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Sodium functions as a negative allosteric modulator of the oxytocin receptor

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ABSTRACT

The oxytocin receptor, a class A G protein coupled receptor (GPCR), is essentially involved in the physiology of reproduction. Two parameters are crucially important to support high-affinity agonist binding of the receptor: Mg^{2+} and cholesterol, both acting as positive modulators. Using displacement assays with a high-affinity fluorescent antagonist (OTAN-A647), we now show that sodium functions as a negative allosteric modulator of the oxytocin receptor. In membranes from HEK293 cells stably expressing the oxytocin receptor, oxytocin binding occurred with about 15-fold lower affinity when sodium chloride was increased from 0 to 300 mM, whereas antagonist binding remained largely unchanged. The effect was concentration-dependent, sodium-specific, and it was also observed for oxytocin receptors endogenously expressed in Hs578T breast cancer cells. A conserved Asp (Asp 85) is known to stabilize the sodium binding site in other GCPRs. Mutations of this residue into Ala or Asn are known to yield non-functional oxytocin receptors. When Asp 85 was exchanged for Glu, most of the oxytocin receptors were localized in intracellular structures, but a faint plasma membrane labeling with OTAN-A647 and the appearance of oxytocin-induced calcium responses indicated that these receptors were functional. However, a sodium effect was not detectable for the mutant D85E oxytocin receptors. Thus, the oxytocin receptor is allosterically controlled by sodium similar to other GPCRs, but it behaves differently concerning the involvement of the conserved Asp 85. In case of the oxytocin receptor, Asp 85 is obviously essential for proper localization in the plasma membrane.

1. Introduction

The oxytocin receptor belongs to the rhodopsin class A of the G protein coupled receptor (GPCR) superfamily [1]. Together with three types of vasopressin receptors (designated as V1A, V1B, and V2), they form a small subfamily. Oxytocin/vasopressin-like peptides and their cognate receptors comprise one of the oldest GPCR signalling systems, with representatives ranging from mammals to arthropods [2,3]. They are involved in many physiological processes related especially to osmoregulation and reproduction.

Oxytocin receptors typically activate the phospholipase Cβ/phosphoinositide pathway that subsequently leads to an increase in the intracellular calcium concentration, activation of various protein kinases, and/or the production of prostaglandin E2 [2,4,5]. Two factors are essentially required for the oxytocin receptor to enable high-affinity oxytocin binding, cholesterol and divalent cations such as Co^{2+}, Mn^{2+}, Mg^{2+} (but not Ca^{2+}). With respect to the cations, most likely Mg^{2+} is the physiological relevant modulator. In dependence on cholesterol, the receptor can reversibly change its affinity state from high- (K_D ~1 nM) to low-affinity (K_D ~100 nM) and *vice versa* (Fig. 1A) [6–8]. The interaction of cholesterol with the oxytocin receptor is of high specificity and induces a more compact receptor conformation [9]. Thus, cholesterol and Mg^{2+} could be regarded as positive allosteric modulators of the oxytocin receptor.

Recently, Na^+ was suggested as a novel allosteric 'co-factor' in the function of class A GPCRs [10]. In high-resolution structures of some GPCRs a partially hydrated sodium ion was observed to be specifically bound in the middle of the transmembrane helix bundle [11–15]. The amino acid residues forming this sodium binding site are highly conserved among class A GPCRs. Fig. 1B shows a sequence alignment of the oxytocin/vasopressin receptors with the adenosine A2A receptor (see red asterisks in Fig. 1B) for which a 1.8 Å resolution structure with sodium pocket is available [13]. Typically, sodium negatively modulates the agonist binding of GPCRs whereas the binding of antagonists was left more or less unaffected [10]. Thus, sodium is assumed to stabilize the inactive receptor state, and there is some evidence that receptor activation and sodium translocation are coupled processes with large physiological impact. However, the sodium dependence of GPCRs

Abbreviations: CCM, cholesterol consensus motif; CRAC, cholesterol recognition amino acids consensus domain; GPCR, G protein coupled receptors; HBS, Hepes buffered saline; MβCD, methyl-β-cyclodextrin; OT, oxytocin; OTR, oxytocin receptor

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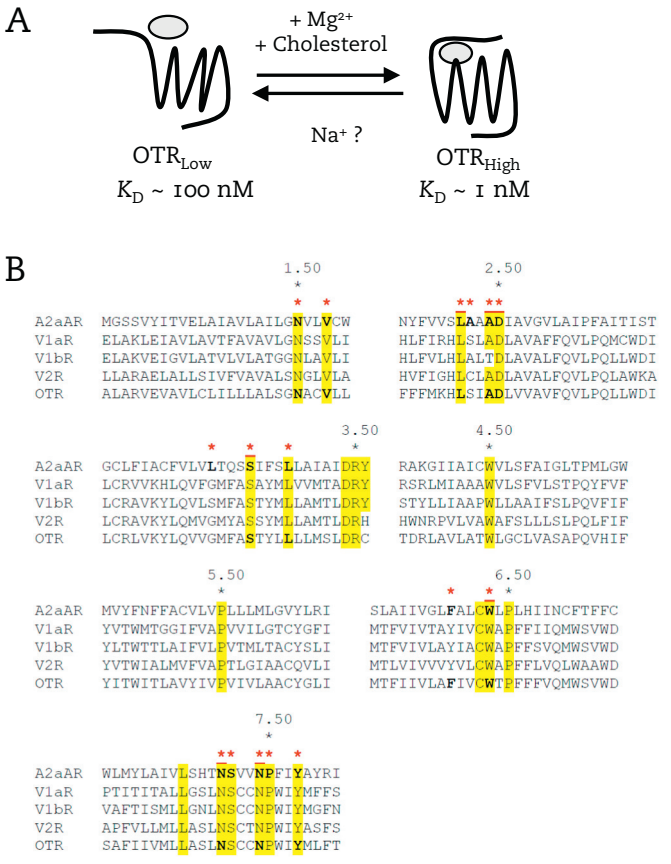

Fig. 1. Allosteric modulation of the oxytocin receptor (A) and sequence alignment (B) of the transmembrane helices for the adenosine A2a receptor and the vasopressin/oxytocin receptor family. The residues of the sodium/water pocket are conserved as shown by the red asterisks. Black stars, most conserved within TM domain (=X.50); yellow, conserved among all of the indicated receptors; red star underlined, conserved residues of the sodium pocket among all class A GPCRs.

has only been demonstrated for a handful of receptors. In view of the profound allosteric regulation of the oxytocin receptor we considered this receptor to be an attractive candidate to analyze its sodium dependence.

Overall, the data show that sodium acts as a negative allosteric modulator of the oxytocin receptor. Our results further substantiate the physiological role of sodium on the function of GPCRs.

2. Materials and methods

2.1. Materials

Oxytocin was supplied by Bachem (Switzerland). The oxytocin antagonist OTAN was a gift from Ferring, Copenhagen. OTAN labeled with Alexa-647 (OTAN-A647) was synthesized in our group. All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.2. Cell culture

All cells used in this study were cultivated in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal calf serum.

2.3. Preparation of membranes

Membranes were prepared from cells similar as described [16]. In brief, cells from confluent 100-mm dishes (~7×10^6 cells/dish) were scraped into 10 ml of PBS, 10 mM EDTA and were pelleted by centrifugation. The cell pellets were resuspended in 1.5 ml of extraction buffer (20 mM Hepes 7.4, 5 mM EDTA, 1 M NaCl; including protease inhibitors) and were homogenized with a loose-fitting Dounce homogenizer. Large cell fragments and cell nuclei were removed by low-speed centrifugation (5 min at 1000 *g*). The supernatant was resuspended in extraction buffer and was centrifuged for 30 min at 32000 *g*. The pelleted membranes were resuspended in binding buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$) and stored at −70 °C.

2.4. Alterations of the cholesterol concentrations in cells and membranes

Extraction of cholesterol from the cells or membranes was carried out as described [16]. Briefly, cells or membranes (~4 mg/ml) were incubated with MβCD (final concentration 30 mM) for various times at 30 °C. To enrich membranes or cells with cholesterol, cholesterol-MβCD inclusion complexes were used. To produce these complexes, cholesterol (final concentration 3 mM or 10 mM) was added to an aqueous solution of MβCD (40 mg/ml). The mixture was overlaid with N_2, and was continuously vortexed under light protection for 24 h at 50 °C in a thermomixer. Cells or membranes were incubated with cholesterol-MβCD (final concentration 0.3 mM of cholesterol in complex) for various times (0–30 min at 30 °C). After the treatments, cells or membranes were washed with indicated medium/buffers and were used for experiments. In HEK293 cells, following a 30-min incubation step with 30 mM MβCD, ~80% of the initial cholesterol in membranes could be removed. When HEK-OTR cells were incubated with 0.3 mM of 10 mM cholesterol-MβCD for 10 min at 30 °C, they were enriched by ~40% cholesterol as compared to untreated cells.

2.5. Binding studies

Binding studies were performed with the fluorescent antagonist OTAN-A647. Membranes (20–100 µg) were incubated with 30 nM OTAN-A647 in a total volume of 100 µl binding buffer for 30 min at 30 °C. Displacement of OTAN-A647 was performed by adding increasing concentrations of OTAN or oxytocin to the labeled probe. In some experiments, the assays included varying concentrations of NaCl (0–300 mM). The binding reaction was stopped by addition of ice-cold binding buffer and subsequent centrifugation at 32000 *g* for 10 min. After a washing step the membranes were resuspended in 80 µl of acetonitrile:water (1:1, vol/vol) and transferred to a 384 well plate. The fluorescence was quantified by Omega reader (BMG).

Binding experiments on intact cells were performed at 14 °C. The cells were harvested and washed twice in basic imaging buffer (BIB) (140 mM NaCl, 10 mM Hepes pH 7.4, 5 mM KCl) in the presence (BIB/5 mM $MgCl_2$) or absence (BIB/5 mM EDTA) of Mg^{2+}. All displacement assays with cells were incubated for 1 h at 14 °C. In a series of experiments, sodium chloride (140 mM) was substituted by various substances: potassium chloride (140 mM), ammonium chloride (140 mM), choline chloride (140 mM), mannitol (280 mM) or sucrose (280 mM). Bound OTAN-A647 was separated from free ligand by centrifugation (2 min at 10,000 × *g*). After a washing step, the cell pellets were resuspended in 80 µl BIB, transferred to a 384 well plate, and counted in the fluorescence reader.

All assays were carried out in triplicate. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled oxytocin or OTAN. For data analysis and graphical output we used Sigmaplot (8.0, Jandel Scientific).

2.6. Measurements of cytosolic Ca^{2+} concentrations

Cells grown on petri dishes to about 80% of confluency were loaded with fura-2 AM (1.5 µM) for 30 min at 37 °C. Thereafter, the cells were scraped from the petri dishes and were resuspended in calcium imaging buffer (BIB plus 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM glucose). In some experiments, the sodium chloride (140 mM) was substituted by

potassium chloride (140 mM), ammonium chloride (140 mM), choline chloride (140 mM), mannitol (280 mM) or sucrose (280 mM). Aliquots of the suspension (6×10^5 cells) were added to prewarmed calcium imaging buffer and transferred into a cuvette which was placed into a thermostated (37 °C) holder. The cell suspension was continuously mixed by a magnetic stirrer. Oxytocin was applied to the cells and the changes of the $[Ca^{2+}]_i$ were monitored spectrofluorimetrically (Quantamaster, PTI, Toronto, Canada). The emission wavelength was set at 510 nm and dual-wavelength excitations were performed at 340 nm and 380 nm, respectively. The $[Ca^{2+}]_i$ was calculated by using the ratio 340/380 nm. The R_{max} value was obtained after Triton X-100 was added to the samples, R_{min} was obtained by chelating calcium with 5 mM EGTA and increasing the pH above 8.3.

2.7. Analytical methods

Lipid extraction was performed with chloroform-methanol according to the method of Bligh and Dyer, with modifications as described [6,17]. Cholesterol was assayed spectrophotometrically using a diagnostic kit (R-Biopharm, Darmstadt, Germany) performed in a micro-scale dimension. Protein was determined by the Bradford assay (Roth, Germany) using bovine serum albumin as a standard.

3. Results and discussion

Here, we address the question whether the binding of allosteric sodium is able to influence the ligand binding behavior of the oxytocin receptor. Studies with other GPCRs showed that the presence of sodium primarily inhibits agonist binding, while leaving unaffected the binding of antagonists [10]. For binding studies of the oxytocin receptor, we selected our previously synthesized and characterized fluorescent oxytocin antagonist (OTAN-A647) as the labeled ligand that can be displaced by unlabeled antagonist (OTAN) or the agonist oxytocin (OT) [9]. To prove whether displacement experiments with these ligands are able to evaluate the allosteric effect of cations at the oxytocin receptor, we first measured the influence of Mg^{2+} on ligand binding in membranes from HEK293 cells stably expressing the human oxytocin receptor (HEK-OTR). It is long known that divalent cations such as Mg^{2+} positively modulate the agonist binding to the oxytocin receptor, without affecting the binding affinity of antagonists [18]. As shown in Fig. 2A, displacement of OTAN-A647 by OTAN was only slightly affected by Mg^{2+}. In the absence of $MgCl_2$, the affinity of OTAN was even ~2 fold higher (IC_{50} ~6 nM) than that in the presence of $MgCl_2$ (IC_{50} ~13nM). In contrast, the binding of oxytocin to the receptor decreased by > 100 fold when Mg^{2+} was omitted from the binding assay, yielding IC_{50} levels of 4.5 µM and 38 nM in the absence and presence of $MgCl_2$, respectively (Fig. 2B). Thus, the strong requirement of Mg^{2+} for the agonist binding of the oxytocin receptor was clearly measurable in displacement assays with OTAN-A647.

Then, we tested the effect of sodium on the oxytocin receptor. For this purpose, membranes from HEK-OTR cells were incubated with OTAN-A647 and its competitor oxytocin in the presence of varying concentrations of NaCl (0–300 mM). As shown in Fig. 3A, increasing amounts of sodium caused a rightward shift in the concentration-response for oxytocin to displace the labeled antagonist. The IC_{50} for oxytocin increased from 10 nM in the absence of NaCl to 151 nM in the presence of 300 mM NaCl (Fig. 3B). This 15-fold lower affinity at 300 mM of NaCl was accompanied with a significant increase in the degree of maximal binding (Fig. 3A). The later effect might be due to the higher hydrophobicity of OTAN-A647 compared with oxytocin. It is expected that hydrophobic interactions of OTAN-A647 with the receptor will be enhanced under high-salt conditions.

Next, we analyzed the effect of sodium *versus* sodium substitutes on the ligand binding of intact cells. HEK-OTR cells were incubated with OTAN-A647 in Hepes-buffered saline either in the presence of NaCl or in solutions in which NaCl was exchanged by KCl, ammonium chloride,

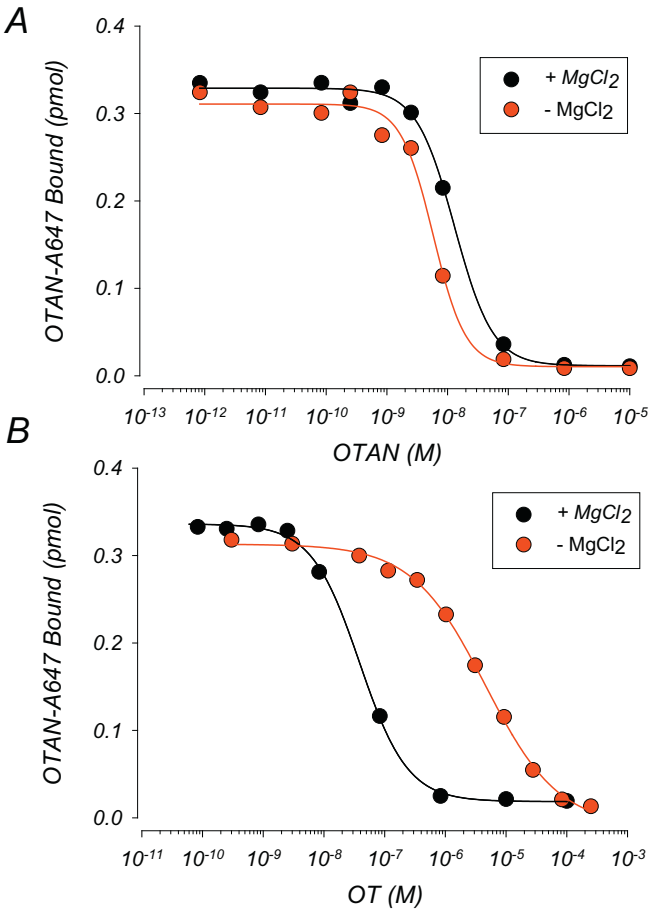

Fig. 2. Displacement of OTAN-A647 by OTAN (A) or oxytocin (B) in the absence or presence of $MgCl_2$. Membranes (50 µg protein) from HEK-OTR cells were incubated in 20 mM Hepes (pH 7.4) buffer plus 5 mM EDTA (●) or 5 mM $MgCl_2$ (●) with OTAN-A647 (30 nM) and displacing concentrations of OTAN (A) or oxytocin (B). Unbound OTAN-A647 was removed and the fluorescence was measured. The binding data were fit by logistic function $y = y_0 + a/(1 + (x/x_0)^b)$ yielding the following parameters a, b, x_0, y_0: A, +$MgCl_2$: 0.317, 1.376, $-1.306 \cdot 10^{-8}$, 0.011; −$MgCl_2$: 0.300, 1.628, $5.803 \cdot 10^{-9}$, 0.01; B, +$MgCl_2$: 0.318, 1.097, $-3.869 \cdot 10^{-8}$, 0.019; −$MgCl_2$: 0.323, 0.719, $4.557 \cdot 10^{-6}$, −0.01. The corresponding IC_{50} values were: A, +Mg: 13 nM, −Mg: 5.8 nM; B, +Mg: 38.7 nM, −Mg: 4.5 µM.

choline chloride (140 mM each), mannitol or sucrose (280 mM each). Mannitol and sucrose were used at higher concentrations to avoid osmotic effects on cells. The highest antagonist binding was observed for NaCl and KCl, while all other substances slightly decreased the binding of OTAN-A647 on the oxytocin receptor of intact cells (Fig. 4). This indicates that all NaCl substitutes except KCl have a slightly disturbing effect on the antagonist binding of the oxytocin receptor. In comparison with NaCl, all substitutes of NaCl exerted a significant leftward shift in the concentration-response for oxytocin in displacement studies with oxytocin on HEK-OTR cells (Fig. 5A and B). The corresponding IC_{50} values were calculated according to the best fit in logistic functions: NaCl (64 nM), mannitol (13 nM), sucrose (4 nM), KCl (26 nM), NH_4Cl (23 nM), choline chloride (21 nM) (Fig. 5C). Thus, in the presence of NaCl, oxytocin had the lowest affinity for the oxytocin receptor.

Taken together, we showed that sodium acts as a negative allosteric modulator of the oxytocin receptor. The binding of the agonist oxytocin occurred with about 15-fold lower affinity when sodium chloride was increased from 0 to 300 mM. The sodium effect was concentration-dependent and of high specificity as sodium substitutes like potassium chloride, ammonium chloride, or choline chloride were significantly less effective in modulating the oxytocin receptor binding compared with sodium chloride. Similarly, oxytocin bound with higher affinity to

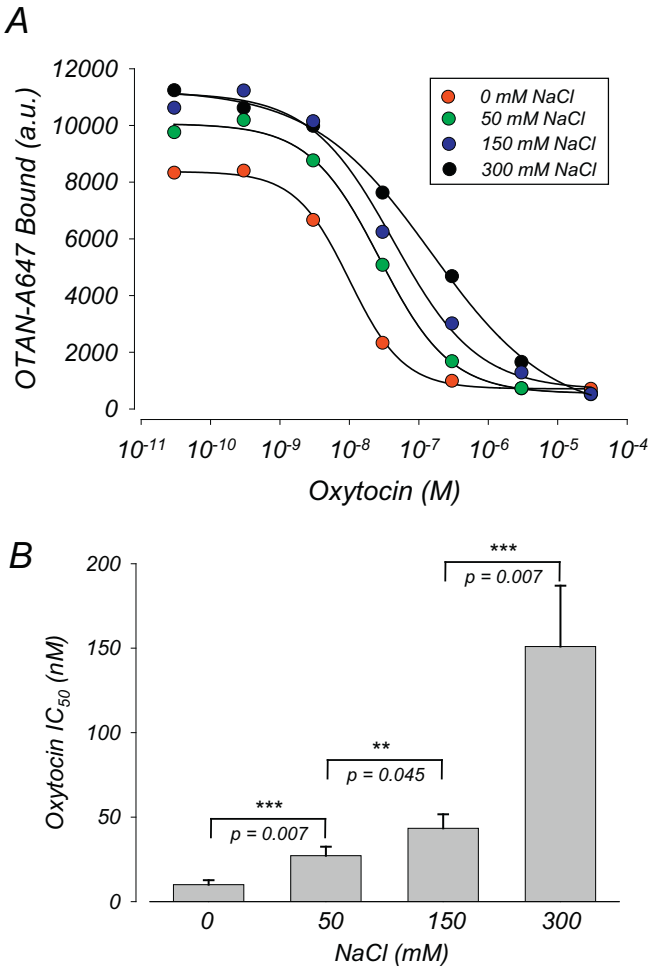

Fig. 3. Displacement of OTAN-A647 by oxytocin in membranes in dependence of varying NaCl concentrations. Membranes (50 µg protein) prepared from HEK-OTR cells were incubated with OTAN-A647 (30 nM) and displacing oxytocin concentrations in the absence or presence of the indicated NaCl concentrations. After washing-off the unbound OTAN-A647 the fluorescence was measured. The binding data for 0, 50, and 150 mM NaCl were best fit by logistic function $y = y_0 + a/(1 + (x/x_0)^b)$, the data for 300 mM NaCl was fit by Hill equation $y = y_0 + ax^b/(c^b + x^b)$ (A). The IC_{50} values (± S.D.) for oxytocin determined in three experiments are shown in panel B. The statistical significance of the data was determined by Student's *t*-test (**, $p < 0.01$; ***, $p < 0.001$).

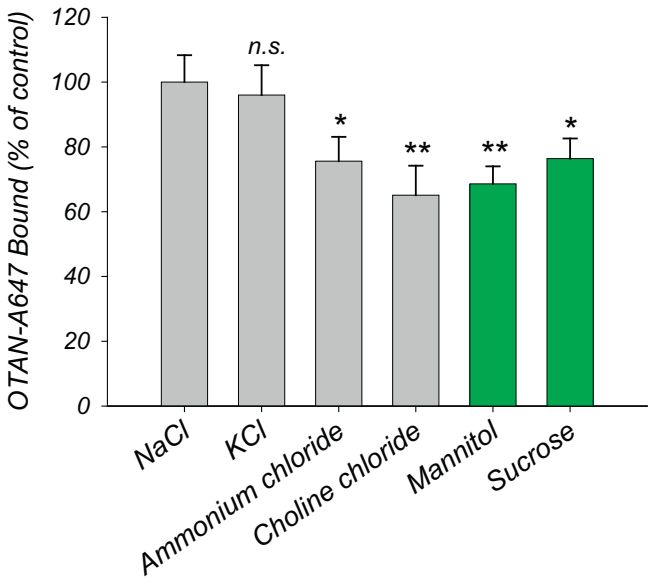

Fig. 4. The influence of the absence of sodium on the binding of OTAN-A647 to HEK-OTR cells. The cells were incubated (30 min, 20 °C) with OTAN-A647 (30 nM) in Hepes buffered (pH 7.4) solutions containing 1.2 mM $MgCl_2$ and one of the following compounds: NaCl (control), KCl, NH_4Cl, cholin chloride (each 140 mM), mannitol, sucrose (each 280 mM). Unbound ligand was removed and the fluorescence was determined. The data are expressed in % of the NaCl control (set to 100%) and represent means ± S.D. ($n = 3$). The data of the NaCl substitutes were analyzed for their differences against the NaCl control using Student's *t*-test yielding *p* values of 0.563 (KCl), 0.019 (NH4Cl), 0.007 (cholin chloride), 0.005 (mannitol), 0.016 (sucrose) (n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

its receptor when sodium chloride was omitted and exchanged by mannitol or sucrose to maintain cellular osmolarity. Additionally, concerning the sodium effect on ligand binding, the oxytocin receptor in cells behaved similar to that in membranes. To thoroughly prove the specificity of the sodium effect for the oxytocin receptor, we used several NaCl substitutes. Clearly, each of the substitutes has its drawbacks. In case of sucrose and mannitol, the chloride anions are missing. Ammonium chloride, a well-known lysosomatotropic substance, as well as choline chloride (possible interference with phospholipid head groups?) might possess more serious side effects in cells. Among all NaCl substitutes used in this study, KCl was the only one that did not interfere with the binding of the oxytocin antagonist to the receptor (Fig. 4) and, at the same time, was effective to displace allosteric sodium from the oxytocin receptor (Fig. 5C). Thus, we assume that in comparison with the other NaCl substitutes, KCl is less disturbing for intact cells. To analyze oxytocin-induced calcium responses in cells, we decided to choose only KCl as substitute of NaCl.

An aspartic acid residue in TM 2 ($D^{2.50}$) that is highly conserved among class A GPCRs plays a crucial role for the stabilization of sodium within its binding site (see Fig. 1B). In case of the human oxytocin receptor, the equivalent residue is D85. Mutation of this critical aspartic acid residue was expected to decrease or abrogate the sodium effect. However, it was observed that exchanges of this residue by alanine (D85A) or by asparagine (D85N) yield non-functional receptors [19,20]. In contrast, when D85 was substituted by glutamic acid (D85E) which keeps the negative charge at this position, oxytocin receptors transiently expressed in COS-7 cells were found to be functional [20]. Therefore, we stably expressed this mutant D85E oxytocin receptor in HEK293 cells and explored its behavior towards sodium ions. The mutant receptor was tagged at its C-terminus with EGFP and was stably expressed in HEK293 cells. This allowed us to visualize receptor expression by fluorescence microscopy. Most of the EGFP fluorescence was localized in tubular-vesicular structures resembling the endoplasmic reticulum (ER) and Golgi compartments (Fig. 6A). Only a very small fraction of the mutant receptors resided in the plasma membrane as shown by its staining with the fluorescent antagonist OTAN-A567 in Fig. 6B (overlay image in Fig. 6C), whereas the non-mutated oxytocin receptors are almost exclusively expressed at the plasma membrane (Fig. 6D). Unfortunately, only negligible amounts of specific OTAN-A647 binding could be measured on D85E cells as well as in membranes derived from these cells. All binding values were close to the detection limit so that no meaningful concentration-response curves could be obtained for the mutant receptors. However, we found that oxytocin was able to induce robust calcium responses in cells expressing the mutant receptor (Fig. 7). We obtained mean EC_{50} levels of 14.1 nM (± 6.4 nM) and 21.8 nM (± 9.6 nM) of oxytocin for the calcium responses of cells cultured in NaCl and KCl, respectively. These two EC_{50} values were not significantly different (*t*-test with $p = 0.36$) from each other. Conclusively, the faint but specific plasma membrane labeling with OTAN-A647 as well as the appearance of oxytocin-induced calcium responses clearly indicated that the mutant oxytocin receptors on the surface of HEK293 cells were functional. However, a sodium effect was not observed in HEK cells expressing the mutant D85E oxytocin receptors. This was unexpected since the charge-sparing mutation $D^{2.50}E$ is able to retain the sodium sensitivity of the receptor

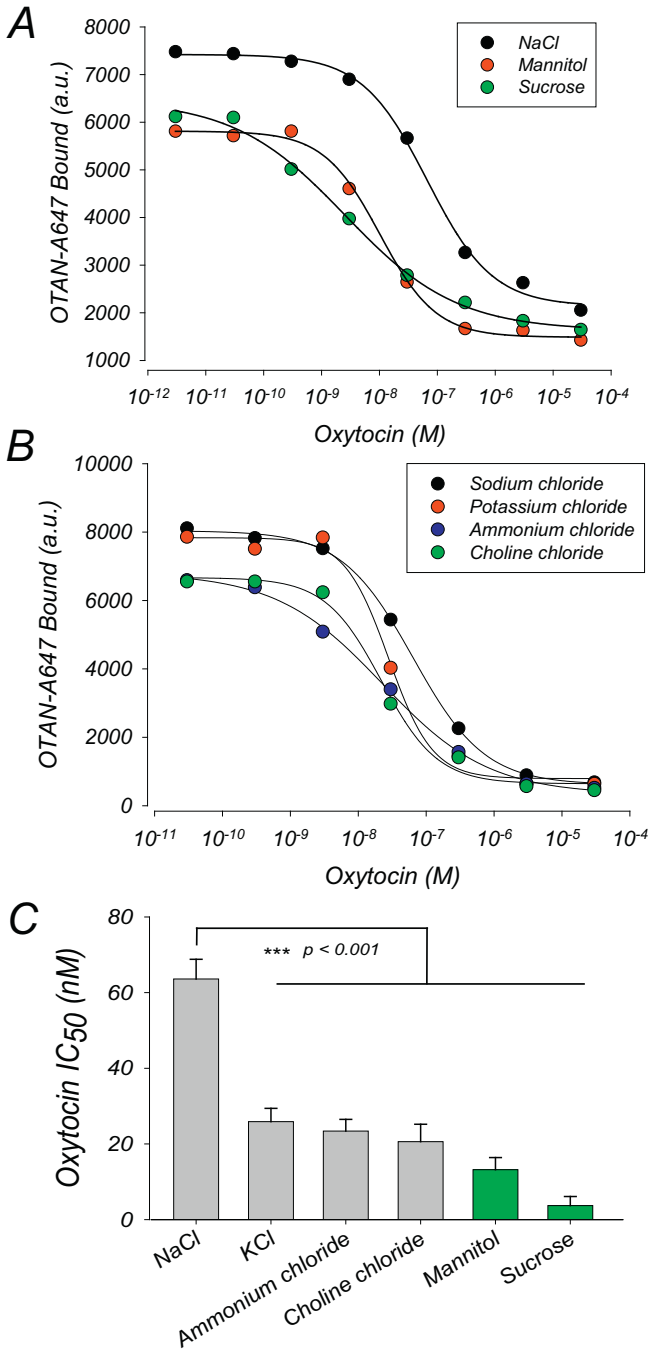

Fig. 5. Displacement of OTAN-A647 by oxytocin on HEK-OTR cells in the presence or absence of NaCl. Cells were incubated with OTAN-A647 (30 nM) and displacing oxytocin concentrations in Hepes-buffered medium containing 1.2 mM $MgCl_2$ and one of the following compounds: NaCl (control), mannitol, sucrose (A), KCl, NH_4Cl, Choline chloride (B). After washing-off the unbound OTAN-A647 the fluorescence was measured. The binding data were best fit by logistic functions. The IC_{50} values (± S.D.) for oxytocin determined in three experiments are shown in panel C. The statistical significance of the data was determined by Student's *t*-test (***, $p < 0.001$).

as shown for the D2 and D4 dopamine receptors [21]. The question arises why each of the substitutions of this aspartate residue leads to either misfunction or partial mistargeting of the oxytocin receptor. Many other GPCRs do not show this behavior [21–25]. Typically, $D^{2.50}$ replacement by an uncharged residue was shown to diminish or abolish agonist-induced G protein activation [26–33]. Since Na^+ contributes to stabilize the inactive receptor state, it is reasonable to assume that a collapse of this sodium site might produce more active but also more unstable receptors with a higher probability for unfolding, mislocalization, or degradation. This might explain the inactivity of the mutant oxytocin receptors D85A and D85N in which the negative charge of D85 has been completely eliminated. The mutation D85E is probably less disruptive for the sodium site and might provoke a milder form of receptor instability comprising prolonged folding and residence in the secretory pathway. On the other hand, the $D^{2.50}N$ mutation has recently been found to improve the structural stability of the A2A adenosine receptor [34]. Thus, to explain the unusual behavior of the D85 mutants of the oxytocin receptor, other reasons have to be considered. Possibly, its sodium sensitivity could be partially caused by D85 independent mechanisms. $D^{2.50}$ independent regulation of the affinity of ligands has been well documented for histamine H1 receptors [35]. Furthermore, it is important to note that structural variations exist among the sodium binding pockets of class A GPCRs [10]. The key polar residues coordinating the Na^+ and water cluster are highly conserved (positions $N^{1.50}$, $D^{2.50}$, $S^{3.39}$, $N^{7.45}$, $S^{7.46}$, $N^{7.49}$, and $Y^{7.53}$) and are also present in the sequence the oxytocin receptor (Fig. 1B). However, amino acid residue 3.35 is also of importance. In the δ-opioid receptor, its side chain ($N^{3.35}$) directly points to the interior of the sodium pocket [11]. In many other GPCR structures, this residue is hydrophobic (*e.g.* $L^{3.35}$ in A2AR) and then points towards the lipidic membrane interface [10]. In contrast, in the oxytocin receptor, the helix breaker glycine occupies this position (Fig. 1B) suggesting a more dynamic structure or environment of its sodium binding site. Finally, it has to be considered that the sodium binding site could interact with the binding site(s) of two other positive allosteric modulators of the oxytocin receptor, cholesterol and Mg^{2+}. They both may counteract the negative allosteric modulation of sodium in an unpredictable manner. Suboptimal amounts of cholesterol and Mg^{2+} could contribute to receptor instability, mistargeting or misfunction. For example, cholesterol supports the function of the oxytocin receptor in several aspects: it guarantees high-affinity state(s), an efficient expression yield, and conformational stability. The continuous presence of a cholesterol-rich environment stabilizes the receptor against thermal inactivation and proteolytic degradation [17,36].

Further experiments should clarify whether the sodium sensitivity of the oxytocin receptor is also observed at the level of signal transduction. For this purpose, the oxytocin-induced calcium responses of HEK-OTR cells cultured in NaCl *versus* KCl containing media were compared. Representative concentration-response curves are shown in Fig. 8A (typical calcium response in inset). Accordingly, a slightly lower EC_{50} was observed for cells under sodium-free conditions. In three experiments, we obtained mean EC_{50} levels of 1.25 nM (± 0.36 nM) and 0.76 nM (± 0.29 nM) of oxytocin for the calcium responses of cells cultured in NaCl and KCl, respectively. According to Student's *t*-test, these EC_{50} values are significantly different ($p = 0.036$).

Finally, the question arises whether this sodium effect is also present in cells endogenously expressing oxytocin receptors. For this purpose, we used Hs578T cells, a human breast cancer cell line in which functional oxytocin receptors have been detected [37]. In these cells, oxytocin-induced calcium responses could be measured. Representative concentration-response curves are shown in Fig. 8B. Notably, the maximum calcium responses obtained in the presence of KCl were about twice as high as those obtained in the presence of NaCl. The corresponding EC_{50} values of these data were 19.1 nM (± 4.9 nM) and 7.0 nM (± 2.6 nM) in the presence of NaCl and KCl, respectively ($n = 3$; *t*-test: $p = 0.018$). Thus, oxytocin receptors endogenously expressed in Hs578T cells revealed a markedly higher calcium response as well as a nearly 3-fold higher affinity towards oxytocin when sodium was absent.

Overall, the sodium effect was not only observed for oxytocin receptors heterologously expressed in stably transfected HEK293 cells but also for oxytocin receptors endogenously expressed in low amounts, such as in the breast cancer cell line Hs578T [37]. Thus, sodium negatively modulates agonist binding whereas antagonist binding

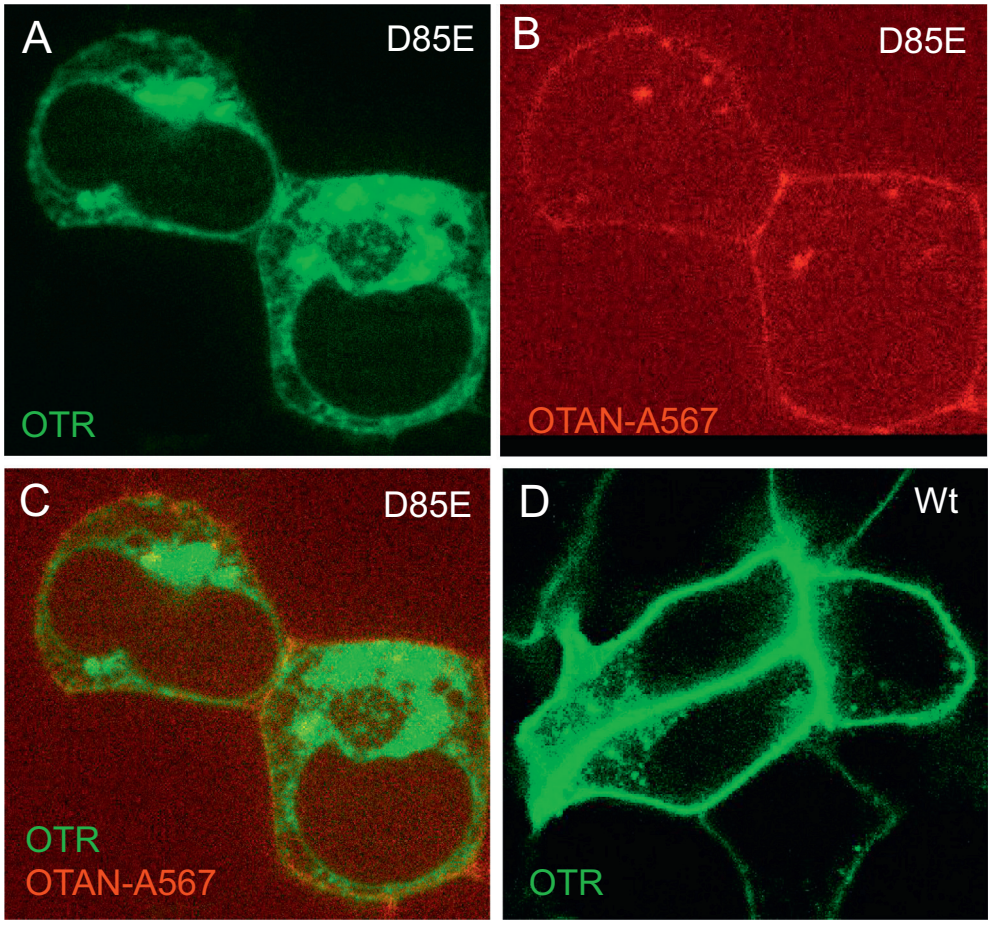

Fig. 6. Fluorescence microscopy of the GFP-tagged D85E mutant (A-C) *versus* non-mutated (Wt) (D) oxytocin receptor in HEK cells. A, mutant receptor (EGFP, green); B, OTAN-A567 binding (red) of cells in A; C, overlay of A and B; D, Wt receptor (EGFP, green).

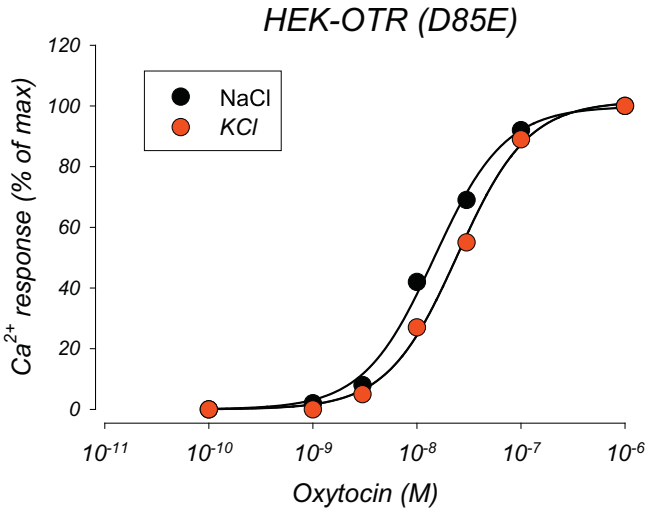

Fig. 7. Calcium responses of HEK cells expressing the mutant D85E oxytocin receptor in the presence of NaCl *versus* KCl. Fura-2 loaded cells were washed with PBS and were resuspended in imaging buffer containing either 150 mM NaCl or KCl. Aliquots (~10^6) of cells were transferred into a cuvette and were stimulated with varying concentrations of oxytocin. Changes of the $[Ca^{2+}]_i$ were monitored spectrofluorimetrically and the peak responses (in % of maximum) from representative experiments are displayed. The data were best fit by Hill function (3 parameters) yielding EC_{50} values of 14.7 nM and 24.6 nM for cells in the presence of NaCl and KCl, respectively. The statistical analysis of the data as determined by Student's *t*-test yielded a *p* value of 0.36 (no significant difference).

remained largely unchanged. This effect was reported for a number of GPCRs (*e.g.* adenosine, adrenergic, opioid, and dopamine receptors) and might be typical for nearly all class A GPCRs [10,12,38–41]. Notably, the sodium effect of the oxytocin receptor was found to be rather strong when analyzed in membranes, but was less pronounced when studied in intact cells. However, this might be due to methodical reasons as the omission or substitution of the external NaCl solution should disturb the membrane potential and several other physiological processes in an unpredictable manner.

In conclusion, the oxytocin receptor is controlled by at least three allosteric modulators, Na^+, Mg^{2+}, and cholesterol. While cholesterol and Mg^{2+} are essential to support the high-affinity state of the oxytocin receptor, sodium negatively modulates the agonist binding of the receptor and presumably stabilizes the inactive receptor state. It is currently unknown whether the modulators cholesterol and Mg^{2+} are located close to the sodium site. Structural data show that cholesterol molecules are able to bind at various positions and clefts of heptahelical GPCRs (reviewed in [42]). The oxytocin receptor possesses two putative cholesterol binding motifs, the CCM site and the CRAC motif, which are both located in the central part of the receptor. Biochemical data also suggest that central or N-terminal parts of the oxytocin receptor may interact with cholesterol [43]. In contrast, virtually nothing in known about the location of the Mg^{2+} binding site of the oxytocin receptor. Thus, further work is required to identify these allosteric receptor sites.

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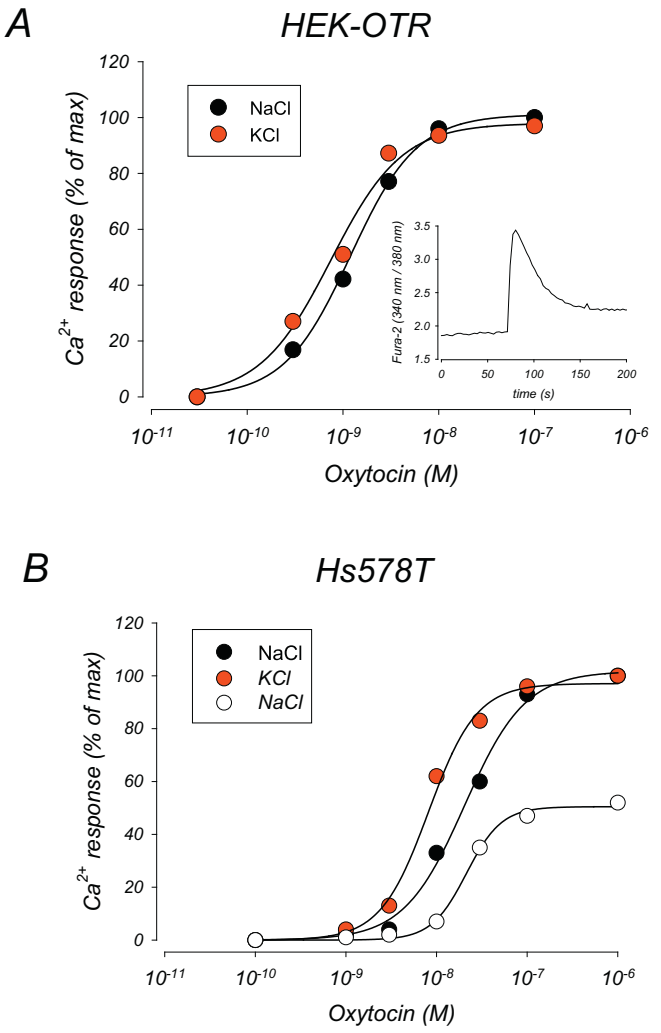

Fig. 8. Calcium responses of HEK-OTR cells (A) and Hs578T cells (B) in the presence of NaCl *versus* KCl. Cells loaded with fura-2 were washed with PBS and resuspended in imaging buffer containing either 150 mM NaCl or KCl. Aliquots (~10^6) of cells were transferred into a cuvette and were stimulated with varying concentrations of oxytocin. Changes of the $[Ca^{2+}]_i$ were monitored spectrofluorimetrically and the peak responses (in % of maximum) from representative experiments are displayed. A typical oxytocin-induced calcium response is shown in the inset of panel A. The data were best fit by Hill function (3 parameters) yielding EC_{50} values of 1.20 nM and 0.75 nM for HEK-OTR cells (A) in the presence of NaCl (black) and KCl (red), respectively. For Hs578T cells (B), EC_{50} values of 20.3 nM and 8.2 nM were obtained in the presence of NaCl (black) and KCl (red), respectively. In Hs578T cells, the maximum calcium response (100%) was obtained in the presence of KCl (red) (100% ± 7.9%, $n = 3$) whereas the responses in the presence of NaCl (open symbols) (51% ± 5.5%, $n = 3$) were significantly lower ($p < 0.001$; *t*-test). For better comparison, the NaCl data were also given normalized to 100% (black symbols).

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