The Oxytocin Receptor Antagonist Atosiban Inhibits Cell Growth via a “Biased Agonist” Mechanism*

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In human myometrial cells, the promiscuous coupling of the oxytocin receptors (OTRs) to Gq and Gi leads to contraction. However, the activation of OTRs coupled to different G protein pathways can also trigger opposite cellular responses, e.g., OTR coupling to Gq inhibits, whereas its coupling to Gi stimulates, cell proliferation. Drug analogues capable of promoting a selective receptor-G protein coupling may be of great pharmacological and clinical importance because they may target only one specific signal transduction pathway. Here, we report that atosiban, an oxytocin derivative that acts as a competitive antagonist on OTR/Gq coupling, displays agonistic properties on OTR/Gi coupling, as shown by specific [35S]GTPγS binding. Moreover, atosiban, by acting on a Gq-mediated pathway, inhibits cell growth of HEK293 and Madin-Darby canine kidney cells stably transfected with OTRs and of DU145 prostate cancer cells expressing endogenous OTRs. Notably, atosiban leads to persistent ERK1/2 activation and p21WAF1/CIP1 induction, the same signaling events leading to oxytocin-mediated cell growth inhibition via a Gi pathway. Finally, atosiban exposure did not cause OTR internalization and led to only a modest decrease (20%) in the number of high affinity cell membrane OTRs, two observations consistent with the finding that atosiban did not lead to any desensitization of the oxytocin-induced activation of the Gq-phospholipase C pathway. Taken together, these observations indicate that atosiban acts as a “biased agonist” of the human OTRs and thus belongs to the class of compounds capable of selectively discriminating only one among the multiple possible active conformations of a single G protein-coupled receptor, thereby leading to the selective activation of a unique intracellular signal cascade.

Oxytocin, a nonapeptide secreted by the neurohypophysis, exerts its biological effects by binding to and activating the oxytocin receptor (OTR), a membrane receptor belonging to the G protein-coupled receptor family (1). As recently reviewed by Gimel (2), OTRs were originally described in the uterus, mammary gland, and central nervous system. In the uterus, the level of OTR expression increases during pregnancy and peaks immediately before the onset of labor, when receptor activation promotes uterine contractility. The OTRs in the mammary gland are located on myoepithelial cells, where they stimulate contractility and promote milk ejection. In the central nervous system, where they modulate hippocampal synaptic plasticity during pregnancy (3), OTRs are involved in regulating a number of reproductive and non-reproductive behaviors.

OTRs have more recently been detected in a vast number of tumor cells of various origin (breast and endometrial carcinomas, neuroblastomas, glioblastomas, Kaposi sarcomas, small-cell lung carcinomas), and their stimulation variably affects cell growth (4). Moreover, it has recently been suggested that OTRs are involved in the differentiation of cardiomyocytes (5) and myoblasts (6).

Despite the biological relevance of OTR signaling, the identity of signaling pathways activated to accomplish these different functions has only been partially clarified. In particular, although human OTRs have been shown to associate to Gq, Gi, and, to a minor extent, to Gαs (7), only the signaling events induced by Gq activation have been extensively investigated and exploited to develop drugs already entered in the clinical use. In myometrial and breast myoepithelial cells, OTR coupling to Gq is responsible for phospholipase C (PLC) activation followed by inositol phosphate (InsP) and diacylglycerol production, increased intracellular calcium, and increased contractility (8). Accordingly, selective oxytocin analogues capable of blocking this pathway are employed in the treatment of preterm labor (9). The physiological role played by OTRs coupled to Gi in myometrial cells is far less clear, although it was shown some years ago to also lead to an increase of cell contractility (10). Recently, a specific role for OTR coupled to Gi has emerged in MDCK and HEK293 cells stably transfected with the human OTR, where OT inhibits cell growth in a pertussis toxin (PTx)-sensitive manner, thus suggesting a key role of OTR-Gi coupling in mediating an anti-proliferative effect (11).

A review of our previous work indicated that atosiban, an...
Oxytocin antagonist that is currently used in the treatment of preterm labor because of its ability to block the G<sub>i</sub>/PLC/calcium signaling pathway in myometrial cells (12–15), inhibits the proliferation of some cancer cells (16). As we have recently shown that the growth of MDCK and HEK293 cells is inhibited via G<sub>i</sub>-coupled OTRs (11), we hypothesized that atosiban inhibited cell growth via a biased agonist mechanism by selectively promoting OTR coupling to G<sub>i</sub>. Biased agonists have been defined as analogues displaying, at a single promiscuous GPCR, agonist properties at one coupling pathway and antagonist properties at another one (17, 18). To check the biased agonist properties of atosiban at the human OTR, we examined atosiban signaling in MDCK and HEK293 cell clones stably transfected with human OTRs, as well as in DU145 human prostate cancer cells endogenously expressing OTRs.

**EXPERIMENTAL PROCEDURE**

**Peptides and Reagents—**OT and Thr<sub>5</sub>Gly<sub>4</sub>OT were obtained from Sigma; atosiban (mpnl-D-Tyr<sub>5</sub>,Thr<sub>8</sub>,Orn<sub>9</sub>)/OT) (12) was synthesized in the laboratory of Dr. M. Manning, Toledo, OH. The sources of the primary antibodies used in this study were as follows: polyclonal anti-GFP (catalogue number sc-397) and anti-caveolin-1 (catalogue number sc-894) from Santa Cruz Biotechnology Inc.; monoclonal anti-green fluorescent protein (GFP) (catalogue number M048–3 clone 1E4) from MBL International Corp.; polyclonal anti-β-tubulin (catalogue number T5293 clone 2–28–33) from Sigma; polyclonal anti-phospho p42/44 MAPK (catalogue number 9101) from Cell Signaling Technology. Peroxidase-conjugated secondary antibodies were from Pierce, and [<sup>35</sup>S]GTP<sub>S</sub> (1250 Ci/mol) was from PerkinElmer Life Sciences.

**Cell Culture—**The human prostate carcinoma DU145 cell line was purchased from ATCC (Manassas, VA) and routinely cultured in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal calf serum (FCS) purchased from ATCC (Manassas, VA) and routinely cultured in RPMI 1640 medium (Invitrogen), in a 5% CO<sub>2</sub> humidified atmosphere, at 37 °C. Production, pharmacological characterization, and culture conditions of MDCK and DU145 cells were then fixed in 2.5% (v/v) glutaraldehyde, stained with 0.1% (w/v) crystal violet in 20% (v/v) methanol, and solubilized with 5 M for the indicated periods of time. Cells were then washed once in ice-cold PBS and lysed in 50 mM Tris-HCl, pH 6.8, containing 2% (v/v) SDS, preheated at 100 °C. After 4–5 cycles of freezing in dry ice and boiling for 2 min, aliquots of the lysates were assayed for protein content using the BCA protein assay reagent (Pierce). Cellular proteins (30 μg) were resolved by Laemmli SDS-PAGE system using 11% acrylamide and blotted onto nitrocellulose membranes (Amersham Biosciences). Blots were incubated overnight at 4 °C in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl), containing 5% (v/v) skimmed milk. The membranes were then incubated for 2 h with primary antibodies diluted in Tris-buffered saline/milk and for 2 h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG (Pierce). When phospho p42/44 MAPK antibody was used, blocking was performed in 5% (w/v) bovine serum album in 1% (w/v) bovine serum albumin/0.2% (v/v) Tween 20 for 1 h at 37 °C, and incubation was performed overnight in Tris-buffered saline/bovine serum albumin at 4 °C. Proteins were detected using the SuperSignal® chemiluminescent substrate (Pierce). For quantification, unsaturated bands were acquired by means of an Arcus II Scanner (Agfa-Gevaert) and analyzed with the NIH Image program Version 1.61 (National Technical Information Service, Springfield, VA).

**Internalization Assays—**Cells were washed twice in serum-free medium and left for 30 min to equilibrate at 37 °C. Cells were then added at a final concentration of 10<sup>−6</sup> M. At fixed time intervals (0, 5, 15, 30, and 60 min), cells were processed for fluorescence microscopy or binding assays.

For fluorescence microscopy, cells grown on glass coverslips placed in 6-well dishes were washed twice with 10 ml sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and fixed for 20 min at room temperature with 4% (v/v) paraformaldehyde in PBS. Fixed cells were rinsed with LS buffer and mounted on glass slides with 90% (v/v) glycerol in PBS. Slides were observed under an MRC1024 Bio-Rad laser scanning confocal microscope.

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remove the unbound radioactivity and then solubilized with 0.5 N NaOH. The samples were transferred to scintillation vials and counted in a /H-counter after the addition of 3.5 ml of scintillation fluid. Specific surface binding at each time was calculated as percentage of specific binding at time 0.

RESULTS

Atosiban-induced Inhibition of MDCK and HEK293 Cells Expressing Human OTRs—To analyze the ability of atosiban to inhibit the growth of cells expressing the human OTR, we checked its effects on MDCK and HEK293 cells stably transfected with the human wild-type (WT) OTR. MDCK cells were fixed and stained with crystal violet, and cell growth was evaluated by measuring the absorbance at 590 nm. Proliferation of HEK293 cells was determined by a methanethiosulfonate-based assay. All of the experiments were done in sextuplicate. B and E, concentration-response curves of atosiban-induced inhibition of cell proliferation in MDCK (B) and HEK293 (E) cells expressing the wild-type OTR. C and F, parental MDCK (C) and HEK293 (F) cells transfected with an empty pEGFP-N3 vector. The concentration-response curves are representative of two independent experiments each performed in triplicate. ***, p < 0.001; **, p < 0.01; *, p < 0.05 versus untreated cells grown for equal periods of time in the absence of the peptide (Student’s t test).

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Atosiban-induced Inhibition of MDCK and HEK293 Cells Expressing Human OTRs—To analyze the ability of atosiban to inhibit the growth of cells expressing the human OTR, we checked its effects on MDCK and HEK293 cells stably transfected with the human OTR fused to EGFP (11, 19). As shown in Fig. 1 (A and D), cell growth was significantly inhibited after 48 h of treatment with OT and atosiban. This effect was concentration-dependent with calculated IC50 values of 20.8 ± 0.2 nM in HEK293 cells and 15.4 ± 0.44 nM in MDCK cells (Fig. 1, B and E), very similar to those calculated for OT (5.76 ± 0.3 nM and 22.0 ± 4.3 nM in HEK293 and MDCK cells, respectively; 13, 18).2 Saturation and competition binding experiments performed in HEK293 cells resulted in the following affinities: $\text{K}_d = 4.9 ± 2.2 \text{nM}$ and $\text{K}_i = 7.15 ± 21.2 \text{nM}$ for OT and atosiban, respectively (data not shown). Finally, as shown in Fig. 1 (C and F), OT and atosiban did not induce any change in cell growth in parental MDCK and HEK293 cells stably transfected with the pEGFP vector alone, thus indicating that their effects on cell growth could only be detected in cells expressing OTRs.

Signaling Pathways Involved in the Atosiban-mediated Inhibition of Cell Growth—As it is known that OTRs couple to Gq and Gi (7) and that receptor coupling to Gi is responsible for the OT-induced inhibition of HEK293 cell growth (11), we investigated the signaling events leading to the atosiban-induced inhibition of cell growth, and particularly, whether Gi is involved. To determine whether atosiban could directly promote OTR coupling to heterotrimeric G proteins, we measured its ability to promote the binding of [35S]GTPγS, according to the paradigm that receptor activation promotes the GDP/GTP exchange on G protein α subunits. This assay utilizes [35S]GTPγS resistance to hydrolysis, its high affinity for the Gα subunits, and its relatively high specific activity to determine receptor-induced Gα activation (22–24). As shown in Fig. 2A, in HEK293 expressing the human OTR, OT and atosiban were both able to induce a percentage increase in [35S]GTPγS binding over basal of 23.84 ± 2.70 and 26.42 ± 4.10, respectively. In contrast, in parental, untransfected HEK293 cells, the two analogues did not induce any significant increase in [35S]GTPγS binding.

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2 A. Reversi, V. Rimoldi, and B. Chini, unpublished results.
These data indicate that atosiban is capable of promoting OTR coupling to G protein, thus displaying agonist properties toward the human OTR. In addition, in OTR-expressing cells pretreated with PTX, the capability of OT and atosiban to increase \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding was completely abolished, indicating a coupling to G\_like proteins. The lack of any residual \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding even in membranes treated with OT indicates that, in our experimental conditions, OTR coupling to G\_q did not significantly contribute to total \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding. We then checked the ability of atosiban to modulate proliferation of PTX-treated cells (Fig. 2B); under these conditions, the atosiban-induced inhibition of cell growth was completely abolished, thus indicating that a PTX-sensitive G protein of the G\_q family mediates the inhibitory effect.

As we have shown that the OT-induced growth inhibition of MDCK and HEK293 cells is associated with a long-lasting activation of MAPK (11), we investigated whether atosiban can also induce MAPK phosphorylation. To this end, we applied atosiban for an increasing length of time and analyzed ERK1/2 activation by means of Western immunoblotting of cell lysates probed with phospho-specific anti-ERK1/2 antibody. As shown in Fig. 2C, atosiban induced a persistent increase in ERK1/2 phosphorylation, a pattern consistent with inhibition of cell growth, as demonstrated previously in HEK293 and MDCK cells (11).

We then verified whether atosiban can promote OTR coupling to G\_q by measuring the atosiban-induced accumulation of total InsPs; as shown in Fig. 2D, atosiban (10^{-7} M) in the absence of atosiban (not shown) fixed at 100%. The amount of InsPs produced at each point is expressed as the percentage of InsPs produced at the maximal OT concentration (10^{-5} M). The data are representative of two independent experiments diverging for <20%.

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Atosiban-induced Inhibition of DU145 Human Prostate Cancer Cell Growth—The human DU145 prostate cancer cell line expresses OTRs but not the endogenous OT ligand, and, in these cells, exposure to OT leads to a significant decrease in cell proliferation, already detectable after 48 h and peaking after 96 h (25). Therefore, this cell line represents a suitable model for testing the ability of atosiban to affect the proliferation of cells endogenously expressing OTRs. When treated with 100 nM atosiban, DU145 cells responded with a significant decrease in proliferation, firstly observed after 48 h and reaching its maximum at 72 h (Fig. 3A). The extent of growth inhibition induced by atosiban was similar to that observed after treatment with equal concentrations of OT or the highly selective OTR agonist Thr⁴Gly⁷OT (Fig. 3B). The anti-proliferative effect of atosiban was concentration-dependent, with a calculated EC₅₀ of 3.9 × 10⁻⁸ M (Fig. 3C). Finally, no additive effect was observed upon combined exposure of cultures to OT and atosiban for 48 h (Fig. 3D).

Finally, we investigated whether atosiban stimulation of OTRs in DU145 cells leads to ERK1/2 phosphorylation. As shown in Fig. 4, both the highly selective OT analogue Thr⁴Gly⁷OT (26) and atosiban elicited a sustained ERK1/2...
activation. Moreover, Western blot analysis showed that the levels of cell cycle inhibitor p21\(^{WAF1/CIP1}\) started to increase after 24 h of exposure and reached the maximum after 48 h, when the cell growth inhibition by Thr\(^4\)Gly\(^7\)OT and atosiban became measurable. These data suggest that the sustained MAPK activation observed after OTR stimulation by Thr\(^4\)Gly\(^7\)OT and atosiban inhibits the growth of DU145 cells by inducing the expression of p21\(^{WAF1/CIP1}\).

OT and Atosiban Induce Different OTR Desensitization and Internalization Patterns—GPCR signaling is generally down-regulated by agonist-induced internalization, a process that sequentially involves agonist binding to the receptor, and subsequent receptor phosphorylation, β-arrestin recruitment, and endocytosis. No data are yet available on the effects of biased agonists on GPCR internalization. To investigate whether atosiban can induce receptor internalization, we used MDCK and HEK293 cells stably transfected with OTR fused to EGFP to monitor receptor distribution by confocal microscopy. Thus, we examined the subcellular distribution of OTRs after incubation with 10\(^{-7}\) M OT or atosiban for increasing lengths of time (Fig. 5, A and B). Before agonist exposure (time 0), confluent monolayers expressing OTRs were homogeneously fluorescent all around the cell edges. A punctate fluorescence (presumably associated with endocytic vesicles) could be seen as early as 5 min after OT application and became more evident after 15 min. After 30 and 60 min, the fluorescent patches seemed to be mainly localized within intracellular compartments of both MDCK and HEK293 cells. In contrast, OTRs remained permanently localized at the cell surface even after 60 min of atosiban treatment, without any signs of endocytic vesicle formation.

We also estimated the number of cell surface OTRs by measuring the high affinity binding of \(^{3}H\)OT to intact cells after different times of peptide exposure. As shown in Fig. 6A, OT treatment led to a progressive and almost complete (75%) loss of high affinity sites in HEK293 cells after only 15 min, whereas atosiban treatment induced the loss of only 20% specific high affinity binding sites even after 120 min.

Finally, we investigated whether the surface OTRs remaining after prolonged atosiban exposure displayed any agonist-induced desensitization of the G\(_i\)/PLC signaling pathway by measuring the OT-induced accumulation of total InsP in the

![Fig. 4. Time course of MAPK and p21\(^{WAF1/CIP1}\) activation in DU145 cells. DU145 cells were treated with the highly specific OTR agonist Thr\(^4\)Gly\(^7\)OT (A) or atosiban (B) for the indicated periods of time. The cells were lysed, and 30 μg of proteins were resolved by SDS-PAGE. MAPK activation was detected by Western blotting with anti-phospho-ERK1/2 antibody, and p21\(^{WAF1/CIP1}\) induction was detected using a specific polyclonal antibody. Immunoblotting with a polyclonal anti-β-tubulin antibody was used to verify that equal amounts of proteins were loaded in all lanes. The blots are representative of two independent experiments.](image)

![Fig. 5. Time course of agonist-induced OTR internalization. Subcellular localization of recombinant wild-type OTR fused to EGFP was visualized by laser scanning confocal microscopy in stably transfected MDCK and HEK293 cells grown on glass coverslips. In these experiments, OT and atosiban were added at time 0, at a final concentration of 10\(^{-7}\) M, and cells were fixed after a further incubation at 37 °C for 5, 15, 30, and 60 min. The images show midsections of the cells. A, stable MDCK cells expressing OTR-GFP. Bas, basal. B, stable HEK293 cells expressing OTR-GFP. Comparable results were obtained with a final concentration of atosiban of 10\(^{-5}\) M, presence or absence of atosiban. As shown in Fig. 6B, a 60-min atosiban treatment did not affect the subsequent accumulation of total InsP induced by OT, thus indicating that atosiban did not induce any measurable desensitization of the canonical G\(_i\)/PLC pathway.](image)
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(Δ-Arg₁,D-Phe⁶,D-Trp⁷,⁹,Leu¹ⁱ) substance P, a neurokinin-1 receptor antagonist that, in small-cell lung carcinoma, inhibits the G₁₂–₁₃-mediated pathway and behaves as an agonist to activate the c-Jun N-terminal kinase. It was subsequently found that the latter effect is mediated by bombesin receptors, i.e. substance P activates bombesin receptor signaling through G₁ and simultaneously blocks receptor coupling to G₁₂. Moreover, it was shown that substance P, via G₁, stimulates the MAPK pathway, leading to cell growth inhibition (28). Another analogue characterized by a biased agonist activity is the bradykinin antagonist dimer CU201 that, in small-cell lung carcinoma and other cancer cell lines, blocks the G₁₂–₁₃ pathways and activates the G₁₁ pathways, once again leading to apoptosis and inhibition of cell growth (29). Biased agonists can have enormous pharmacological and clinical importance because they can trigger, for each single receptor subtype, only the “appropriate” downstream signaling pathway, thus representing a novel class of highly selective compounds. The existence of biased agonists is consistent with a multistate model of receptor activation in which single ligands can promote specific receptor conformations capable of differentially activating distinct signaling partners. This phenomenon, termed “agonist-directed trafficking of receptor stimulus” by Kenakin (30, 31), has recently gained further support by the finding that inverse agonists acting on β-adrenergic and vasopressin receptors can lead to ligand-selective receptor activation (32).

Agonist-directed trafficking of receptor stimulus should not be confused with an “agonist strength-based mechanism.” In this case, a powerful agonist may produce a stimulus sufficient to simultaneously activate two intracellular pathways, whereas a weaker agonist may only stimulate the most sensitive one. As quoted from a recent review of Kenakin: “The most sound evidence for the demonstration of receptor-selective states with a ligand is either: (1) thorough clear differences in efficacy (maximal response in which affinity is not an issue) or (2) actual reversal of relative potency in which there can be no question of differences as a result of a different strength of signal” (18). Here, comparing the potencies of OT and atosiban would be questionable because potency at G₁₂–₁₃-coupled OTRs is evaluated as increased generation of InsPs, whereas potency at G₁-coupled OTRs is measured as degree of cell growth inhibition, an effect downstream of the proximal second messenger generation step. The evidence of stimulus trafficking comes, instead, from the very different efficacies of OT and atosiban in promoting OTR/G₁₂–₁₃ coupling, despite binding affinities differing by only 1 order of magnitude. Notably, atosiban was devoid of any agonist activity on OTR/G₁ coupling when compared with the full agonist oxytocin, demonstrating the induction of ligand-selective receptor states. Furthermore, atosiban was confirmed to be a competitive antagonist on OTR/G₁ coupling, indicating biased agonist properties.

It is worth noting that a number of other peptidic antagonists have been found to interfere with cell proliferation in a complex and only partially understood manner, including the bradykinin B2 antagonist Hoe140 (33), the “dipeptoid” CCK-B/gastrin agents (34), the neurotensin receptor antagonist SR48692 (35), the leukotriene B₄ receptor antagonist LY293111 (36), and the somatostatin receptor antagonist cSSTA (37). It will be interesting to verify whether the complex effects on cell growth by these peptides may also be explained by a biased agonist action on their receptors. In this case, their classification as antagonists will need to be re-evaluated.

Atosiban acts on a GPCR activated by OT, the small peptide from which it was originally derived. Since a vast number of peptidic analogues of oxytocin have been developed over the last 40 years (38, 39), it would be very interesting to evaluate the structural-functional relationships leading to biased agonist properties. The OTR has been subjected to molecular modeling by several groups (40), and recently, the atosiban-OTR interactions leading to putative high affinity binding have been investigated (41). A combined molecular modeling and site-directed mutagenesis study could greatly improve our understanding of the molecular basis underlying receptor activation as well as the specificity of receptor-G protein interaction. It will also be interesting to determine the amino acid residues of OT that are responsible for the induction of a selective coupling state of the receptor, as done for the human parathyroid hormone, where the regions responsible for the dual coupling to adenyl cyclase and PLC have been mapped (42).

As atosiban acts as an agonist to promote G₁₂–₁₃-mediated signaling, we investigated whether, like endogenous OT, it could induce agonist-induced receptor internalization (19, 43). On the basis of our confocal microscopy data, we can conclude that atosiban, at the concentrations used, does not induce any receptor internalization. Likewise, no desensitization of G₁ PLC signaling occurred. However, as atosiban treatment led to a small but significant 20% decrease in the number of high affinity binding sites, it is tempting to speculate that this OTR...
population may represent the agonist-activated OTRs coupled to G\(\alpha\) that undergo desensitization, a hypothesis that will require further investigation. It has been recently shown that recombinant human OTRs expressed in HEK293 cells undergo agonist-dependent phosphorylation by GRK2, an event that ultimately leads to receptor internalization (44), but it is still unclear whether receptor coupling to different G proteins affects the recruitment of the kinase and the subsequent events involved in receptor trafficking. To clarify this issue, atosiban could represent a very valuable tool.

Finally, it is worth noting that atosiban inhibits cell growth not only in cell lines transfected with the human OTR but also in DU145 human prostate cancer cells endogenously expressing OTRs (this study) and mammary carcinoma cells (16); this occurs in the absence of G\(\alpha\)/PLC stimulation and thus represents a very specific downstream signaling event. Given that atosiban has already been approved as an antagonist for the treatment of preterm delivery, a pilot clinical trial of its use in the treatment of cancer cells expressing high human OTR levels warrants consideration.

In conclusion, our data indicate that atosiban, a peptide derivative of the posterior pituitary OT hormone, possesses biased agonist activity on the human OTRs and represents a very valuable tool for investigating the molecular basis of ligand-induced coupling specificity.

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REFERENCES

1. Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., and Okayama, H. (1992) Nature 356, 526–529
2. Gimpl, G., and Fahrenholz, F. (2001) Physiol. Rev. 81, 629–683
3. Tomizawa, K., Iga, N., Lu, Y. F., Moriwaki, A., Matsushita, M., Li, S. T., Miyamoto, O., Itano, T., and Matsu, T. (2003) Nat. Neurosci. 6, 384–390
4. Bussolati, G., and Cassoni, P. (2001) Endocrinology 142, 1130–1136
5. Paquin, J., Danalache, B. A., Jankowski, M., McCann, S. M., and Gutkowska, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9550–9555
6. Breton, C., Hauggi, C., Barberis, C., Heitz, F., Bader, C. R., Bernheim, L., and Tribollet, E. (2002) J. Clin. Endocrinol. Metab. 87, 1415–1418
7. Strakova, Z., and Soloff, M. S. (1997) J. Clin. Endocrinol. Metab. 82, 1094–1104
8. Takasu, H., Gardella, T. J., Luck, M. D., Potts, J. T., Jr., and Bringhurst, F. R. (1994) Trends Pharmacol. Sci. 15, 197–207
9. Coomarasamy, A., Knox, E. M., Gee, H., and Khan, K. S. (2002) Lancet 359, 1553–1560
10. Bestervelt, L., Barr, B., and Dethloff, L. (2000) Cell. Signal. 12, 53–61
11. Moody, T. W., Chiles, J., Casibang, M., Moody, E., Chan, D., and Davis, T. P. (2001) Peptides (N. Y.) 22, 109–115
12. Tong, W. G., Ding, X. Z., Hennig, R., Witt, R. C., Standop, J., Pour, P. M., and Slusarz, M. J. (2000) Exp. Physiol. 85, 899–904
13. Manning, M., Miteva, K., Pancheva, S., Stoev, S., Wo, N. C., and Chan, W. Y. (1995) J. Biol. Chem. 270, 2176–2182
14. Phaneuf, S., Asboth, G., MacKenzie, I. Z., Melin, P., and Lopez Bernal, A. (1994) J. Biol. Chem. 269, 6177–6182
15. Cassoni, P., Sapino, A., Papotti, M., and Bussolati, G. (1996) Int. J. Cancer 82, 817–820
16. Jarpe, M. B., Knall, C., Mitchell, F. M., Buhl, A. M., Duize, E., and Johnson, D. L. (1998) J. Biol. Chem. 273, 1627–1634
17. Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., and Okayama, H. (2003) J. Biol. Chem. 278, 3097–3104
18. Phaneuf, S., Asboth, G., MacKenzie, I. Z., Melin, P., and Lopez Bernal, A. (1994) Am. J. Obstet. Gynecol. 171, 1627–1634
19. Jarpe, M. B., Knall, C., Mitchell, F. M., Buhl, A. M., Duize, E., and Johnson, D. L. (1998) J. Biol. Chem. 273, 1627–1634
20. Kirk, C. J., Guil1, G., Balestre, M., and Jard, S. (1996) Biochem. J. 320, 197–204
21. Chini, B., Mouillac, B., Ala, Y., Balestre, M., Trumpp-Kallmeyer, S., Hoffack, J., Elands, J., Hibert, M., Manning, J., Jard, S., and Barberis, C. (1995) EMBO J. 14, 2176–2182
22. Wieland, T., and Jakobs, K. (1994) Methods Enzymol. 237, 3–27
23. Harrison, C., and Traynor, J. R. (2003) Life Sci. 74, 498–508
24. Milligan, G. (2003) Trends Pharmacol. Sci. 24, 87–90
25. Cassoni, P., Marrocco, T., Sapino, A., Allia, E., and Bussolati, G. (2004) Int. J. Oncol. 25, 899–904
26. Elands, J., Barberis, C., Jard, S., Tribollet, E., Dreifuss, J., Bankowski, K., Manning, M., and Sawyer, W. H. (1997) Eur. J. Pharmacol. 346, 197–207
27. Cosnarrusamy, A., Knox, E. M., Ge, H., and Khan, K. S. (2002) Med. Sci. Monit. 8, RA268–RA273
28. MacKinnon, A. C., Waters, C., Jodrell, D., Haslett, C., and Sethi, T. (2001) J. Biol. Chem. 276, 28083–28091
29. Chan, D. C., Gera, L., Stewart, J. M., Helfrich, B., Zhao, T. L., Feng, W. Y., Chan, K. K., Covey, J. M., and Bunn, P. A., Jr. (2002) Clin. Cancer Res. 8, 1280–1287
30. Kenakin, T. (1995) Trends Pharmacol. Sci. 16, 232–256
31. Kenakin, T. (1995) Trends Pharmacol. Sci. 16, 188–192
32. Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kuroto, T., Bouvier, M., and Pineyro, G. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11406–11411
33. Drue, S., and Liebmann, C. (2000) Br. J. Pharmacol. 131, 1553–1560
34. Bestervelt, L., Barr, B., and Dethloff, L. (2000) Cell. Signal. 12, 53–61
35. Moody, T. W., Chiles, J., Cashbang, M., Moody, E., Chan, D., and Davis, T. P. (2001) Peptides (N. Y.) 22, 109–115
36. Tong, W. G., Ding, X. Z., Hennig, R., Witt, R. C., Standop, J., Pour, P. M., and Adrian, T. E. (2002) Clin. Cancer Res. 8, 3232–3242
37. Stirnweis, J., Boehmer, P. D., and Liebmann, C. (2002) Peptides (N. Y.) 23, 1503–1506
38. Berde, B., and Boissonnas, R. A. (1968) in Handbook of Experimental Pharmacology (Berde, B., ed) Vol. 23, pp. 802–870, Springer-Verlag New York Inc., New York.
39. Manning, M., and Sawyer, W. H. (1993) J. Recept. Res. 13, 195–214
40. Chini, B., and Fanelli, F. (2000) Exp. Physiol. 85, (special number), 598–665
41. Slusarz, M. J., Slusarz, R., Kazmierkiewicz, R., Trojan, J., Wisniewski, K., and Ciarkowski, J. (2003) Protein Pept. Lett. 10, 295–302
42. Takasu, H., Gardella, T. J., Luck, M. D., Potts, J. T., Jr., and Bringhurst, F. R. (1999) Biochemistry 38, 13453–13460
43. Oakley, R. H., Laporte, S. A., Halt, J. A., Barak, L. S., and Caron, M. G. (2001) J. Biol. Chem. 276, 19452–19460
44. Haishi, A., Devost, D., Laporte, S. A., and Zimgg, H. H. (2004) Mol. Endocrinol. 18, 1277–1286
45. Laemi, U. K. (1970) Nature 227, 680–685