Essential role of magnesium in oxytocin-receptor affinity and ligand specificity

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We have analysed, with the aid of a novel radioiodinated oxytocin (OT)-receptor antagonist, the role of Mg^{2+} in uterine OT-receptor function. The antagonist-receptor interaction was characterized by high affinity, reversibility and stereospecificity in Tris/HCl buffer containing 3 mmol of Mg^{2+} /litre as well as buffer free of Mg^{2+} . By contrast, omission of Mg^{2+} decreased the affinity of the receptor for OT by about 1500-fold; moreover, the stereospecificity of agonist, but not antagonist, binding was lost. Since guanine nucleotides had relatively minor effects in this system (≤ 2 -fold decrease in OT affinity), we suggest that the agonist-binding site of OT receptors is directly modulated by Mg^{2+} , unlike other receptors, where the effects of bivalent cations are exerted via guanine-nucleotide-binding (G-) proteins. Thus the ligand recognition mechanism of OT receptors may be novel in this respect.

INTRODUCTION

Oxytocin (OT), like other peptide hormones, exerts its cellular actions by interacting with specific high-affinity receptors on the surface of target cells in various organs of the body. Previous studies have demonstrated that OT is bound by its receptor only in the presence of Mg^{2+} or related cations, which were postulated to modulate the affinity, as well as the availability, of the receptors for interaction with the ligand (cf. [1,2]). More recently, antagonist analogues of OT have been synthesized that are suitable for radioiodination and which open up new possibilities in investigating the properties of OT receptors [3]. In the present study we have used such a novel OT analogue to examine the role of Mg^{2+} in the recognition of ligands by the OT receptor in rat uterus.

MATERIALS AND METHODS

Female Wistar rats (200–300 g body wt.; Harlan Olac, Bicester, Oxon., U.K.) were used. Unless indicated otherwise, the animals received subcutaneous implants made of silastic tubing which contained 50 mg of diethylstilboestrol (Sigma, Poole, Dorset, U.K.) 5–6 days before they were killed by decapitation. A crude membrane fraction of uteri was prepared as described previously [4,5]. The final pellet was resuspended in Tris/HCl (0.05 mol/l), pH 7.4, containing bacitracin and bovine serum albumin [0.01 and 0.1 % (w/v) respectively; from Sigma], EGTA (1 mmol/l) and various concentrations of bivalent cations (Mg^{2+} as $MgSO_4$, the others as chlorides) or Na_2EDTA (2.5 mmol/l). The concentration of protein in the binding assay was 60 μ g/ml (Coomassie Brilliant Blue method [6]). The reaction was at room temperature for 60 min unless otherwise indicated; separation was performed by vacuum filtration over GF/C filters (Whatman) [4,5].

The OT agonist analogues were: oxytocin (OT), [Arg-8]-

vasopressin (AVP) (Ferring A.B., Malmö, Sweden), [Thr-4,Gly-7]OT (TG-OT) (M. Manning, Toledo, OH, U.S.A.) [7], [deamino-7,Lys-4]AVP (dLAVP) (G. Toth, Szeged, Hungary) [4], [D-Tyr-2]OT free acid (V. Hruby, Tucson, AZ, U.S.A.) [15]. The antagonists used were [β -mercapto- $\beta\beta$ -cyclopentamethylenepropionic acid-1,*o*-methyl-Tyr-2,Thr-4,Orn-8,TyrNH₂-9]vasotocin (OTA) (M. Manning) [3], [β -mercapto- $\beta\beta$ -cyclopentamethylenepropionic acid-1,*o*-methyl-Tyr-2]AVP (d[CH₂]₅Tyr[Me]-AVP), [deaminopenicillamine-1,*o*-methyl-Tyr-2]AVP (dPenTyr[Me]AVP), both from Bachem U.K. [7], [penicillamine-1,*p*-methyl-Phe-2,Thr-4,Orn-8]OT (PPT-OT) (V. Hruby) [8,8a].

Somatostatin (Sigma), bradykinin and [sarcosine-1, isoleucine-8]angiotensin II (Saralasin) (Bachem) were also used. Radioiodination of OTA was performed by minor modifications of the Iodogen method [3,9]. The specific radioactivity of the ¹²⁵I-OTA obtained was estimated to be 0.6 nCi/fmol [10].

Ligand-displacement curves were linearized by logit-log transformation, the IC_{50} value was estimated by linear-regression analysis, and the inhibitor constant (K_i) was calculated [11]. All slopes were between -0.9 and -1.1 , and coefficients of correlation (r) were less than -0.95 .

RESULTS AND DISCUSSION

Characterization of the sites binding ¹²⁵I-OTA in rat uterus membrane suspensions in EDTA-containing buffer

The binding of ¹²⁵I-OTA to uterus membrane preparations in the presence of EDTA (92.5 mmol/l) was time-dependent, reaching a steady state at about 60 min. Addition, at 60 min, of 5 ml of assay buffer, containing 10^{-6} mol of OTA/l, reversed the association of radioligand with the particulate fraction (Fig. 1). Estimation of the 'on-' and 'off-' rates [12] of this reaction gave a K_d of 0.16 nmol/l. Binding-saturation experiments, per-

Abbreviations used: OT, oxytocin; G-proteins, guanine-nucleotide-binding proteins; abbreviations for OT agonist analogues and antagonists are given in the Materials and methods section; IC_{50} , concentration causing 50 % of maximal inhibition; K_i , inhibitor constant; B_{max} , maximal binding capacity; p[NH]ppG, guanosine 5'-[β -imidotriphosphate ('GMP-PNP').

Fig. 1. Time course of association (Δ) and dissociation (▲) of ^{125}I -OTA in uterus membranes

Incubation was performed at room temperature, and dissociation was induced by adding 3 ml of incubation buffer containing OTA (10^{-6} mol/l). The ordinate shows the amount of radioactivity specifically bound out of a total of 30000 c.p.m. added; non-specific binding was 950 c.p.m.; the results are means of triplicates.

formed by adding increasing amounts of OTA in the presence of a constant amount of radiolabel, showed that the specific component of binding was saturable. Scatchard plots of these data suggested the presence of a single population of sites with high affinity ($K_d = 0.16 \pm 0.05$ nmol/l; mean $\pm$ S.E.M., $n = 3$) and limited capacity ($B_{\text{max.}} = 519 \pm 68$ fmol/mg of protein, $n = 3$). Finally, injection of $5 \mu\text{g}$ of 17β -oestradiol per rat to ovariectomized rats increased the concentration of sites binding OTA by about 5-fold 48 h later (results not shown).

These findings indicate that, in the presence of EDTA, ^{125}I -OTA labels physiologically relevant oxytocin receptors in the uterus membrane suspension (cf. [1–4]).

Effects of bivalent cations on ligand affinity and stereospecificity

The bivalent cations Mg^{2+} , Mn^{2+} , Co^{2+} and Ca^{2+} (all at 3 mmol/l) failed to have any effect on the total amount of specifically bound ^{125}I -OTA in buffer free of EDTA. Moreover, Scatchard plots of binding isotherms of OTA in the presence of EDTA (2.5 mmol/l) or Mg^{2+} (3 mmol/l) were identical (with Mg^{2+} in two experiments: $K_d = 0.1$ and 0.27 nmol/l; $B_{\text{max.}} = 595$ and 414 fmol/mg of protein). By contrast, Mg^{2+} , Mn^{2+} and Co^{2+} , but not Ca^{2+} , enhanced the ability of OT (250 nmol/l) to inhibit the binding of the tracer (results not shown). The effects of Mg^{2+} on OT potency were dose-dependent, maximum displacement of the tracer occurring at 1 mmol/l or higher (Fig. 2).

In summary, Mg^{2+} , Mn^{2+} and Co^{2+} are all effective in promoting the interaction of OT with the receptor labelled with antagonist, whereas Ca^{2+} is ineffective. This is also the case when $[^3\text{H}]\text{OT}$ is the tracer, and has been also reported for vasopressin isoreceptors (cf. [1,13,14,16]).

The potency of several oxytocin analogues to compete for OT receptors with ^{125}I -OTA was estimated in both

the presence and absence of Mg^{2+} (Table 1). The cation did not affect the K_i of any of the antagonists compounds tested. In marked contrast, in Mg^{2+} -free buffer, the agonists OT and TG-OT were more than 1000-fold less potent than in Mg^{2+} at 1 mmol/l (Table 1). However, both peptides completely displaced the tracer at high concentrations (up to 10^{-4} mol/l), along curves that were parallel with those for the respective peptides in Mg^{2+} -containing medium, indicating that all sites labelled with ^{125}I -OTA are available for interaction with OT and TG-OT. The extent of the right-shift was not the same for all agonists: it was proportional to the potency of the compound in Mg^{2+} -containing buffer. Thus, AVP and the partial-agonist peptide $[\text{D-Tyr-2}]\text{OT}$ [15] became virtually equipotent with OT, the range of relative potencies being markedly narrowed from 2300-fold to 2.3-fold.

Given the striking change in specificity, we tested three oligopeptides, namely bradykinin, somatostatin and Saralasin in the binding assay. In the presence of Mg^{2+} we found all peptides to be at least 10000-fold less potent than OT. However, in Mg^{2+} -free buffer, somatostatin and bradykinin displaced ^{125}I -OTA only 48- and 279-fold less potently than OT respectively, and this was largely due to the decrease in the potency of OT. Saralasin was ineffective up to 1 mmol/l. Thus, while there is a marked decrease in the specificity of ligand recognition in Mg^{2+} -free medium, preference for some traits of the oxytocinergic stereochemistry is still apparent.

Agonist-specific modulation of receptor affinity by Mg^{2+} has been shown previously in other receptor systems (cf. [18,19]), but the degree of the change found in the present study is unprecedented. Furthermore, no systematic studies of specificity have been reported for the low-affinity state of the receptors under study. Our data indicate that Mg^{2+} is involved in stabilizing a high-affinity high-specificity conformation of the receptor, which is necessary for the binding of agonists. In contrast, antagonists are not affected, possibly because they stabilize another conformation of the receptor that is not capable of signal transduction. This notion is supported by previous findings showing that different structural features are required for the high-affinity binding of antagonists than for agonists (cf. [8,8a]).

A decrease in receptor affinity and specificity for agonists with no change in the binding of antagonists can be brought about in the presence of Mg^{2+} by genetic engineering of β -adrenergic receptors [17]. Thus, in at least two different ligand-receptor systems, agonist affinity and specificity change in parallel, while the binding of antagonists is unaffected. The molecular mechanisms underlying this phenomenon remain to be explored.

Are the effects of Mg^{2+} on agonist affinity mediated via G-proteins?

Since most receptor-ligand interactions are modulated by Mg^{2+} in association with G-proteins (cf. [18,19]), the effects of Mg^{2+} were examined in the presence of the GMP analogue guanosine 5'-[β -imido]triphosphate (p[NH]ppG; 'GMP-PNP') at a concentration of 10^{-4} mol/l, which is maximally effective in most systems [18,19]. As shown in Fig. 2, pp[NH]pG decreased the maximal effect of Mg^{2+} and tended to increase the half-effective concentration of Mg^{2+} (0.04 as against 0.07 mmol/l and without p[NH]ppG respectively).

Fig. 2. Effect of various concentrations of Mg²⁺ on the ability of OT (250 nmol/l) to compete with ¹²⁵I-OTA in the presence (▲) or absence (△) of pp[NH]pG (10⁻⁴ mol/l)

The ordinate shows specific binding expressed as a percentage of radiolabel specifically bound without OT; the results are means of triplicates. The potency of OT to compete with ¹²⁵I-OTA for uterus OT receptor in Tris/HCl buffer containing MgSO₄ (1 mmol/l) (△), MgSO₄ (1 mmol/l) and p[NH]ppG (10⁻⁴ mol/l) (▲), and Na₂EDTA (2.5 mmol/l) (◇). The ordinate shows specific binding as a percentage of the amount of radiolabel bound in the absence of OT. Results are means of duplicates.

Table 1. Effect of Mg²⁺ on the potency of OT analogues to compete with ¹²⁵I-OTA for binding to OT receptors in rat uterus

Data are geometrical means with the retransformed standard error (in parentheses, *n* = 3) or results from two separate experiments (separated by semi-colon). The 'magnesium index' for antagonists was calculated to indicate the magnitude of the effect of the cation on ligand affinity by dividing the *K_i* obtained in the absence of Mg²⁺ by that in the presence of the cation. For abbreviations, see Materials and methods section.

Peptide	<i>K_i</i> (mmol/l)					
	With Mg ²⁺	Without Mg ²⁺				
OT antagonists						
OTA	0.1 (1.10)	0.1; 0.1				
PPT-OT	1.4 (1.19)	2.2; 3.9				
dPenTyr[Me]AVP	14.2; 16.8	12.9; 12.6				
d(CH ₂) ₅ Tyr[Me]AVP	19.5; 20.6	28.6; 22.8				
OT agonists						
OT	1.4 (1.15)	2438; 1814				
TG-OT	2.3 (1.17)	2065; 3571				
[2-D-Tyr]OT	59.3; 65.9	2000; 5100				
AVP	13.9 (1.26)	1360 (1.22)				
dLAVP	2495; 4401	4786 (1.16)				
Rank order of agonists						
(a) With Mg ²⁺						
Agonist ...	OT	TG-OT	AVP	[D-Tyr-2]OT	dLAVP	
Relative potency ...	1	0.61	0.1	0.02	0.0004	Range 2370-fold
Magnesium index ...	1500	1180	98	51	1.9	
(b) Without Mg ²⁺						
Agonist ...	AVP	OT	TG-OT	[D-Tyr-2]OT	dLAVP	
Relative potency ...	1.5	1	0.77	0.66	0.44	Range 2.3-fold

Further experiments indicated that, at 1 mmol of Mg^{2+} /l, p[NH]ppG causes an apparent 1.3–2-fold decrease (right-shift) in the potency of OT to displace ^{125}I -OTA (Fig. 2, inset). Because of the small change caused by the guanine nucleotide, we cannot say with certainty whether all or only some receptor sites were in the low-affinity state.

The amount of ^{125}I -OTA bound was not altered by p[NH]ppG in any of the experiments, and there was no detectable effect on the potency of OTA to displace the tracer or on the number of receptor sites as deduced from Scatchard plots (results not shown). Finally, it should be added that the effect of p[NH]ppG was variable between various batches of membrane preparation; the maximal right-shift of the OT curve was 2-fold. This is possibly due to the presence of variable amounts of endogenous GDP and/or G-proteins in different batches of membrane preparation.

The activity of various types of G-proteins is destroyed by alkylation with *N*-ethylmaleimide (cf. [20]). Treatment of the membrane preparation for 30 min at 37 °C with this reagent at 1 mmol/l had no appreciable effect on the binding of ^{125}I -OTA or on the potency of OT to displace ^{125}I -OTA (results not shown).

In summary, the effects of manipulating G-protein function in this system are minimal compared with the changes brought about by altering the concentration of Mg^{2+} .

Taken together, these observations support the notion [2] that Mg^{2+} interacts with the ligand-binding site of the OT receptor to stabilize a conformation favouring high affinity and specific binding of agonists. Given a number of analogous observations with V_1 - and V_3 -type receptors for vasopressin, it appears that the requirement for Mg^{2+} is a generic mechanism for ligand recognition by these receptors. Further studies with purified receptor and G-protein preparations are required to establish this hypothesis.

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