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Thermodynamics of the interaction between oxytocin and its myometrial receptor in sheep: A stepwise binding mechanism

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Oxytocin
Arginine vasopressin
Tritium-labeled oxytocin: [2-tyrosine-2,6-^{3}H]oxytocin
Tritium-labeled arginine vasopressin: [8-arginine, 3-phenylalanine-3,4,5-^{3}H]vasopressin
Atosiban: [1-mercaptopropionic acid, 2-*D*-(O-ethyl)-tyrosine,4-threonine, 8-ornithine]oxytocin
GTP-γS: guanosine-5′-O-(3-thiotriphosphate)

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ABSTRACT

Entropy (ΔS), enthalpy (ΔH) and heat capacity (ΔC_p) changes attending the oxytocin interaction with its two binding sites on myometrial cell membranes in sheep were derived from the temperature dependence of K_d values. The high affinity oxytocin site (K_d on the order of 10^{-9} mol l^{-1}, 25 °C), ascribed to the oxytocin receptor (OXTR), is entropy-driven in the temperature range 0–37 °C. Enthalpy component prevails as a driving force in the binding to the low affinity site ($K_d \approx 10^{-7}$) within the higher temperature range. ΔC_p values in both cases do not differ significantly from zero but become highly relevant in the presence of a GTP analog (10^{-4} M GTP-γS). Under these conditions, ΔC_p in the low site interaction becomes negative and ΔS is shifted toward negative values (enthalpy drift); ΔC_p of the high affinity site rises to a high positive value and the interaction is even more strongly entropy driven. Atosiban, a competitive antagonist of oxytocin at OXTR displays a single significant binding site on myometrial cells (K_d about 10^{-7} mol l^{-1}). Thermodynamic profiles of atosiban and the low affinity oxytocin site show conspicuous similarities, indicating that the inhibitor is bound to the low affinity site, and not, with a lower affinity, to the putative receptor protein. It is suggested that the interaction of oxytocin with its responding system on myometrial membranes follows in two distinct steps that are likely to be associated with several independent binding domains in the GPCR receptor.

1. Introduction

The contractile response of uterus to oxytocin is initiated on receptor sites located in the membrane of myometrial smooth muscle cells. In human uterus, this membrane receptor (OXTR) has a heptahelical structure [1,2] and is associated, via G_q or G_i protein, with the inositol phosphate signaling system. High and low affinity oxytocin binding sites occur in rat, sheep, and calf uterus [3–5] differing in their dissociation constants (K_d) and binding capacities (B). Studies on cultured myometrial cells and their membrane fractions indicate high affinity binding sites with a K_d of 8×10^{-10} to 3×10^{-9} mol l^{-1}, and low ones with K_d in the ranging from 5×10^{-8} to 5×10^{-7} mol l^{-1}. A clear-cut effect of GTP on both K_d's and B's in experiments on rat myometrial membranes could not so far be demonstrated [5].

Receptor systems displaying several sites with different binding affinities are well known from the literature, and models attempting to describe some of their specific classes were thoroughly reviewed [6–10]. Interdependent receptor forms in equilibrium were suggested in several models, most recently in a ternary model involving G-protein-activated receptor forms [11–13]. However, outcomes of binding experiments based on measurements of the total bound ligand (not of individual complexes) cannot be matched by models in which a rapid transition between individual forms of a single receptor is

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presumed [12]. Such a finding rather indicates either an involvement of a single receptor in several "semi-stable", virtually independent forms: different phosphorylation states, pre-coupling with the corresponding G protein, binding on distinct receptor domains, dimeric or oligomeric receptor complexes, etc. [14,15]. Alternatively, two or more independent binding sites that regulate distinct cellular responses may form complexes with several conformational forms of the ligand. And naturally, some of the identified binding regions, without supporting pharmacological evidence, may be associated with non-receptor sites like various kinds of ligand-membrane components, ligand transport forms, etc.

It was suggested earlier that both high and low oxytocin binding sites are involved in the uterotonic response to oxytocin [16,17]. We infer from temperature profiles that binding of oxytocin in sheep myometrium receptor may proceed in two steps via formation of a ligand–mediator complex, as proposed in the model by Sargent and Schwyzer [18]: in the first step a ligand is bound to the lipid membrane phase, and in this form catalyses the formation of the final ligand–receptor complex. In this model mediator and receptor molecules are virtually independent. In our case, atosiban, a competitive inhibitor of oxytocin [19,20] seems to be bound preferentially to the mediator.

2. Materials and methods

2.1. Preparation of myometrial cell membranes

Buffer A: pH 7.4, 10 mM Tris, 1.5 mM EDTA, 0.01% Bacitracin (Sigma B-0125), 1 mM benzamidine (Sigma B-6506), 0.002% soybean trypsin inhibitor (Sigma T-9003).

Buffer B: pH 7.4, 50 mM Tris–HCl, 1.2 mM magnesium sulphate; Bacitracin, benzamidine and soybean trypsin inhibitor as in buffer A.

White Alpine Sheep (WAS) in oestrus (September–mid December) were used. Uteri were dissected about 10 min after slaughtering. Uterus horns were cut off and stored in ice-cold buffer A. Similar to the protocol of Ayad and Wathes [21], the endometrium was removed by scraping. The myometrial tissue was mechanically separated from the perimetrial membrane and broken up with a scalpel. After adding 9 ml of the buffer A per 1 g of wet tissue, the homogenization followed in an Ultraturax mixer (20,000 rpm, for 30 s) and by three strokes in a Potter–Elvehjem homogenizer. Differential centrifugation consisted of two steps (both at 4 °C): $1000 \times g$, 10 min, and $160{,}000 \times g$, 30 min. The pellet from the last step was re-suspended and re-homogenized in buffer B (same volume as used for the initial homogenization). The differential centrifugation was repeated under the same conditions. The final pellet was re-suspended in buffer B and protein concentration was adjusted to roughly 4 mg/ml. Membranes were stored in small aliquots at −80 °C. Proteins were estimated using the BioRad Protein Assay kit (Bradford) in the presence of 0.2% sodium dodecyl sulphate (Fluka 717 29).

2.2. Peptides

Oxytocin was purchased from Sigma (O-6379). Arginine vasopressin and atosiban, [1-mercaptopropionic acid, 2-*D*-(O-ethyl)-tyrosine,4-threonine, 8-ornithine]oxytocin, were donated by Ferring AB, Malmö, Sweden. Tritium-labeled oxytocin, [2-tyrosine-2,6-^{3}H]oxytocin (various batches 32–48.5 Ci/mmol) and tritium-labeled arginine vasopressin, [8-arginine, 3-phenylalanine-3,4,5-^{3}H]vasopressin (75.8 Ci/mmol) were products of New England Nuclear, Boston, MA.

2.3. Binding experiments

2.3.1. Basic protocol

Experiments were carried out with tritium-labeled oxytocin in various isotopic dilutions with non-labeled oxytocin. Total oxytocin concentration ranged from 2×10^{-10} to 10^{-4} M (8 probes within the range 2×10^{-10} and 10^{-8} M, 6 within 3×10^{-8} and 10^{-4} M). Oxytocin in 150 μl buffer B was placed in covered Eppendorf plastic centrifugation tubes. Measurements were carried out in the temperature range from 0 °C to 37 °C. The tubes were annealed to the desired temperature in a thermostatic bath for 30 min and pH was re-adjusted. Constant temperature was achieved in a Höppler thermostatic bath (Colora Messtechnik GmbH, Lorch, Württenberg, G.F.R.) equipped with an external cooling system (eutectic mixture water–ice–NaCl). The whole equipment and attached tubing were thermally isolated. The estimated accuracy was roughly 0.1-0.3 °C at both lower and higher temperatures. Incubation was started by the addition of 50 μl of the equally annealed membrane stock suspension (final dilution was ca. 500 μg/ml protein) and incubated under constant shaking for 60 min. Preliminary experiments indicated that, depending on the temperature, steady-state of ligand binding was reached after a short initial transient phase in 25–45 min [22] (similar results were earlier reported for rat and sow uterus cells [23]). 1 and 2 μl aliquots were used for estimation of the total radioactivity. Membranes were then separated by filtration on glass microfiber filters GF/C (Whatman 1822 915) pre-treated by immersion in 0.1% bovine serum albumin for 45 min at 4 °C. A 24-Well Plate Cell Harvester Inotech AG, CH-5605 Dottikon, Switzerland, was used for separation. Filters were quickly rinsed by filtration of three 2 ml portions of cold buffer B; each rinsing lasted less than 1 s. Filters were placed in scintillation vials to which 5 ml scintillation cocktail was pipetted (Ultima Gold High Flashpoint Liquid Scintillation Cocktail, Canberra-Packard No. 6013329). β-Counting (dpm) followed after 24 h at 4 °C for 10 min. An identical experimental setup, using medium without membranes, was used for estimation of the radioactivity retention on the filter. The difference of these two dmp values was assigned as the bound fraction on the incubated cell membranes. A similar protocol was used for the binding of arginine vasopressin to the myometrial membranes (25 °C).

Another set of measurements was carried out accordingly in the presence of 10^{-4} M guanosine-5′-O-(3-thiotriphosphate) (GTP-γS, Sigma G 6018). Membranes were pre-incubated for 10 min in buffer B containing GTP-γS in the same concentration.

2.3.2. Evaluation of binding isotherms

Equilibrium between concentrations of bound (c_b) and free (c_f) ligand in a system of superimposed, mutually independent binding sites can be matched by a sum of Hill-type binding isotherms related to distinct sites (subscript j),

$$c_b = \sum_j \frac{B_j c_f^{h_j}}{K_{d,j}^{h_j} + c_f^{h_j}} + N c_f, \tag{1}$$

where B_j stands for the binding capacity, $K_{d,j}$ for dissociation constant of the ligand–receptor complex, h_j is a power (Hill) coefficient of a jth-binding site, N represents low affinity ("nonspecific") binding. The total number of j-sites is virtually unknown and binding profiles described by Eq. (1) need to be analyzed by a stepwise strategy. In the STEP procedure [3,24] applied in our experiments, such a strategy rests upon optimization of the power coefficient h (by the modified bisection root-finding method) in partial segments of continuous c_f, c_b-data along the entire ligand concentration range employed. Estimates of B and K_d within these segments follow from the regression