmic transformation of the dissociation curve only the rapidly reversible component was considered.

total volume was filtered through a Millipore filter previously washed with 5 ml of cold solution containing 10 mM $MgCl_2$, 100 mM Tris-HCl pH 8, 0.1% bovine serum albumin (w/v). The filter was then washed three times with 10 ml of this same solution. The value of the control samples without enzyme was proportional to the [$^3$H]oxytocin concentration (0.07% of the radioactivity filtered).

These two methods of binding assay gave the same results. Oxytocin binding was expressed in pmoles [$^3$H]oxytocin bound per mg protein. Each value is the mean of two separate experimental data. Protein was determined according to Lowry [11] using bovine serum albumin as standard.

### 3. Results and discussion

The binding of [$^3$H]oxytocin to particulate fractions of frog bladder epithelial cells was linear with the enzyme concentration in the medium and was a rapid process saturable with time. At $2.5 \times 10^{-7}$ M of labelled hormone an equilibrium state was reached within 10 min at 30° (fig. 1). At 0° and for the same hormonal concentration the equilibrium value was unchanged. However, the half time for association increased to 17 min at 0°, instead of 1 min at 30°. The interaction between [$^3$H]oxytocin and its receptors was a partially reversible process (fig. 1 right part). About 40% of the bound hormone was not released by reducing the hormonal concentration to a subthreshold value. The remaining bound radioactivity might correspond to [$^3$H]oxytocin covalently bound through disulfide bridges since 95% of the irreversibly bound hormone was released in the presence of $10^{-2}$ M dithiothreitol. Such covalent binding of [$^3$H]oxytocin was earlier observed on intact frog skin epithelial cells [1]. There is no experimental evidence suggesting that this binding could be involved in the biological response.

Two categories of binding sites were detected in broken cell preparations (fig. 2, left part); these sites had different affinities for oxytocin and different maximum capacities. Saturation of the second category of binding sites could not be obtained with [$^3$H]oxy-

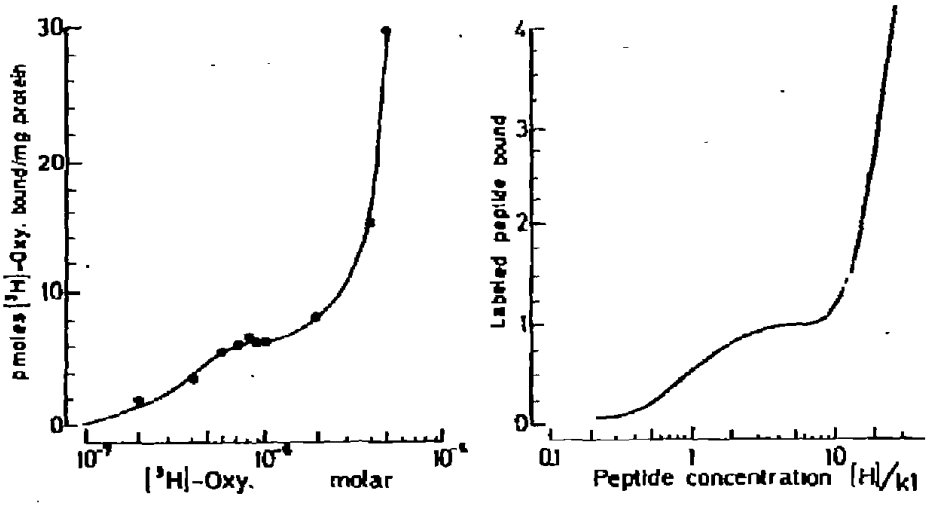


Fig. 2. [$^3$H]Oxytocin binding as a function of hormonal concentration. Binding was measured after 5 min incubation at 30° as a function of [$^3$H]oxytocin concentration using the centrifugation technique (see Methods). Enzyme concentration was 1.08 mg/ml. Note the presence of a saturation plateau between 7.0 to $15.0 \times 10^{-7}$ M on the experimental binding curve (left part.) The experimental curve can be adequately described by a cooperative binding of [$^3$H]oxytocin to 2 categories of binding sites (1 and 2) with different affinities ($K_{m_1}$ and $K_{m_2}$) and maximum capacities ($RT_1$ and $RT_2$):

$$RH = RT_1 \times \frac{H^{n1}}{K_{m_1} + H^{n1}} + RT_2 \times \frac{H^{n2}}{K_{m_2} + H^{n2}}$$

The theoretical curve (right part) was constructed using the following parameters: $K_{m_2}/K_{m_1} = 40$, $RT_2/RT_1 = 30$, $n_1 = 2$, $n_2 = 4$. H varied from 0.1 to 30 $K_{m_1}$ (explanations in the text).

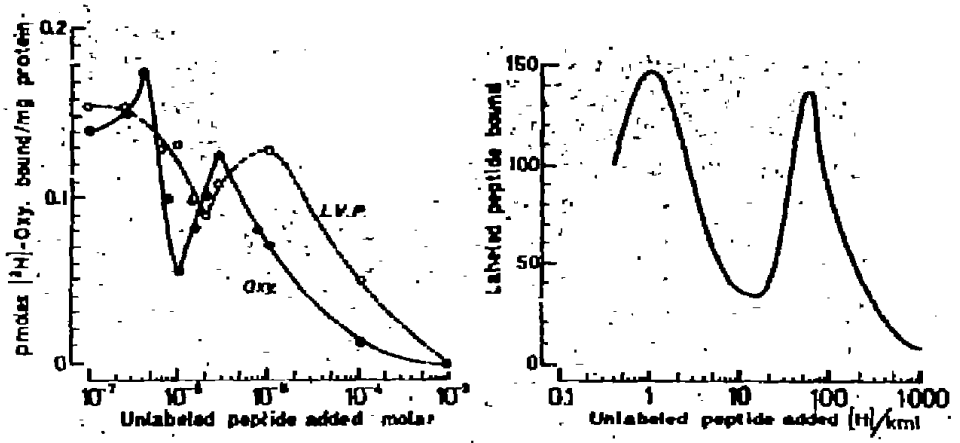


Fig. 3. Competition for binding between [$^3$H]oxytocin and unlabelled oxytocin or lysine-vasopressin. Binding was measured after 5 min incubation at 30°. Enzyme concentration was 0.96 mg/ml. Each tube contained [$^3$H]oxytocin ($10^{-7}$ M) and increasing amounts (0 to $10^{-3}$ M) of either unlabelled oxytocin (oxy) or lysine vasopressin (L.V.P.). All experimental values were corrected for non-specific binding (24% of binding at $10^{-7}$ M) and plotted as a function of unlabelled peptide concentration (left part). The theoretical curve (right part) was calculated from the model presented in the legend of fig. 2 using the same set of parameters, the [$^3$H]oxytocin concentration corresponds to 0.2 $K_{m_1}$. Binding is expressed as % of binding when no unlabelled peptide is added.

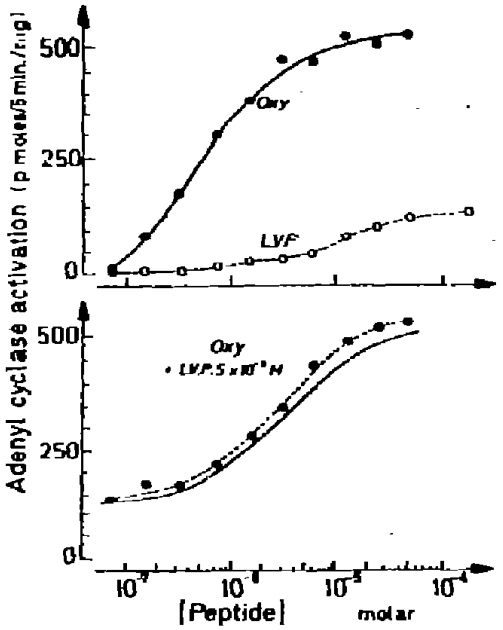


Fig. 4. Activation of frog bladder adenylate cyclase by oxytocin and lysine vasopressin. Adenylate cyclase activity was assayed for 5 min at 30° as indicated under Methods in the presence: 1) of increasing amounts of either oxytocin (oxy) or lysine vasopressin (L.V.P.) upper curves; 2) of a constant amount of lysine vasopressin ($5 \times 10^{-5}$ M) plus increasing amounts of oxytocin (lower curve, dotted line). Adenylate cyclase activation (increase in activity over basal activity: 163 μmole cyclic AMP/5 min/mg) is plotted as a function of peptide concentration in the incubation medium. Solid line in lower part of figure was constructed assuming that L.V.P. and oxytocin compete for the same receptors and using the respective $K_m$ values and maximal velocities for oxy and L.V.P., as deduced from the two upper curves.

tocin alone. Saturation was evident when [$^3$H]oxytocin binding is measured in the presence of increasing amounts of unlabelled oxytocin.

The binding curves for both categories of binding sites are S shaped. Hill coefficients were 1.5 for the high affinity and 4 for the low affinity sites, respectively.

The ratio of the apparent $K_m$ values for the two categories of sites ($K_{m_2}/K_{m_1}$) = 40; their maximum capacity ratio ($RT_2/RT_1$) = 30. Cooperativity was also apparent when [$^3$H]oxytocin binding at $10^{-7}$ M (a concentration lower than $K_{m_1}$) was measured as a function of the concentration of unlabelled oxytocin or lysine vaopressin in the incubation medium (fig. 3). Lysine vaopressin was able to compete with oxytocin for binding. Competition between oxytocin and lysine vasopressin was also apparent from adenylate cyclase experiments.

Thus as shown in fig. 4, lysine vasopressin stimulated frog bladder epithelial cell adenylate cyclase (apparent $K_m$ $10^{-5}$ M), but maximum stimulation was four times lower than that produced by oxytocin.

When the responses to increasing amounts of oxytocin were measured in the presence of $10^{-5}$ M lysine vasopressin, the experimental curve was the same as the theoretical curve calculated for pure competitive interaction of the two peptides on the same receptors. Despite the fact that lysine-vasopressin and oxytocin

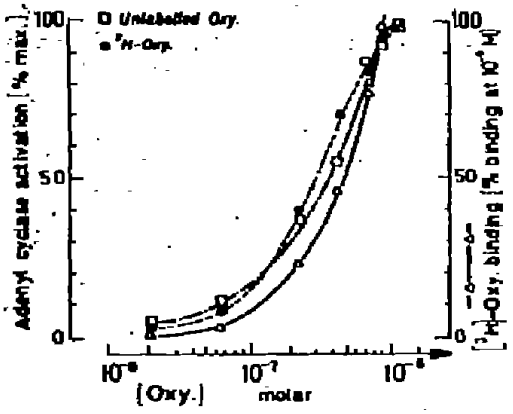


Fig. 5. Correlation between binding and adenylate cyclase activation. [$^3$H]Oxytocin binding was measured after 5 min incubation at 30° and expressed as % of the maximum capacity of high affinity binding sites (binding at $10^{-6}$ M [$^3$H]oxy: 0.53 pmoles/mg protein). Cyclase activity was assayed for 5 min at 30° as indicated under Methods in the presence of increasing amounts of either [$^3$H]oxytocin or unlabelled oxytocin. Activation due to hormone is expressed as % maximum activation. Basal activity was 140 pmoles cyclic AMP/5 min/mg and maximum hormone-stimulated activity, 637 pmoles cyclic AMP/5 min/mg. Enzyme conc. was 0.98 mg/ml.

produced different maximum stimulation of the adenylate cyclase system, they were able to elicit the same maximum response in the intact frog bladder. This suggests the presence of spare receptors in the structure in accordance with earlier observations [6, 12, 13]. Estimation of the association and dissociation constants $k_1$ and $k_2$ for the binding sites coupled to the adenylate cyclase system gave the following values (see fig. 1): $k_1 = 7.2 \times 10^5$ M$^{-1}$ $\ell^{+1}$ min$^{-1}$ and $k_2 = 0.18$ min$^{-1}$. The value of the ratio $k_2/k_1 = 2.5 \times 10^{-7}$ agrees with the $K_m$ values deduced either from the binding curves or dose–response curves of the adenylate cyclase system (see fig. 5).

Table 1
Effect of calcium on [$^3$H]oxytocin binding.

| $Ca^{2+}$ added (M) | [$^3$H]Oxytocin bound (pmoles/mg protein) |
|---|---|
| 0 | 0.71 |
| $5 \times 10^{-6}$ | 0.70 |
| $10^{-4}$ | 0.77 |
| $10^{-3}$ | 0.76 |
| $10^{-2}$ | 0.99 |

Enzyme II (0.96 mg/ml) was used and incubation carried out in the presence of increasing $Ca^{2+}$ concentrations. Binding was measured, using the centrifugation technique, after 5 min incubation at 30° in the presence of [$^3$H]oxytocin: $2.2\ 10^{-7}$ M (non-maximum concentration for the high-affinity binding sites).

It was not possible to detect binding sites on the broken cell preparation with an affinity as high as that of the receptor sites present in the intact structure and involved in the initiation of the biological response. The most likely explanation for the difference observed between intact cells and the broken cell preparation is that the homogenization procedure deteriorates the properties of the receptor and leads to a great loss in their apparent affinity for the hormone. This loss is similar for both categories of binding sites.

As previously discussed [5] traces of calcium are necessary for stimulation of frog bladder adenylate cyclase by oxytocin whereas higher concentrations ($10^{-5}$ to $10^{-3}$ M) inhibit such stimulation.

It was observed (table 1) that [$^3$H]oxytocin binding measured at a hormone concentration giving half saturation of adenylate cyclase was not modified by increasing $Ca^{2+}$ concentration in the incubation medium from 0 to $10^{-3}$ M. This observation indicates that the calcium effect is located at the coupling step and has no connection with the accessibility of the hormone to receptor sites.

An increase in the amount of bound [$^3$H]oxytocin was observed at $10^{-2}$ M $Ca^{2+}$. Similar results were obtained by Cuatrecasas on the case of the insulin receptor of isolated fat cell membranes [14].

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