Molecular biology of the oxytocin receptor: a comparative approach
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Introduction
The nonapeptides oxytocin and vasopressin belong to an ancient peptide hormone family with representatives in both protostome and deuterostome lineages. The two mammalian hormones themselves probably diverged from a common ancestor at about the time that vertebrates evolved on the earth. There are discrete oxytocin-like and vasopressin-like peptides in telost fish; only in cyclostomes does it appear that there is only one member of this family [1].

Correspondingly, the receptors for these hormones must also have retained structural and functional features reflecting the balance between conservative and divergent evolution. We and others have cloned genes from several different species, which encode receptor proteins for members of the oxytocin-like nonapeptide hormones. Although representing a broad range of species, the physiologies in which these receptors are involved are very similar, and to date all species analysed appear to have a single homologous oxytocin receptor gene, the encoded receptor protein of which is linked to the inositol trisphosphate-protein kinase C signal-transduction pathway. We have taken advantage of this situation to apply a comparative molecular approach to analyse those structural features of the oxytocin receptor and its gene that are subject to high conservative selective pressure and hence belie an important role essential for the functional or structural integrity of the receptor.

Amino acid and nucleotide (mRNA) sequence information is available for several eutherian species, e.g. human [2], rat [3], pig [4], bovine [5], sheep [6], mouse [7], vole [8], and marmoset monkey [9]. In addition, genomic sequence is available for human [10], rat [3], bovine [5], mouse [7] and vole [8]. Recently, we have begun studying the equivalent receptor in a marsupial, the tammar wallaby [11], and preliminary cDNA and protein sequence is available for this species (L. Parry, R. Bathgate and R. Ivell, unpublished work); there is also a partial sequence available for another marsupial, the eastern grey kangaroo [12]. Marsupials may have either oxytocin or the reptilian oxytocin-like peptide, mesotocin. Amphibians and birds on the other hand possess only mesotocin. One amphibian mesotocin receptor sequence has been cloned [13], and we have recently begun looking at the mesotocin receptor expressed in chicken.

Comparative physiology of the oxytocin receptor
Unlike the vasopressin-like peptides, very little is known about the physiology of oxytocin or mesotocin in non-mammalian species. In birds it would appear that both pressor and reproductive-related functions are subserved by a single hormone, vasotocin. Although this is the representative of the vasopressin-like branch of the hormone family, it differs from mesotocin by only a single amino acid residue and is responsible not only for maintaining osmotic balance but also is involved in oviposition, the avian equivalent of birth. Nevertheless, birds do have an oxytocin-like peptide, mesotocin, but its function remains obscure.

In both eutherian and marsupial mammals, oxytocin or mesotocin are consistently involved in similar acute reproductive processes. These include, in the female, uterine contraction related to parturition, contraction of myoid cells during lactation, control of sexual and maternal behaviour within the central nervous system, and probably also regulation of the oestrous cycle [14]. In the male, oxytocin is involved in the ejaculatory response, and as a local factor regulating testicular steroidogenesis and prostatic function [15]. In addition to these reproductive-related functions, oxytocin appears also to be involved centrally in the control of appetite and Na+ balance.

In a detailed study of the tammar wallaby, a marsupial, we have recently been able to show that the pattern of oxytocin receptor expression during pregnancy is very similar to that in eutherian mammals, with a high up-regulation of uterine receptor numbers immediately before labour and birth [11].
Comparative pharmacology of the oxytocin receptor

The availability of appropriate receptor ligands, and in particular \[^{[25]}\text{OTA}\] (d(CH₂)₄[Tyr-(Me)₂,Tyr⁴,0rns,Tyr-NH₂]vasotocin), has permitted several detailed studies of oxytocin receptor pharmacology in different species. Figure 1 illustrates similar analyses for the oxytocin receptor from the human myometrium, the bovine endometrium and from the tammar myometrium. Irrespective of the species, the receptor shows very comparable binding affinities for several different oxytocin analogues. These are summarized in Table 1, together with equivalent data for the oxytocin receptors of the rat and the possum, additional eutherian and marsupial representatives showing that these data are indeed similar across most mammalian species.

The pharmacological data suggest that the molecular shape of the ligand-binding site on the receptor has been conserved across mammalian evolution, which in this case means approximately 130 million years, the time when it is assumed that marsupials split off from the eutherian branch [19]. The amino acid sequence of the oxytocin receptor is very highly conserved among modern eutherians, so that little can be deduced from a comparison of these sequences (Figure 2), except that variation is tolerated in the extreme N-terminus of the molecule, in the third intracellular loops and in parts of the C-terminus. Figure 2 also indicates those amino acids that are conserved in addition in the known mesotocin receptors (open circles; here the toad [13] and partial sequences from the chicken, tammar wallaby, (R. Bathgate, L. Parry and R. Ivell, unpublished work) and kangaroo [12]). Seeing that the pharmacology is probably equivalent for these receptors to that of the eutherian receptors, then the added evolutionary distance now emphasizes several regions that are conserved and others where variation is tolerated without unduly influencing ligand binding. The transmembrane domains TM1, TM2, TM3, TM5 (particularly the extracellular half), TM6 and TM7 are highly conserved, but not TM4. All the intracellular loops are poorly conserved. Parts of all extracellular loops are well conserved, although variations are tolerated. Interestingly, there is also a highly conserved region close to the end of the C-terminal domain, which could well be involved in phosphorylation or interaction with members of the signal-transduction pathway. For comparison, those residues that are

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**Figure 1**

Competitive displacement curves of various oxytocin- and vasopressin-related ligands displacing \[^{[25]}\text{OTA}\] for binding to the oxytocin receptor from (a) bovine endometrium, (b) human and (c) tammar myometrium

All assays were performed exactly as described in [11] and [16]. Oxytocin; □, OTA; ○, arginine vasopressin; ●, Manning compound [17] (courtesy of Dr. Maurice Manning, Toledo, OH, U.S.A.); x, Atosiban (courtesy of Dr. Per Melin, Ferring Pharmaceuticals).
IC₅₀ values of various oxytocin- and vasopressin-related ligands displacing [³²P]OTA binding to oxytocin receptors

AVP-A is a specific vasopressin antagonist ([deamino-Pen, O-methyl-Tyr⁵,Arg⁷]vasopressin) [18]. Results in parentheses are percentages. n.d., not determined. Data are derived as in [11] and [16].

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxytocin</th>
<th>OTA</th>
<th>Mesotocin</th>
<th>Arginine vasopressin</th>
<th>Manning compound</th>
<th>Atosiban</th>
<th>AVP-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.83 (100)</td>
<td>0.56 (147)</td>
<td>n.d.</td>
<td>9.20 (9.0)</td>
<td>8.86 (9.4)</td>
<td>27.6 (3.0)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Bovine</td>
<td>0.78 (100)</td>
<td>0.49 (160)</td>
<td>n.d.</td>
<td>3.99 (19.6)</td>
<td>6.42 (12.2)</td>
<td>14.2 (5.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rat</td>
<td>0.58 (100)</td>
<td>0.14 (430)</td>
<td>1.42 (40.8)</td>
<td>1.24 (46.7)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.40 (10.7)</td>
</tr>
<tr>
<td>Tammar</td>
<td>2.93 (100)</td>
<td>0.79 (370)</td>
<td>4.91 (161)</td>
<td>59.11 (4.9)</td>
<td>14.10 (20.8)</td>
<td>63.1 (4.6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Possum</td>
<td>1.05 (100)</td>
<td>0.15 (690)</td>
<td>1.10 (95.5)</td>
<td>4.20 (25.0)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.50 (19.1)</td>
</tr>
</tbody>
</table>

The regions that are conserved across all oxytocin and mesotocin receptors are homologous to those that have been pinpointed in oxytocin and vasopressin receptors using site-directed mutagenesis and by analysis of naturally occurring mutations causing nephrogenic diabetes insipidus [21–27], and confirm that the ligand-binding site probably comprises the extracellular halves of most transmembrane domains, as well as parts of extracellular loops 1, 2 and 3, but not the N-terminus or intracellular regions. The N-terminus is glycosylated in the oxytocin receptor at two or three different sites, depending upon the species. By site-directed mutagenesis of the human oxytocin receptor, we have recently shown that individual elimination of these sites has no influence on ligand binding, confirming that most of this domain is probably not involved in the formation of the ligand-binding pocket (T. Kimura and R. Bathgate, unpublished work).

Regulation of the oxytocin receptor gene in vivo

Very little is known about the regulatory mechanisms directly influencing the expression of the oxytocin receptor. In mammals in which the role of oxytocin in female reproductive physiology has been best studied, it has been found that there is a considerable up-regulation of the oxytocin receptor and its specific mRNA in the myometrium immediately before birth (e.g. [28]) and in the mammary gland at the same time, although here a high receptor level is maintained through lactation. This up-regulation correlates in vivo with a decline in serum progesterone and a concomitant increase in oestradiol. Whether these steroids have any direct effect on receptor expression is not known, although they may indirectly modulate receptor gene expression [29]. In the promoter regions of the oxytocin receptor genes that have been sequenced to date, palindromic motifs of the type known to correspond to oestradiol- or progesterone-response elements have only been found in the mouse and vole [7,8], and not in the rat. Half-palindromes, however, occur in most sequences. Similar stimulatory effects of oestradiol in vivo have been reported for oxytocin receptors in the rat brain [30], although interestingly oestradiol has the opposite effect in vivo, down-regulating oxytocin receptors in the kidney of the same species [31]. This apparent contradiction serves to strengthen the argument that oestradiol probably does not have a direct effect on the oxytocin receptor gene.

Possibly the most impressive information to date on whether or not steroids directly regulate the oxytocin receptor comes from a study of the tammar wallaby receptor during pregnancy and parturition [11]. In this species, the two halves of the double uterus continue as discrete entities...
up to and including the cervix, and in normal gestation only one uterus is gravid, the other remaining empty. There is thus no direct communication between the two uteri, although both

Figure 2
Deduced amino acid sequence of the human oxytocin receptor

Above the sequence, the extracellular (extra-loops 1–3), transmembrane (TM1–TM7) and intracellular (intra-loops 1–3) domains are indicated by the relative positions of the horizontal lines. Below the sequence, filled circles mark amino acids conserved across all known eutherian oxytocin receptor sequences; open circles indicate those amino acids additionally conserved in all known mesotocin receptor sequences (tamar wallaby, kangaroo, chicken, toad); crosses indicate amino acids shared also with the rat V1a, rat V1b and human V2 receptors.

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extracellular N-terminus

MEGALAAANSAAAANSAAPGAEGRHATGPPRNEALREVEAVLCLLALLASGNACV

extracellular domain

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intra-loop 1

LLALRTSQEHRLPPHEHLSDLADLVAVFQVLPPDMLDITPRTFYGPDLCLTWLVLQV

---

extra-loop 1

---

TM1

LAELTSEMLLSLNLKGALICQPFLRLSLRETDRLAVLATGWICVALAPQVHFSLREV

---

TM3

VGAFASTILLLMSLNLKGALICQPFLRLSLRETDRLAVLATGWICVALAPQVHFSLREV

---

intra-loop 2

---

TM2

LALALRTTRQKESRLFFFELKSLADLWAVFQVPQQLDTE REYGPDDL~VKYLQV

---

TM4

LAELTSEMLLSLNLKGALICQPFLRLSLRETDRLAVLATGWICVALAPQVHFSLREV

---

intra-loop 3

---

TM5

ADGVEDCWBFQPGWPEAYITWITALYV1EPV1LACLYGLISFKWQLSLEKAAAA

---

intra-loop 4

---

TM6

AAPEGAADGGHRVALRVSVLISKLKKARTVTVFFFIIFIVLAFIVCCTPFFTVPQHFWV

---

extracellular domain

---

intra-loop 3

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TM7

DANAFKSAFIIIYMLAALNSCNPWMTGHLFHELVQMLCCLSCASTLKGKGLGET

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intracellular C-terminus

SASKKSSSS5FLSHSR5SSQRMCSQQPSTA

1997
(A) Regions of more than 75% homology between the promoter region of the human oxytocin receptor gene promoter and equivalent regions of the bovine (B, boxes above the line) and rat (R; boxes below the line) genes, and (B) nucleotide sequence of part of the bovine oxytocin receptor gene promoter indicating (asterisks) nucleotides that are absolutely conserved between the bovine, rat and human gene promoters.

The horizontal arrows in (B) indicate the principal transcription-start site.
are subjected equally to the same systemic influences conveyed via the circulation. Shortly before birth there is a marked up-regulation of oxytocin receptors in the gravid uterus, just as in eutherian mammals. In the non-gravid uterus, not only are oxytocin receptors not up-regulated, they are in fact suppressed below the levels in the normal cycle and early pregnancy [11]. This suggests that circulating steroids are not influencing receptor levels, rather there is a marked ipsilateral effect due to the presence of the foetus. Since in this species the young are born at an extremely small and immature stage, uterine distention, hydrodynamic or other stress-associated parameters are not involved.

Ruminants offer another animal model which throws light on oxytocin receptor regulation in vivo. Ruminants are unusual among mammals in having an active oxytocin endocrine feedback loop regulating the oestrous cycle. Oxytocin, produced in large amounts by the ruminant corpus luteum, interacts with receptors on endometrial epithelial cells, causing the release from this tissue of substantial levels of prostaglandin F2α (PGF2α). This prostaglandin in turn stimulates the release of more oxytocin from the ovarian luteal cells and ultimately causes luteolysis and hence the termination of the luteal cycle and oestrus. This positive feedback loop is absolutely dependent upon the up-regulation of the endometrial oxytocin receptors shortly before the end of the oestrous cycle, following the lutropin surge. Just as during parturition, this up-regulation correlates with a decline in circulating progesterone and an increase in oestradiol from the preovulatory follicle [32]. Two aspects of this physiology are significant in the present context. First, although in vivo oxytocin receptors may be down-regulated, for example, in the endometrium of the mid-cycle, tissue pieces from any stage of the cycle explanted and cultured in vitro spontaneously up-regulate the oxytocin receptors in those tissue pieces [33]. Thus in vivo there would appear to be a systemic suppression of the oxytocin receptors during the mid-cycle, which is removed upon explanting the tissue. We have made similar observations for dispersed primary cultures of pure bovine endometrial epithelial cells (S. Horn, C. Lioutas, R. Bathgate and R. Ivell, unpublished work). The second aspect concerns what is referred to as maternal recognition of pregnancy. In the event that fertilization occurs and a blastocyst is formed, then the normal oxytocin-mediated feed-

back loop leading to luteolysis must be interrupted, so that the corpus luteum can be retained to continue the production of progesterone essential for the maintenance of pregnancy in the first trimester. In ruminants this occurs via production in large amounts by the blastocyst of an interferon-α-like molecule, referred to as interferon τ [34,35]. Interferon τ interacts with specific receptors on the endometrial epithelial cells, specifically down-regulating the oxytocin receptors and their mRNA in the same cells. Whether this is a direct effect on the oxytocin receptor gene or whether intermediate molecules are involved is not yet known. However, these experiments have focused attention on a possible role for cytokines in oxytocin receptor gene regulation.

Taking these observations together, an impression is created of a receptor that, in certain cell types, is constitutively up-regulated to a basal level, with specific suppression under certain circumstances in vivo. At particular times and in specific tissues, there may be an additional but highly specific further up-regulation to attain the very high levels of receptors found in, for example, the myometrium of the term uterus. Some of these effects are likely to be induced by local paracrine factors, rather than by circulating steroids or other endocrine hormones.

**Regulation of the oxytocin receptor gene in vitro**

Unfortunately, no cell lines are available that express high levels of oxytocin receptors. There are, however, several cell lines that have low but measurable levels of oxytocin receptors or their specific mRNA. These include immortalized human myometrial cells [36], porcine kidney cells [4], human breast carcinoma cells [37] and human prostatic carcinoma cells [15]. Several studies have used primary cultures of myometrial cells [38] or endometrial epithelial cells [39] or of complex tissue fragments [33]. Apart from the demonstration of a clear inhibitory effect of interferon τ on ruminant endometrial epithelial cells [39] and of interferon α on human myometrial cells [40], for none of these cells has any regulation of the oxytocin receptor gene been shown. For human myometrial cells in primary culture it has been possible to show a desensitization of the oxytocin receptor. This appears to occur at both the post-receptor level and the level of the specific mRNA [38].
**Structure and regulation of the oxytocin receptor gene**

The complete genomic structure of the oxytocin receptor gene has been elaborated for humans [10], cattle [5], rats [3], mice [7] and voles [8]. The protein-coding region itself is split by only a single intron, with a splice site separating the sequences for the sixth and seventh transmembrane domains. The remaining intron(s) are within the 5' untranslated region. Except for the human and the mouse there is only a single intron here. In these species this intron appears to allow the splicing in of a further very small exon within this intronic region. For the bovine gene it has been shown that the splicing in this first intron is not consistent; there are several splice variants both at the acceptor and donor intron margins, which lead to small sequence variations in the 5' untranslated region [5].

 Primer extension sequencing and RNase-protection assays indicate that, for all the species for which genomic sequence is available, there are multiple transcription-start sites, consistent with the lack of a Goldberg-Hogness box to orientate the RNA Pol II transcription complex. For all the genes sequenced, there is in excess of 1000 bp of the genomic region upstream of the transcription-start site, the presumed promoter region. Many researchers have submitted this region to a computer search for putative transcription-factor-binding motifs; however, this has in general been frustrating since there is no consistency in the results from one species to the next, or in comparison with actual nuclear protein binding.

Since the regulation of the oxytocin receptor in *vivo* appears to follow similar patterns of temporal- and tissue-specificity in most mammals, we have argued that regulatory sequences in the promoter region are probably conserved following the rules of natural selection, just as protein motifs involved in receptor–ligand binding are conserved across species. In Figure 3(A) we have plotted the promoter region of the human oxytocin receptor gene as a straight line. Above and below this line, boxes represent regions of at least 75% homology to either the bovine promoter (B, above the line) or the rat promoter (R, below the line). What is immediately evident is that there is no good correlation on a box-to-box basis, although there is a clear region of high homology to both species in the general region from -1 to -750. In Figure 3(B) we have plotted the equivalent region from the bovine oxytocin receptor gene, and have marked with asterisks those nucleotides that are conserved across all three species. There are numerous blocks of ten or more nucleotides with a high degree of homology across all three species. Whether these blocks represent true transcription-factor-binding motifs, or whether they represent conserved structural DNA motifs necessary for bending or other secondary DNA conformation, or possibly for nucleosome spacing, is not yet clear.

In order to obtain a better picture of the functionality of this promoter region, we have undertaken DNA–protein binding studies using nuclear extracts from various tissues in electrophoretic mobility-shift assays. Dividing the promoter regions of both the human and the bovine genes into consecutive approx. 150–200 bp segments, we could show specific binding of nuclear proteins to all regions of both promoters. For the bovine gene, a comparison between expressing and non-expressing bovine myometrial tissue failed to show any specific differences in protein binding correlating with an *in vitro* up-regulation of the gene (R. Bathgate and R. Ivell, unpublished work). This could mean that nuclear protein extracts were missing important cofactors or modifications, or that important regulatory sequences are not necessarily within the immediate (2000 bp) upstream region of the gene. Further research is required to clarify this issue.

As an alternative to such nuclear protein–DNA binding studies, the functionality of a promoter region can be defined by transfection of different deletion constructs of the specific gene promoter coupled to an appropriate reporter gene. Unfortunately, this approach is hindered for the oxytocin receptor gene by the lack of adequately expressing homologous cell lines. By default therefore researchers have resorted to transfection of such reporter constructs into heterologous cell systems, which are not expressing the endogenous gene. It could be shown for the rat gene that a reporter construct comprising up to 3.1 kb of the promoter responded to Ca²⁺-ionophore stimulation in Syrian hamster myometrial cells [41], although not to interleukins or oestradiol. Whether this represents what may be happening *in vivo* is clearly highly speculative, but at least such experiments do imply that a given region of DNA is functional and able to transduce information entering the cell. An alternative to using heterologous cell
lines is to transfec similar reporter constructs into homologous primary cultures of cells that do naturally express the endogenous oxytocin receptor in vitro. This approach has not been successful to date, although we are currently developing the bovine endometrial epithelial cell system to this end.

Conclusions

A comparative approach to the study of gene promoter regions appears to be just as profitable as a similar study at the level of primary protein sequence. In particular, where we can demonstrate that the essential physiology, i.e. pharmacology and receptor regulation, have been conserved through evolution, then we can expect conservation of nucleotide motifs where there is conservation of functionality. For a gene that appears to be as highly conserved as that for the oxytocin receptor, then recourse to studying species outside the eutherians, especially marsupials or even birds, would appear to offer many benefits. It may, however, turn out that regulation of this most interesting receptor is more complex than we imagine, with a mixture of constitutive and specific regulatory features. To date, most lines of evidence tend to point to constitutive elements, in contradiction of the marked temporal pattern of receptor expression. Only when better and more appropriate cell systems are available will we be able to clarify these issues. One final feature of the oxytocin receptor that distinguishes it from most other membrane receptors is that the majority of functions in which it is involved appear to require a positive feedback situation. This is true for the myometrial receptor at birth, the endometrial receptor at luteolysis, as well as for the hypothalamic receptor during the milking reflex. Unlike most other receptors, the oxytocin receptor may have encoded within its gene information that prevents the usual type of down-regulation or desensitization upon repeated ligand-dependent stimulation, thus permitting the continued signal transduction required for a positive feedback loop.

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Simulations on dimeric peptides: evidence for domain swapping in G-protein-coupled receptors?

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Introduction

It is known that peptides derived from intracellular loop 3 of G-protein-coupled receptors are able to activate G-proteins [1], and these studies have shed some light on the mechanism of activation. Recently, however, it has been shown that dimers of these peptides show enhanced potency, suggesting that multiple interaction sites on both the receptor and the G-protein are important in activation. Here we use molecular-dynamics simulations on a peptide dimer to investigate whether these observations are consistent with an activation process involving receptor dimers.

The simulations were performed on Q\textsubscript{dimer}, where peptide Q was taken from the C-terminus of intracellular loop 3 of the \(\alpha_\text{2A}\)-adrenergic receptor. (Intracellular loop 3 is known to play a key role in receptor activation [2].) This peptide dimer has been shown to be 100-fold more effective than the monomer at inhibiting high-affinity \(\alpha_2\) agonist binding and substantially more potent in inhibiting \(\alpha_2\) agonist-stimulated GTPase activity. In CHO cell membranes, the potency of Q\textsubscript{dimer} was found to be about two orders of magnitude greater than that of Q for GTPase activity with G\textsubscript{G}\textsubscript{i} [3]. Since there have been a number of recent reports suggesting that G-protein-coupled receptor activation may involve dimerization [4–7], these observations on the Q\textsubscript{dimer} would appear to be consistent with this.

However, closer examination of the structure of Q\textsubscript{dimer} with the electron-cryomicroscopy studies of rhodopsin [8,8\textsubscript{a}], which also show the presence of 1,2-dimers, suggest that the associa-