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Oxytocin receptors expressed and coupled to Ca^{2+} signalling in a human vascular smooth muscle cell line

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1 In a human vascular smooth muscle cell line (HVSMC), binding experiments with [^{3}H]-arginine^8-vasopressin (AVP) have shown the existence of a homogeneous population of binding sites with affinity (K_d value) of 0.65 nM and a maximum number of binding sites (B_{max}) of 122 fmol mg^{-1} protein.

2 Nonlabelled compounds compete for [^{3}H]-AVP binding in the HVSMC membrane with an order of potency of oxytocin > lyspressin $\geq$ AVP > Thr^4, Gly^7-oxytocin > (β-mercapto-β-β-cyclopentamethylenepropionyl-*O*-Me Tyr^2, Arg^8) vasopressin > desmopressin > OPC21268 > OPC31260. This order was markedly different from that observed in rat vascular smooth muscle cells (A10), a well-established V_{1A} receptor system.

3 In HVSMC both oxytocin and AVP increased inositol 1,4,5-trisphosphate (IP_3) production and $[Ca^{2+}]_i$ response, but the efficacy of the responses was greater for oxytocin than AVP.

4 Reverse transcription-polymerase chain reaction (RT-PCR) assay detected only oxytocin receptor but not V_{1A} or V_2 receptors in HVSMC, whereas only V_{1A} receptors were found in A10 cells.

5 In conclusion, in HVSMC only oxytocin receptors are expressed among the vasopressin receptor family, and they coupled to phosphatidyl inositol (PI) turnover/Ca^{2+} signalling. This unexpected observation should provide new insight into the functional role of the oxytocin receptor in a human vascular smooth muscle cell line.

Keywords: Human vascular smooth muscle cell line; oxytocin receptor; intracellular Ca^{2+}; inositol (1,4,5)-trisphosphate; reverse transcription polymerase chain reaction

Introduction

Arginine^8-vasopressin (AVP) and oxytocin were first recognized as hypothalamo-neurohypophysial peptides which exert a variety of hormonal effects. Extensive pharmacological studies have classified AVP receptors into two major categories, AVP-V_1 and AVP-V_2 receptors. AVP-V_1 receptors mediate the contractile effects of AVP on vascular smooth muscle and the glycogenolytic response in liver by activating phosphatidyl inositol (PI) turnover/Ca^{2+} pathway, while the AVP-V_2 receptor in the kidney controls and anti-diuretic action of AVP by stimulating adenosine 3':5'-cyclic monophosphate (cyclic AMP) production (Share, 1988; Manning & Sawyer, 1991). Oxytocin, in contrast, plays a role in the expression of maternal behaviour in the brain, the milk-ejection reflex and contraction of the pregnant uterus (Pedersen & Prange, 1979; Richard *et al.*, 1991). The oxytocin receptor is reported to be expressed only in the pregnant uterus, lactating mammary gland and brain, and is not expressed in other peripheral tissues. AVP and oxytocin receptors mediating the effects in human tissues are similar to those in the rat (Scala-Guenot & Strosser, 1992; Briner *et al.*, 1992; Chan *et al.*, 1993). Furthermore, cDNAs encoding the rat and the human vasopressin V_{1A} receptors (Morel *et al.*, 1992; Thibonnier *et al.*, 1994), the rat and the human V_2 receptors (Lolait *et al.*, 1992; Birnbaumer *et al.*, 1992) and the human oxytocin receptor have been cloned recently (Kimura *et al.*, 1992), and all are found to be members of the G protein-coupled receptor family of seven putative transmembrane spanning domains. While information on the vasopressin/oxytocin receptor family is accumulating, little is known as yet regarding the effects of oxytocin in vascular smooth muscle, mainly because selective ligands for each receptor have been lacking. Oxytocin is also known to contract the vascular smooth muscle; however, the effect is thought to result from the activation of the vasopressin V_{1A} receptor as oxytocin can interact with these receptors (Altura & Altura, 1984; Nakano, 1973).

Recent pharmacological studies indicated a marked species difference in the effects of several newly developed antagonists selective for each receptor of the vasopressin/oxytocin receptor family (Pettibone *et al.*, 1992). In the rat, vasopressin-induced contraction of vascular smooth muscle cells is found to be mediated by V_{1A} receptor (Plevin *et al.*, 1992; Thibonnier *et al.*, 1991), and the same receptor subtype is thought to be present in human vascular smooth muscle. In the present study, we have examined the vasopressin/oxytocin receptor subtype expressed in a human vascular smooth muscle cell line (HVSMC) by comparing binding characteristics of [^{3}H]-AVP with that in A10 rat vascular smooth muscle cells (VSMC).

Methods

Cell culture

A human vascular smooth muscle cell line (HVSMC) imported from Clonetics (San Diego, California, U.S.A.) was purchased from Kurabo (Osaka, Japan). Cells were grown to confluence in monolayers on 225 cm^2 culture dish in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum and 0.4 μg ml^{-1} of gentamicin in a 5% CO_2-95% air atmosphere at 37°C. The medium was changed every 3 days, and the cells

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were subcultured every 5–7 days after trypsinization. All cells were used in these experiments only between passages 5–15. For experimental purposes, confluent cells were serum-deprived by culture in DMEM containing 0.3% BSA for 18 h. A10 rat vascular smooth muscle cells were purchased from ATCC and maintained under the same conditions as the HVSMC.

Membrane preparation

HVSMC and A10 cells in a 500 cm^2 plastic dish were washed twice with phosphate buffered saline (PBS), scraped into a solution containing 250 mM sucrose, 10 mM $MgCl_2$, and 50 mM Tris/HCl (pH 7.4), and homogenized with a polytron (Kinematica, Lucerne, Switzerland) at 4°C. After centrifugation at 2,000 *g* for 10 min at 4°C, the supernatant was centrifuged at 40,000 *g* for 20 min at 4°C with a 70Ti-fixed angle rotor (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ and stored at −80°C until use.

Binding assay

Incubations were carried out at 30°C for 60 min in a final volume of 0.5 ml containing 50 mM Tris-HCl (pH = 7.4), 10 mM $MgCl_2$ and 10% glycerol From preliminary experiments, binding was rapid, reaching equilibrium in less than 30 min at 30°C. After equilibrium had been achieved, binding remained stable for at least another 60 min. Incubations were started by the addition of [^{3}H]-AVP to membranes from HVSMC (100 μg protein). In the competition experiments, a single concentration (1.0 nM) of [^{3}H]-AVP and at least 3 to 6 concentrations of antagonists were used. Incubation was terminated by rapid filtration through 0.1% polyethyleneimine-presoaked Whatman GF/B filters using a Brandel cell harvester (Gaithersburg, Maryland, U.S.A.). The filter was then rinsed 3 times with 3 ml of 50 mM Tris-HCl (pH = 7.4) and 10 mM $MgCl_2$. Radioactivity retained on filters was counted with a liquid scintillation counter (2000CA, Packard, Meriden, Connecticut, U.S.A.). Nonspecific binding was determined in the presence of 1 μM AVP. Protein content of each membrane suspension was measured by the method of Bradford (1976).

Measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells were incubated for 30 min with 5 μM Fura-2/AM in Hank's balanced salt solution (HBSS) containing 0.05% BSA and 10 mM glucose. They were washed twice with HBSS, and the slides were secured in a quartz cuvette in a CAF/100 fluorescence spectrometer (Japan Spectroscopic Co., Ltd. Tokyo, Japan) equipped with a thermostatically controlled cell holder. Excitation wavelengths were set at 340 and 380 nm and the emission wavelength at 500 nm. Fluorescence was monitored for 1–3 min until the $[Ca^{2+}]_i$ signals stabilized and basal $[Ca^{2+}]_i$ measurements were obtained. The cells were then subjected to specific agonists (e.g. AVP) or experimental perturbations, and $[Ca^{2+}]_i$ signals were recorded for an additional 10 min. Ca^{2+} calibration was performed on each coverslip by exposing cells to 10 μM ionomycin, followed by 300 mM EGTA/ 3 M Tris buffer (pH 9.0). $[Ca^{2+}]_i$ was determined from the ratio of fluorescence at 340 and 380 nm as described by Grynkiewicz *et al.* (1985) using the following equation:

$$[Ca^{2+}]_i(nM) = K_d \times [(R - R_{min})/(R_{max} - R)] \times \beta$$

Where β is the ratio of fluorescence of Fura-2 at 380 nm in zero and saturating Ca^{2+} and K_d is the dissociation constant of Fura-2 for Ca^{2+}, assumed to be 224 nM.

Inositol phosphate assay

HVSMC were grown to confluence on 6-well plates, then serum-deprived for 18 h. IP_3 was measured quantitatively in AVP and oxytocin-stimulated HVSMC by IP_3 competition assay. Briefly, before stimulation with AVP or oxytocin, HVSMC were preincubated with HBSS containing 10 mM LiCl for 5 min at 37°C. The reaction was terminated at designated times by addition of 10% (w/v) $HClO_4$. The mixture was kept on ice for 20 min, then neutralized with ice-cold 1.53 M KOH/ 75 mM HEPES for 20 min. The sample was then centrifuged at 2,000 *g* for 10 min to remove the $KClO_4$ precipitate. The supernatant samples (10 μl each) were assayed for IP_3 with an IP_3 assay kit (Du Pont New England Nuclear, Boston, Maryland, U.S.A.). The standard curve was linear from 0.12 to 12.0 pmol of IP_3.

Reverse transcription polymerase chain reaction

Total cellular RNA was extracted by complete lysis with 5.5 M guanidine isocyanate, and purified from a 2.0 ml portion of lysate per tube by centrifugation through 0.8 ml of a 5.7 M caesium chloride cushion in a TL100.3 fixed-angle rotor (Beckman Instruments, Inc.,) at 350,000 *g* for 4 h. The RNA pellet was collected in 200 μl of 0.1 M Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS solution, extracted with phenol/chloroform (1:1 v/v), ethanol-precipitated, dried, resuspended in 50 μl of RNase-free water, and quantified by absorbance measurement at 260 nm. In preliminary experiments, the integrity of the purified RNA collected with this method was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1% agarose formaldehyde gel. To eliminate contaminating genomic DNA, prepared total cellular RNA samples were further treated with RNase-free DNase I (Strategene, La Jolla, California, U.S.A.). RNA samples (10 μg each) were incubated for 30 min at 37°C with 20 u of DNaseI in DNase buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM $CaCl_2$), 2 u of placental RNase inhibitor (TaKaRa, Kyoto, Japan) in a 30 μl volume. The reaction was stopped by an extraction with phenol/chloroform (1:1 v/v), and RNA samples were ethanol-precipitated, then vacuum-dried, and resuspended in RNase-free water.

RNA samples were reverse transcribed as follows: each sample contained 10 μg of total cellular RNA, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 0.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 20 u of RNase inhibitor, 100 pmol random hexamer (TaKaRa, Kyoto, Japan) and 200 u Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Life Technologies, Inc., Gaithersburg, Maryland, U.S.A.) in a final volume of 20 μl. After incubation at 37°C for 60 min, the samples were heated at 94°C for 5 min to terminate the reactions, and were stored at −20°C until use. As controls for template contamination, mock cDNA synthesis reactions were carried out in the absence of either reverse transcriptase, or RNA as indicated in the results.

The primers were synthesized on a model 391A (Applied Biosystems, Inc., Foster City, California, U.S.A.) DNA synthesizer (using β-cyano-methylphosphoramidate derivatives), quantified by absorbance measurement at 260 nm, and stored at −20°C. Oligonucleotide primers were constructed from the published cDNA sequences of rat vasopressin V_{1A} receptor, rat and human V_2 receptor, and human oxytocin receptor cDNA. In all assays, β-actin served as a control for assessing the efficacy of RNA isolation and cDNA synthesis.

The sequences of the vasopressin V_{1A} receptor primers were: 5′-ATGCTGGTGGTGATGACAGCCGACCGCTAC-3′ (coding sense), corresponding to bases 667-696, 5′-CATCTGGACAATGAAGAAAGGCGCCCAGCA-3′ (anticoding sense), which anneals to bases 1200-1171 of the cloned rat V_{1A} receptor full-length sequence (Morel *et al.*, 1992). The primers correspond to the third and sixth trans-

membrane domains which are highly conserved between species (Morel *et al.*, 1992; Thibonnier *et al.*, 1994; Hirasawa *et al.*, 1994b).

The sequences of the vasopressin V_2 receptor primers were: 5'-ATGGTGGGCATGTATGCCTCCTCCTACAT-3' (coding sense), corresponding to bases 399–427 of the cloned rat and human V_2 receptor full-length sequence, respectively. 5'-AGTGTCATCCTCACGGTCTTGGCCA-3' (anticoding sense), which anneals to bases 859-835 of the cloned rat and human V_2 receptor full-length sequence, respectively (Birnbaumer *et al.*, 1992).

The sequences of the oxytocin receptor primers were: 5'-GTGGTGGCAGTGTTTCAGGTGCTGCCGCAG-3' (coding sense), corresponding to bases 627-655, 5'-CCAGGGCTGGATGAAGACGGCCCAGCAGTC-3' (anticoding sense), which anneals to bases 952-923 of the cloned human oxytocin receptor full-length sequence, respectively (Kimura *et al.*, 1992).

The sequences of β-actin primers were 5'-ATCATGTTTGAGACCTTCAACACCCCAGCC-3' (coding sense), corresponding to bases 2158-2187 of the cloned full-length sequence, 5'-AAGAGAGCCTCGGGGCATCGGAACCGCTCA-3' (anticoding sense), corresponding to bases 2579-2550. The predicted sizes of the amplified vasopressin V_{1A}, V_2 receptor, oxytocin receptor and β-actin PCR products were 534, 461, 326 and 422 base pairs, respectively.

Each reverse transcription mixture was diluted 1:5 in RNase-free water and 2 μl aliquots were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 mM each primer) spanning the given sequence for amplification, 200 μM each of dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 0.01% (v/v) gelatin, and 2.5 u of Taq DNA polymerase (Perkin Elmer Cetus Corporation, Norwalk, Connecticut, U.S.A.) in a final volume of 25 μl. The reaction mixture was then overlaid with 3 drops (~50 μl) of mineral oil and amplified for 30 cycles in a Perkin Elmer Cetus thermal cycler (Norwalk, Connecticut, U.S.A.). The amplification profiles consisted of denaturation for 1 min at 94°C; primer annealing for 30 s at 55°C, and extension for 1 min at 72°C. Negative control reactions without template were routinely included in PCR amplifications with both primer sets.

Using the cDNA of HVSMC or A10 cells, or genomic DNA as templates, we confirmed that all primer sets we used in the present study could amplify the expected DNA in both cells in preliminary experiments. Also, the amount of PCR products were quantified by adding the α-[^{32}P]dCTP in PCR reaction, and we found the amounts of PCR products increased exponentially depending on PCR cycle number in our PCR conditions for vasopressin V_{1A}, V_2, oxytocin receptors and β-actin (data not shown).

DNA sequence analysis

Each PCR product was purified after agarose gel electrophoresis by the SUPREC-01 (TaKaRa, Kyoto, Japan), blunted by T4 polymerase and subcloned into the EcoRV site of Bluescript II KS(+) plasmid. They were sequenced by the GENESIS 2000 DNA analysis system (Du Pont Medical Products, Wilmington, Delaware, U.S.A.) to confirm the PCR products obtained were the same as the published cDNA clone.

Analysis of data

Results are expressed as the mean ± s.e.mean or the mean with 95% confidence limits. Analysis of binding data was performed as previously described (Yazawa *et al.*, 1992). Dose-response curves were fitted to a logistic equation by an iterative fitting procedure (Delean *et al.*, 1980). Statistical difference between two means was determined by Student's unpaired *t* test and *P* values less than 0.05 were considered to be significant. Regression lines were calculated by the least squares method.

Materials

[^{3}H]-arginine^8 vasopressin (2893.4 GBq mmol^{-1}) was obtained from Du Pont-New England Nuclear, oxytocin from Peptide Inst. (Osaka, Japan), and desmopressin, lyspressin, (β-mercapto-β-β-cyclopentamethylene-propionyl-*O*-Me Tyr^2, Arg^8) vasopressin (V1-ANT) and Thr^4, Gly^7-oxytocin were from Sigma (St. Louis, Missouri, U.S.A.). OPC21268, (1-(1-(4-(3-acetylaminopropoxy)benzoyl)-4-piperidyl)-3,4-dihydro2(1H)-quinolinone) and OPC31260, (5-dimethylamino-1-(4-(2methylbenzoylamino)benzoyl)-2,3,4,5-tetrahydro-1H-benzazepine) were prepared at Yamanouchi Pharmaceutical Co. Ltd. All culture reagents were from Gibco Laboratories (Paisley, Scotland) and culture flasks and plates were purchased from Costar (Cambridge, Maryland, U.S.A.). All other chemicals were of reagent grade.

Results

Figure 1 shows specific binding of [^{3}H]-AVP on membranes prepared from HVSMC was greater than 70% of total binding at the concentrations tested and was a saturable process. Scatchard analysis revealed a single class of high affinity binding site with K_d of 0.65 ± 0.08 nM and B_{max} of 122 ± 16 fmol mg^{-1} protein, $n = 3$ (Figure 1). Competition for [^{3}H]-AVP binding sites by the nonlabelled vasopressin and oxytocin analogues were compared in HVSMC and A10 cells (Figure 2 and Table 1). The rank order of potency sites was markedly different between the two cell lines, particularly for oxytocin (1.7 vs. 100 nM) and Thr^4, Gly^7-oxytocin (14 vs. > 10,000 nM). Furthermore, a V_1-specific antagonist, (β-mercapto-β-cyclopenta methylene-propionyl-O-Me Tyr^2, Arg^8) vasopressin was found to be markedly weaker in HVSMC compared to rat A10 cells. These binding data suggested that [^{3}H]-AVP binding sites in HVSMC are of the character of the oxytocin rather than the vasopressin V_1 or V_2 receptors.

The effects of AVP and oxytocin upon the $[Ca^{2+}]_i$ response and also on the Ins(1,4,5)P_3 production were examined in HVSMC. As shown in Figure 3a, in the presence of 1.3 mM extracellular $[Ca^{2+}]$, baseline $[Ca^{2+}]_i$ in HVSMC was 58.6 ± 9.7 nM ($n = 24$) and the addition of 100 nM AVP induced a prompt increase in $[Ca^{2+}]_i$ which peaked within 15 to 30 s after AVP addition with a peak value of 128.8 ± 11.6 nM

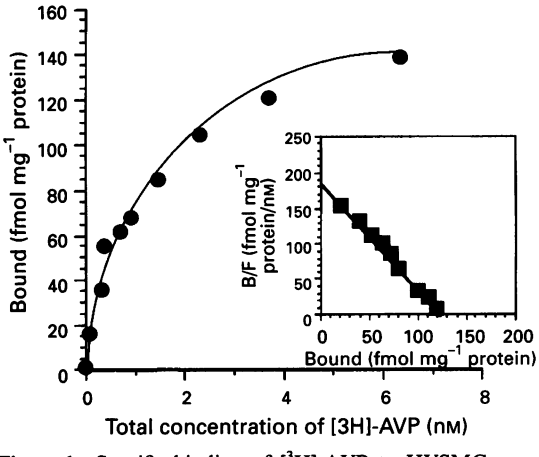

Figure 1 Specific binding of [^{3}H]-AVP to HVSMC membrane and Scatchard plot (Inset). Increasing concentrations of [^{3}H]-AVP (0–6.0 nM) were added to membranes, which were then incubated for 90 min at 37°C. Specific binding is shown. Similar results were obtained from at least three different experiments performed in duplicate.

($n=6$). Also, in Ca^{2+}-free medium, both AVP and oxytocin caused $[Ca^{2+}]_i$ responses in HVSMC, suggesting that AVP- and oxytocin-induced $[Ca^{2+}]_i$ mobilization comes mainly from the intracellular Ca^{2+} stores (data not shown). Oxytocin (100 nM) was found to be more potent than AVP (100 nM) in causing $[Ca^{2+}]_i$ response in HVSMC (Figure 3a). By contrast, AVP and lyspressin (100 nM each) increased $[Ca^{2+}]_i$ more effectively than oxytocin (100 nM) in A10 cells (Figure 3b). Similar effects of AVP and oxytocin on the Ins(1,4,5)P_3 production were obtained in HVSMC (Figure 4). As shown in Figure 4, both AVP and oxytocin (either 1 or 100 nM) significantly ($P<0.05$) increased Ins(1,4,5)P_3 production. However, the maximal Ins(1,4,5)P_3 production was not significantly different between the two hormones or between 1 and 100 nM of each hormone (Figure 4).

We next performed a RT-PCR study to determine the vasopressin/oxytocin receptor subtype expressed in HVSMC. As shown in Figure 5, in A10 cells PCR product of the expected size was visible only for the V_{1A} receptor but not for either V_2 or oxytocin receptor, whereas in HVSMC that was visible only for the oxytocin receptor. The sequences of the PCR products obtained in A10 and HVSMC were confirmed to be identical to the nucleotide positions 667–1200 bp of rat vasopressin V_{1A} receptor cDNA and 627–952 bp of human oxytocin receptor cDNA, respectively (data not shown). Also, DNA sequencing confirmed that the human or rat genomic PCRs gave identical PCR products as obtained by RT-PCR reaction; however, the V_{1A} receptor PCR product obtained when the rat V_{1A} receptor primer sets were used with the human genome as a template which was found to be a part of the human V_{1A} receptor (Thibonier *et al.*, 1994; Hirasawa *et al.*, 1994b). No products were detected with negative controls performed in the absence of cDNA, or without the reverse transcription reaction, assuring that the amplified products all originated from mRNA rather than from contaminating genomic DNA (Figure 5). In both HVSMC and A10 cells, similar amounts of β-actin PCR products were obtained in each of the RT-PCR samples (Figure 5).

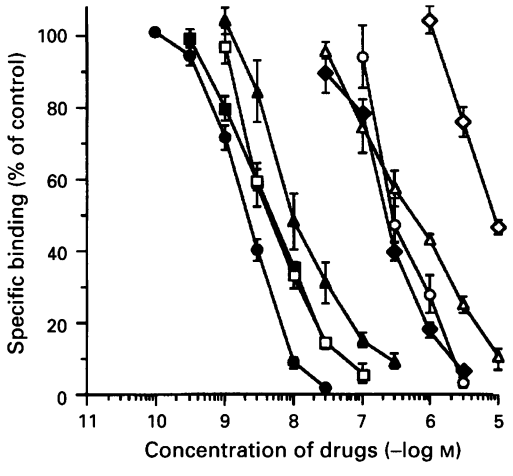

Figure 2 Competitive inhibition curves for [^{3}H]-AVP binding to HVSMC membrane. HVSMC membranes were incubated with [^{3}H]-AVP (1 nM) in the presence of the indicated concentration of unlabelled AVP (■), oxytocin (●), Thr^4, Gly^7-oxytocin (▲), (β-mercapto-β-β-cyclopentamethylenepropionyl-*O*-Me Tyr^2, Arg^8) vasopressin (◆), lypressin (□), desmopressin (○), OPC21268 (△), or OPC31260 (◇). Each point represents the mean ± s.e.mean of three or four experiments.

Discussion

The present study shows for the first time the presence of oxytocin receptor rather vasopressin V_{1A} receptor in the human vascular smooth muscle cell line. Radioligand binding with [^{3}H]-AVP in HVSCM and A10 revealed a single class of binding site in both cell lines. The K_d values obtained in the two cell lines are in good agreement with those reported for the vasopressin receptor (Plevin *et al.*, 1992). However, the rank order of potency for several ligands, oxytocin and TG-oxytocin in particular, were markedly different between HVSCM and A10; thus, K_i values of oxytocin and TG-oxytocin are 1.7 nM and 14 nM in HVSMC, respectively, while these are 100 fold weaker in A10, suggesting that the [^{3}H]-AVP binding site in HVSMC is characteristic of oxytocin receptor. Furthermore, the [^{3}H]-AVP binding site in HVSMC appeared to be coupled to PI turnover/Ca^{2+} signalling, and exhibits the character of an oxytocin receptor. In contrast to HVSCM, we found that vasopressin-stimulated PI turnover/$[Ca^{2+}]_i$ response in A10 cells were mediated by the V_{1A} receptor, as previous reported by Plevin *et al.* (1992); thus, in A10 cells, AVP and a number of related analogues stimulated the accumulation of [^{3}H]-inositol phosphate with the similar rank order of potencies (AVP > > oxytocin). These binding and functional results were further substantiated by RT-PCR assay with specific primers. Together the data showed that the [^{3}H]-AVP binding site of HVSMC was different from the V_{1A} receptor expressed in rat A10 vascular smooth muscle cells, and can be classified as the oxytocin receptor.

Although the cells we used in the present study were obtained from human aorta, they are plainly a stable cell line. Thus, our demonstration that such cells lack the expected V_{1A} receptors may not truly reflect the behaviour of native human aortic smooth muscle; however, a physiological role of oxytocin receptors in HVSMC as shown in the present study was of interest. In preliminary studies we have observed that oxytocin has no growth promoting effect as measured by [^{3}H]-

Table 1 K_i values of vasopressin analogues on [^{3}H]-AVP binding to membrane from HVSMC and A10 cells

	HVSMC		*A10*	
Ligand	*K_i (nM)*	*Hill coefficient*	K_i (nM)	*Hill coefficient*
AVP	4.5 (4.4–4.6)	0.89	1.9 (1.8–1.9)	0.84
Oxytocin	1.7 (1.5–1.9)	0.88	100 (93–108)	0.66
TG-oxytocin	14 (10–18)	0.69	>10,000	
V1-ANT	230 (220–250)	1.06	1.1 (1.0–1.3)	0.94
Lyspressin	4.6 (4.0–5.3)	0.86	0.61 (0.57–0.65)	0.87
Desmopressin	480 (410–570)	0.88	700 (590–830)	0.88
OPC21268	610 (550–670)	0.69	110 (95–120)	0.92
OPC31260	9,700 (9,100–10,300)	0.91	740 (710–780)	0.89

The competitive binding experiments were performed on membranes from HVSMC and A10 cells. The K_i values and Hill coefficient were calcuated as described under Methods. K_i values represent mean and 95% confidence limits from three different experiments.

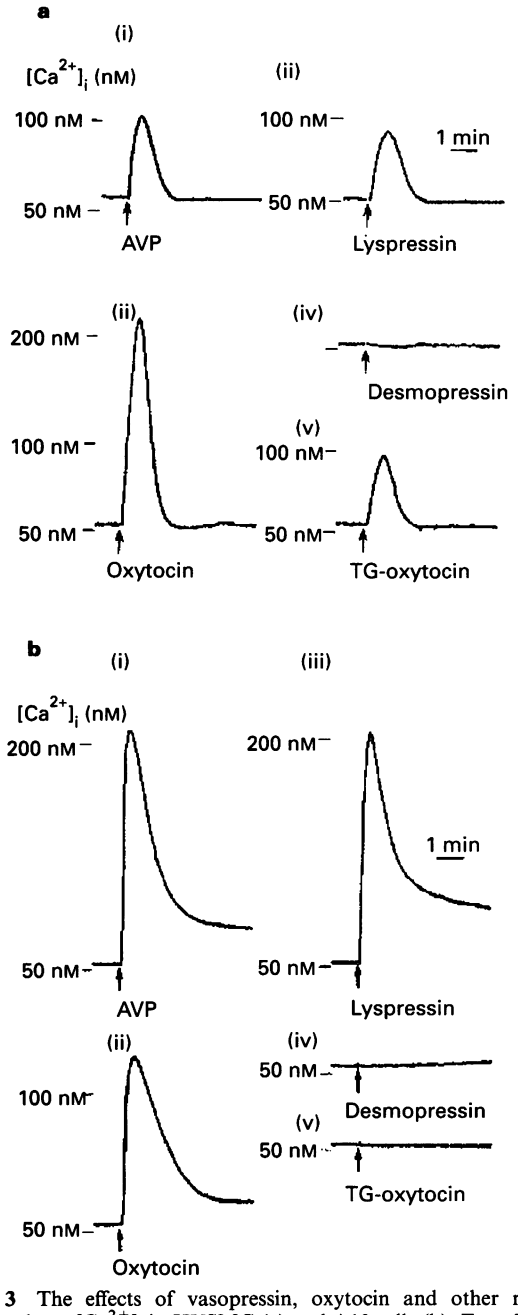

Figure 3 The effects of vasopressin, oxytocin and other related compounds on $[Ca^{2+}]_i$ in HVSMC (a) and A10 cells (b). Fura-2/AM-loaded HVSMC or A10 cells were exposed to 100 nM of (i) AVP, (ii) oxytocin, (iii) lypressin, (iv) desmopressin or (v) TG-oxytocin. Test substances were applied at the arrows and were present throughout each recording. Representative tracings are shown; similar results were obtained from at least four separate experiments.

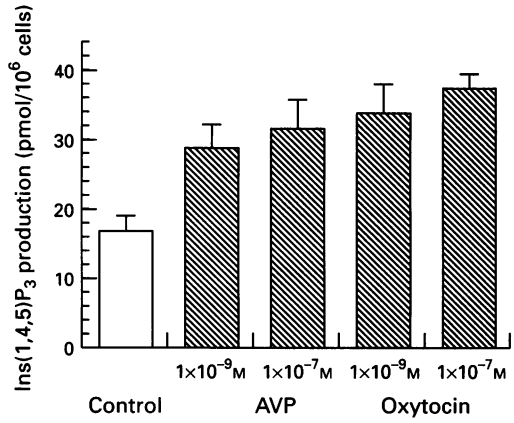

Figure 4 Effect of AVP and oxytocin on inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$) production. HVSMC were cultured in 6-well plates, and $Ins(1,4,5)P_3$ production was measured 30 s after stimulation with the indicated ligand. Both hormones significantly ($P<0.05$) increased $Ins(1,4,5)P_3$ production; however, the maximal $Ins(1,4,5)P_3$ production was not significantly different between the two hormones or between 1 and 100 nM. Basal level of $Ins(1,4,5)P_3$ production was 17.2 ± 2.2 pmol/10^6 cells ($n=5$), and $Ins(1,4,5)P_3$ production by 1 and 100 nM AVP was 28.8 ± 3.4 and 31.7 ± 3.8 pmol/10^6 cells ($n=3$ each), respectively, while those by 1 and 100 M oxytocin were 33.9 ± 3.6 and 37.1 ± 2.0 pmol/10^6 cells ($n=3$ each), respectively. The data represent the mean $\pm$ s.e.mean.

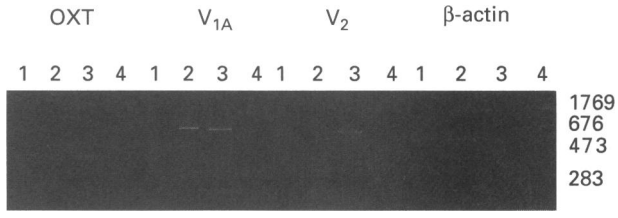

Figure 5 Vasopressin V_{1A}, V_2 receptor and oxytocin receptor mRNA expression in A10 cells and HVSMC. RT-PCR was performed with 100 ng of total RNA from A10 cells and HVSMC using the primer set as described under Methods. PCRs using as templates cDNAs from HVSMC (lane 1) and A10 cells (lane 2) are shown. Also shown are PCRs with human genomic DNA as a positive control (lane 3) and without template as a negative control (lane 4). PCR products were electrophoresed on 4% polyacrylamide gel and stained with ethidium bromide. A 534-base pair, 461-base pair and 326-base pair PCR products specific for human vasopressin V_{1A}, V_2 receptor and oxytocin receptor, respectively were detected. PCR was performed at 30 cycles.

thymidine uptake in HVSMC (data unpublished). Altura & Altura (1984) reported that oxytocin appears to be a much more potent constrictor than AVP in placental-umbilical vascular cells, and suggested a role for oxytocin in effecting closure of the umbilical vessels at birth and in parturition. However, the study did not examine which subtype of the vasopressin/oxytocin receptor family mediates this response, since the selective ligands were lacking. Several antagonists that have been recently developed to be selective for oxytocin receptor would be potentially valuable in further studying the expression and functional role for oxytocin receptor in the intact human vascular system (Gavras, 1990; Laszlo *et al.*, 1991).

Our study showed the usefulness of the RT-PCR assay in determining the receptor mRNA expression in HVSMC and A10 cells. cDNAs encoding the rat vasopressin V_{1A} (Morel *et al.*, 1992), rat vasopressin V_2 (Lolait *et al.*, 1992), human vasopressin V_1 (Thibonnier *et al.*, 1994; Hirasawa *et al.*, 1994b), human vasopressin V_2 (Birnbaumer *et al.*, 1992) and human oxytocin (Kimura *et al.*, 1992) receptors have been cloned, and these receptors are found to be members of the G protein-coupled receptors with seven putative transmembrane domains. This information made it possible to determine the distributions of these receptor subtypes transcripts. We have recently demonstrated that the RT-PCR method is advantageous in detecting rare transcripts from limited sources and identifying specific mRNA among the homogeneous populations (Horie *et al.*, 1993; Hirasawa *et al.*, 1994a). Using the sensitive and specific method, we could also determine the existence of oxytocin receptor at the transcription level in HVSMC.

This is the first report of the existence of an oxytocin receptor in a human vascular smooth muscle cell line. Activation of this receptor by AVP and oxytocin involves Ca^{2+} mobili-

zation from intracellular Ca^{2+} stores. This unexpected observation would provide new insight into the functional role of oxytocin receptor in human vascular smooth muscle.

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