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Pharmacologic characterization of the oxytocin receptor in human uterine smooth muscle cells

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1 [^{3}H]-oxytocin was used to characterize the oxytocin receptor found in human uterine smooth muscle cells (USMC). Specific binding of [^{3}H]-oxytocin to USMC plasma membranes was dependent upon time, temperature and membrane protein concentration.

2 Scatchard plot analysis of equilibrium binding data revealed the existence of a single class of high-affinity binding sites with an apparent equilibrium dissociation constant (K_d) of 0.76 nM and a maximum receptor density (B_{max}) of 153 fmol mg^{-1} protein. The Hill coefficient (n_H) did not differ significantly from unity, suggesting binding to homogenous, non-interacting receptor populations.

3 Competitive inhibition of [^{3}H]-oxytocin binding showed that oxytocin and vasopressin (AVP) receptor agonists and antagonists displaced [^{3}H]-oxytocin in a concentration-dependent manner. The order of potencies for peptide agonists and antagonists was: oxytocin > [Asu^{1,6}]-oxytocin > AVP = atosiban > d(CH$_2$)$_5$Tyr(Me)AVP > [Thr^4,Gly^7]-oxytocin > dDAVP, and for nonpeptide antagonists was: L-371257 > YM087 > SR 49059 > OPC-21268 > SR 121463A > OPC-31260.

4 Oxytocin significantly induced concentration-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and hyperplasia in USMC. The oxytocin receptor antagonists, atosiban and L-371257, potently and concentration-dependently inhibited oxytocin-induced $[Ca^{2+}]_i$ increase and hyperplasia. In contrast, the V_{1A} receptor selective antagonist, SR 49059, and the V_2 receptor selective antagonist, SR 121463A, did not potently inhibit oxytocin-induced $[Ca^{2+}]_i$ increase and hyperplasia. The potency order of antagonists in inhibiting oxytocin-induced $[Ca^{2+}]_i$ increase and hyperplasia was similar to that observed in radioligand binding assays.

5 In conclusion, these data provide evidence that the high-affinity [^{3}H]-oxytocin binding site found in human USMC is a functional oxytocin receptor coupled to $[Ca^{2+}]_i$ increase and cell growth. Thus human USMC may prove to be a valuable tool in further investigation of the physiologic and pathophysiologic roles of oxytocin in the uterus.

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Abbreviations: [Asu^{1,6}]-oxytocin, Deamino-dicarba-oxytocin; Atosiban, 1-deamino-2-D-Tyr-(O-ethyl)-4-Thr-8-Orn-oxytocin; AVP, vasopressin; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CHO, Chinese hamster ovary; d(CH$_2$)$_5$Tyr(Me)AVP, [β-mercapto-β,β-cyclopentamethylenepropionyl^1, O-Me-Tyr^2, Arg^8]-Vasopressin); dDAVP, [deamino-Cys^1, D-Arg^8]-Vasopressin; DMSO, dimethyl sulphoxide; FCS, foetal calf serum; Fura 2-AM, Fura 2-acetoxymethyl ester; IP$_3$, inositol-1,4,5-triphosphate; L-371257, 1-{1-[4-[(*N*-Acetyl-4-piperidinyl)oxy]-2-methoxybenzoyl]piperidin-4-yl}-4*H*-3,1-benzoxazin-2(1*H*)-one; MAP, mitogen-activated protein; OPC-21268, 1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1*H*)-quinolinone; OPC-31260, 5-dimethylamino-1-{4-(2-methylbenzoylamino)benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine; PBS, phosphate-buffered saline; SmBM, Smooth Muscle Cell Basal Medium; SR 121463A, 1-[4-(N-tert-butyl-carbamoyl)-2-methoxybenzene sulphonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy) cyclohexane]indol-2-one; equatorial isomer; SR 49059, (2S) 1-[(2R 3S)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulphonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide; [Thr^{4}Gly^7]-oxytocin, [4-Threonine,7-glycine]oxytocin; USMC, uterine smooth muscle cells; YM087, [4′-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-d][1]benzoazepine-6-carbonyl)-2-phenylbenzanilide monohydrochloride

Introduction

The neurohypophysial hormones oxytocin and vasopressin (AVP), despite close structural similarities, have distinct biological functions. AVP plays important physiologic roles in vasoconstriction and antidiuresis (Sawyer & Manning, 1985). In contrast, oxytocin has important functions during parturition: to contract the uterine myometrium during labour and the mammary myoepithelium postpartum to elicit milk letdown (Soloff *et al*., 1979). Oxytocin exerts its biological effects through binding to a specific receptor, which has been identified, cloned and found to belong to a family of seven membrane-spanning receptors that transduce signals through G proteins (Kimura *et al*., 1992; Jasper *et al*., 1995; Rozen *et al*., 1995). Oxytocin activates phospholipase C-mediated hydrolysis of polyphosphoinositides *via* the oxytocin receptors to generate second messengers, inositol-1,4,5-triphosphate (IP_3), which induces an increase of free intracellular calcium (Tasaka *et al*., 1991; Jasper *et al*., 1995; Holda *et al*., 1996), and 1,2-diacylglycerol, which activates protein kinase C (Morrison *et al*., 1996; Schrey *et al*., 1988). Oxytocin receptors have been

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shown to be present in uterus, mammary gland, brain and ovarian tissues (Soloff, 1975; Soloff *et al*., 1977; Barberis & Audigier, 1985) and mediate contraction, milk letdown and induction of specific mating and maternal behaviours (McCarthy *et al*., 1992; 1994).

In most target sites, the number of oxytocin binding sites is highly regulated (Schumacher *et al*., 1993). In the uterus of each mammalian species studied, oxytocin binding sites undergo a 10 to >100 fold (rat: 10 fold, rabbit: 17 fold, human: 100–125 fold) upregulation during pregnancy, reaching the maximum prior to the onset of parturition (Soloff *et al*., 1979; Fuchs *et al*., 1982; 1984; Maggi *et al*., 1988; 1990), indicating oxytocin may play a key role in the initiation of labour, both before and at term. Therefore, oxytocin receptor antagonists may be potential agents to prevent premature and preterm labour (Soloff *et al*., 1979; Fuchs *et al*., 1982; Lefebvre *et al*., 1992; Thornton & Smith, 1995). For over three decades, scientists in a number of different laboratories have worked to design peptide analogue of oxytocin and several peptide oxytocin receptor antagonists have been discovered (Pettibone *et al*., 1989; Melin *et al*., 1986; Lopez Bernal *et al*., 1989; Manning *et al*., 1989; 1995). Among them, atosiban, which has high affinity for both oxytocin and V_{1A} receptors and is a competitive oxytocin and V_{1A} receptor antagonist, exhibited a therapeutic effect in preterm labour and primary dysmenorrhea (Akerlund, 1987; Andersen *et al*., 1989; Goodwin *et al*., 1996). Recently, several non-peptide and orally bioavailable oxytocin receptor antagonists, including L-371257, with potential utility for managing preterm labour have been discovered (Bell *et al*., 1998; Williams *et al*., 1994; 1995).

Although, previous investigations have characterized oxytocin receptors in rat and human uterine and mammary gland tissue, the characterization of oxytocin receptors in human uterine smooth muscle cells (USMC) has not been undertaken. Furthermore, a cellular system expressing human oxytocin receptors would be very useful for the development of new oxytocin receptor ligands, and would also allow detailed investigation of the regulation of oxytocin receptor function. In the present study, the first direct characterization of oxytocin binding sites in human USMC is reported. Furthermore, USMC were used to characterize and compare the affinity, selectivity and potency of oxytocin and AVP receptor agonists and antagonists using receptor binding, second messenger and proliferation assays.

Methods

Materials

AVP and oxytocin were obtained from Peptide Institute Inc. (Osaka, Japan). [Asu[1,6]]-oxytocin (Deamino-dicarba-oxytocin) (Yamanaka *et al*., 1970), [Thr[4]Gly[7]]-oxytocin ([4-Threonine, 7-glycine]oxytocin) (Lowbridge *et al*., 1977), d$(CH_2)_5$Tyr (Me)AVP ([β-mercapto-β,β-cyclopentamethylenepropiony-l[1],O-Me-Tyr[2],Arg[8]]-Vasopressin) (Kruszynski *et al*., 1980) and dDAVP ([deamino-Cys[1],D-Arg[8]]-Vasopressin) (Zaoral *et al*., 1967) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Atosiban (1-deamino-2-D-Tyr-(O-ethyl)-4-Thr-8-Orn-oxytocin) (Lopez Bernal *et al*., 1989), YM087 (4′-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-d][1]benzoazepine-6-carbonyl)-2-phenylbenzanilide monohydrochloride) (Tahara *et al*., 1997), OPC-21268 (1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl} -3,4-dihydro-2(1*H*)-quinolinone) (Yamamura *et al*., 1991), OPC-31260 (5-dimethylamino-1-{4-(2-methylbenzoylamino)benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine) (Yamamura *et al*., 1992), SR 49059 ((2S) 1-[(2R 3S)-(5-chloro-3- (2- chlorophenyl)- 1 -(3,4- dimethoxybenzene- sulphonyl) -3 -hydroxy -2,3 - dihydro- 1H-indole- 2- carbonyl]- pyrrolidine-2-carboxamide) (Serradeil-Le Gal *et al*., 1993), SR 121463A (1-[4-(N-tert-butyl-carbamoyl)-2-methoxybenzene sulphonyl] - 5- ethoxy- 3-spiro - [4- (2-morpholinoethoxy)cyclohexane]indol-2-one; equatorial isomer) (Serradeil-Le Gal *et al*., 1996) and L-371257 (1-{1-[4-[(*N*-Acetyl-4-piperidinyl)oxy]-2-methoxybenzoyl]piperidin - 4 - yl} - 4*H* - 3,1-benzoxazin -2(1*H*)-one) (Williams *et al*., 1995) were synthesized at Yamanouchi Pharmaceutical Co. (Ibaraki, Japan). The structures of these compounds were determined by ^{1}H-nuclear magnetic resonance, mass spectrometry and elemental analysis. Their purity was measured by high-pressure liquid chromatography and was >98%. These antagonists were initially dissolved in dimethyl sulphoxide (DMSO) at 10^{-2} M and diluted to the desired concentration with the assay buffer. The final concentration of DMSO in the assay buffer did not exceed 1%, which did not affect [^{3}H]-oxytocin binding. [^{3}H]-oxytocin (specific activity, 43.5 Ci mmol^{-1}) was obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). Foetal calf serum (FCS) and trypsin-EDTA were from Gibco (Grand Island, NY, U.S.A.). Bovine serum albumin (BSA) was from Nacalai Tesque Inc. (Kyoto, Japan). Fura 2-acetoxymethyl ester (AM) was from Dojindo Laboratories (Kumamoto, Japan) and EGTA and ionomycin were from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest reagent grade available.

Cell culture

Human USMC imported from Clonetics (Lot No. 16372; San Diego, CA, U.S.A.) were purchased from IWAKI (Tokyo, Japan). The cells were grown at 37°C in Smooth Muscle Cell Basal Medium (SmBM) (Clonetics) supplemented with 0.5 μg ml^{-1} human epidermal growth factor, 5 mg ml^{-1} insulin, 1 μg ml^{-1} human fibroblast growth factor, 5% FCS and antibiotics (GA-1000) in a humidified atmosphere of 5% CO_2 in air. USMC were subcultured every 7 days into a 150 cm^2 culture dish using 0.05% trypsin-0.53 mM EDTA, with culture medium changed every 3 days. Cells were arranged in bundles forming a highly organized hill-and-valley structure, which is characteristic of smooth muscle cells in culture. All experiments were performed between passages 5–10 and cells were identified histochemically by anti-α-actin and factor VIII antibody.

[^{3}H]-oxytocin binding assay

Human USMC in a 500 cm^2 culture dish was 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 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. The resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ and stored in small aliquots at −80°C until use. Protein was determined by the Coomassie blue method using BSA as a standard. For saturation binding studies, membrane preparations were incubated with various concentrations of [^{3}H]-oxytocin (0.1–6.0 nM). For competition studies, [^{3}H]-oxytocin (0.7 nM) was added to membrane preparations, which was then incubated with various concentrations of compounds in 250 μl of assay buffer (Tris-HCl 50 mM, pH 7.4, containing $MgCl_2$ 10 mM and 0.05% BSA). Binding reactions were

initiated by the addition of the membrane preparations and assay mixtures were incubated for 60 min at 30°C, which allowed equilibrium to be established. After incubation, the reaction was terminated by addition of 3 ml of ice-cold Tris buffer (Tris-HCl 50 mM, pH 7.4, and $MgCl_2$ 10 mM) followed immediately by rapid filtration through Whatman GF/C filters. The radioactivity retained on filters was counted with a liquid scintillation counter (Packard Tricarb scintillation counter, Packard Instrument Co., Inc., CT, U.S.A.). Non-specific binding was determined in the presence of an excess oxytocin (1 μM). The inhibitory dissociation constant (K_i) was calculated from the following formula: $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ is the concentration of radioligand present in the tube and K_d is the dissociation constant of radioligand obtained from Scatchard plot analysis (Cheng & Prusoff, 1973). Data were analysed using GraphPad PRISM software (GraphPAD Software, Inc.: San Diego, CA, U.S.A.).

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

Serum-deprived monolayer cultures of USMC were grown on coverglasses (13.5 mm in diameter) and were assayed 1 day later. Cell monolayers were loaded with Fura 2-AM (2 μM/coverglass) in Krebs-Henseleit-HEPES buffer (containing in mM: NaCl 130, KCl 5, $CaCl_2$ 1.25, $MgSO_4$ 0.8, glucose 5.5, HEPES 20 and 0.1% BSA, pH 7.4) for 30 min at 37°C. They were then washed with PBS, transferred to Fura 2-free Krebs-Henseleit-HEPES buffer and incubated for an additional 30 min at 37°C. The coverglass was placed into a quartz cuvette containing 2 ml Krebs-Henseleit-HEPES buffer and maintained at 37°C with continuous stirring. When thermal equilibrium was reached, the fluorescence signal was recorded with a CAF-110 spectrofluorometer (Japan Spectrometer Co., Tokyo, Japan) at both 340 and 380 nm excitation wavelengths, and 500 nm emission wavelength. After recording the baseline signal for 3 min, oxytocin was added to the cuvette to stimulate the mobilization of intracellular calcium in the presence or absence of antagonists (preincubation of 3 min). Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (R_{max}) with the nonfluorescent Ca^{2+} ionophore, ionomycin (25 μM), after which minimal fluorescence (R_{min}) was obtained by adding 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, the $[Ca^{2+}]_i$ was determined using the following equation: $[Ca^{2+}]_i$ (nM) $= K_d \times [(R - R_{min})/(R_{max} - R)] \times b$ (Grynkiewicz *et al.*, 1985). The term b is the ratio of fluorescence of Fura 2 at 380 nm under zero Ca^{2+} conditions to saturated Ca^{2+} conditions. K_d is the dissociation constant of Fura 2 for Ca^{2+}, taken from Grynkiewicz *et al.*'s measurement of 224 nM.

Determination of cell number

Human USMC were seeded into 48-well culture plates at 60–80% confluence, washed with PBS, and incubated in SmBM containing 0.5% FCS for 48 h. The cultures were then incubated for 72 h in SmBM containing 0.5% FCS and 0.05% BSA containing vehicle alone or various concentrations of agonists, antagonists, or both. To determine the number of cells per culture well, the cells were exposed to AlamarBlue (IWAKI, Tokyo, Japan) (Ahmed *et al.*, 1994) during the last 3 h of the incubation, and the absorbencies of the wells at 570 and 600 nm were measured with SPECTRAmax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). Control experiments showed a linear relation between absorbance and cell number up to a cell density of 30,000 cells well^{-1}.

Analysis of data

Experimental results are expressed as the mean ± standard error of the mean (s.e.mean) or the mean with 95% confidence limits. All experiments were repeated at least three times, and comparable results were obtained.

Results

Saturability of [^{3}H]-oxytocin binding

Specific binding of [^{3}H]-oxytocin to USMC plasma membranes was dependent upon time, temperature and membrane protein concentration. The saturability of [^{3}H]-oxytocin binding was measured by incubating the USMC membranes with various concentrations of [^{3}H]-oxytocin from 0.1–6.0 nM (Figure 1a). The saturation isotherm is a rectangular hyperbola, suggesting that a single population of saturable high affinity oxytocin binding sites exists. Scatchard plot analysis of specific binding of [^{3}H]-oxytocin to USMC membranes gave an apparent

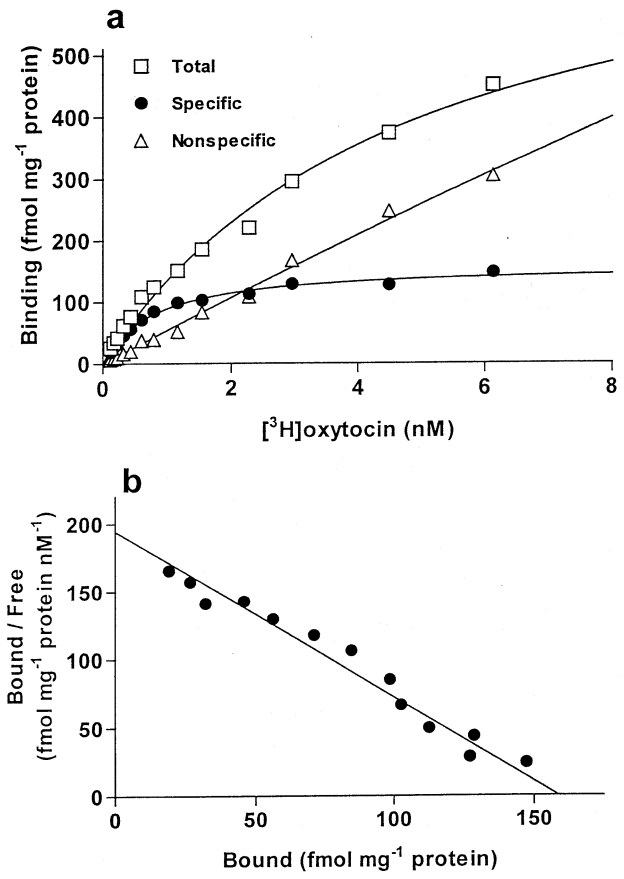

Figure 1 (a) Saturation equilibrium specific binding of [^{3}H]-oxytocin to human USMC membranes. Increasing concentrations of [^{3}H]-oxytocin were incubated with USMC membranes (0.1 mg protein) for 60 min at 30°C. Specific binding was determined as the difference between total and nonspecific binding in the absence and presence of 1 μM unlabelled oxytocin. (b) Scatchard plot of specific [^{3}H]-oxytocin binding data in (a). X-intercept of least-squares fit to Scatchard plot is a measure of maximal receptor density (B_{max}); negative reciprocal of the slope is the dissociation constant (K_d). Results are representative data from eight independent experiments performed in duplicate.

dissociation constant (K_d) of 0.76 ± 0.04 nM and a maximal receptor density (B_{max}) of 153 ± 4 fmol mg^{-1} protein (6560 receptors cell^{-1}) (Figure 1b). A plot of binding data for [^{3}H]-oxytocin according to the Hill equation gave a straight line with a Hill coefficient (n_H) of 1.02 ± 0.02, suggesting the existence of one population of binding sites.

Specificity of [^{3}H]-oxytocin binding

Pharmacologic specificity of binding was assessed by studying the inhibition of [^{3}H]-oxytocin binding by oxytocin and AVP receptor agonists and antagonists. Naturally occurring neurohypophysial hormones, oxytocin and AVP, showed high affinities for USMC membranes, with K_i values of 0.75 ± 0.08 nM for oxytocin and 2.99 ± 0.39 nM for AVP (Figure 2a). Synthetic analogues selective for oxytocin, V_{1A} or V_2 receptors were then tested for their ability to displace [^{3}H]-oxytocin binding. The oxytocin receptor agonists, [Asu^{1,6}]-oxytocin and [Thr^4,Gly^7]-oxytocin, and the antagonist, atosiban, had high affinity for USMC with K_i values of 1.40 ± 0.24 nM for [Asu^{1,6}]-oxytocin, 17.9 ± 2.8 nM for [Thr^4,Gly^7]-oxytocin and 3.55 ± 0.52 nM for atosiban (Figure 2a,b). In contrast, the V_{1A} receptor selective antagonist, d(CH$_2$)$_5$Tyr(Me)AVP, exhibited moderate affinity for USMC with K_i value of 7.43 ± 0.54 nM, and the V_2 receptor agonist, dDAVP, exhibited much lower affinity with K_i value of 141 ± 11 nM (Table 1). Nonpeptide oxytocin and AVP receptor antagonists, L-371257, SR 49059, OPC-21268, SR 121463A, OPC-31260 and YM087, were then assayed for their ability to inhibit binding of [^{3}H]-oxytocin (Figure 2b, Table 1). The oxytocin receptor selective antagonist, L-371257, showed high affinity for USMC membranes with a K_i value of 2.21 ± 0.23 nM. The V_{1A} receptor selective antagonists, SR 49059 and OPC-21268, exhibited moderate affinity with K_i values of 69.3 ± 7.3 nM and 209 ± 10 nM, respectively. However, the V_2 receptor selective antagonists, SR 121463A and OPC-31260, exhibited much lower affinity with K_i values of 1940 ± 110 nM and 2490 ± 480 nM, respectively. On the contrary, the V_{1A}/V_2 receptor antagonist, YM087, showed moderate affinity with a K_i value of 29.8 ± 4.1 nM. For the entire series of oxytocin and AVP receptor agonists and antagonists tested, there was a highly significant correlation between the pK_i values determined on human USMC membranes and the corresponding values measured on human myometrium oxytocin receptors (Figure 3a). No such correlation was found when comparing the ligand specificity of USMC to those of human V_{1A}, V_{1B} and V_2 receptor-expressing Chinese hamster ovary (CHO) cell membranes (Figure 3b,c,d).

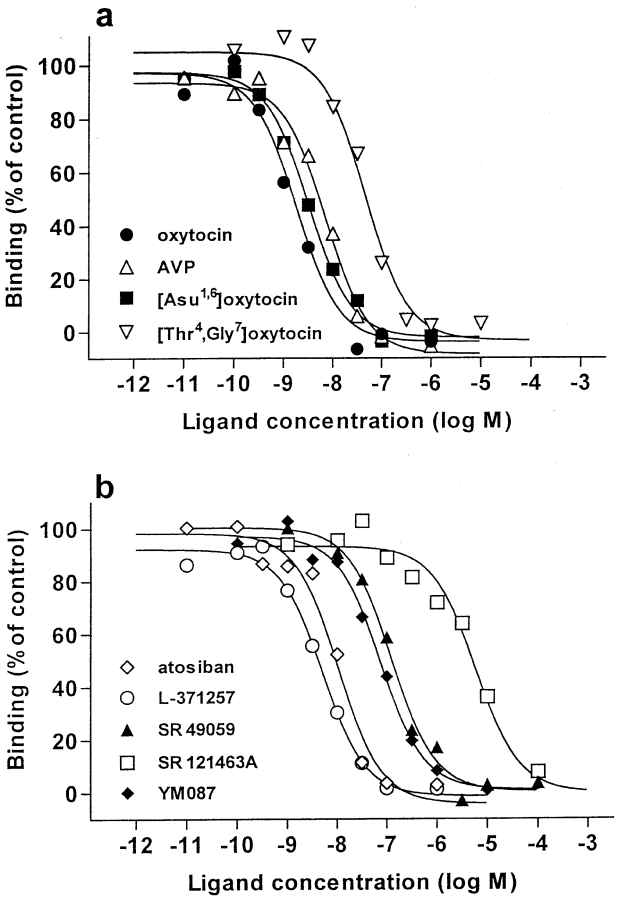

Figure 2 Displacement of specific [^{3}H]-oxytocin bound to human USMC membranes by oxytocin, AVP and oxytocin receptor agonists (a) and nonpeptide oxytocin and AVP receptor antagonists (b). Membranes (0.1 mg protein) were incubated with 0.7 nM of [^{3}H]-oxytocin in the presence or absence of increasing concentrations of unlabelled compounds for 60 min at 30°C. Specific binding of [^{3}H]-oxytocin is expressed as percentage of the control binding. Results are representative data from four independent experiments performed in duplicate.

Oxytocin-induced [Ca^{2+}]$_i$ increase

Because a prominent action of oxytocin in several cell types is to increase [Ca^{2+}]$_i$, the coupling of oxytocin receptors present in human USMC to second messenger pathways was tested. The basal [Ca^{2+}]$_i$ in human USMC was 66.2 ± 0.6 nM. Addition of oxytocin, AVP, [Asu^{1,6}]-oxytocin and [Thr^4,Gly^7]-oxytocin to USMC resulted in an increase in [Ca^{2+}]$_i$ in a concentration-dependent manner (Figure 4a, Table 2). Agonist-induced rise of [Ca^{2+}]$_i$ was biphasic and an immediate and transient spike was observed (time to peak < 10 s), followed by a prolonged plateau of elevated [Ca^{2+}]$_i$ which was still above basal values 2 min after addition

Table 1 K_i values of various oxytocin and AVP receptor agonists and antagonists measured using human uterine smooth muscle cell (USMC) plasma membranes and human oxytocin receptors

	USMC	*Oxytocin receptors*
Oxytocin	0.75 ± 0.08	19 ± 0.1[a]
AVP	2.99 ± 0.39	7.0 ± 1.6[a]
[Asu^{1,6}]-oxytocin	1.40 ± 0.24	
[Thr^4, Gly^7]-oxytocin	17.9 ± 2.8	22 ± 4[a]
Atosiban	3.55 ± 0.52	34 ± 2[a]
d(CH$_2$)$_5$Tyr(Me)AVP	7.43 ± 0.54	5.9 ± 1.0[a]
dDAVP	141 ± 11	140 ± 16[a]
L-371257	2.21 ± 0.23	4.6 ± 0.3[d]
SR 49059	69.3 ± 7.3	130[b]
OPC-21268	209 ± 10	170 ± 49[a]
SR 121463A	1940 ± 110	1213 ± 383[c]
OPC-31260	2490 ± 480	1077 ± 319[c]
YM087	29.8 ± 4.1	

Inhibitory constant (K_i) values were calculated according to the equation of Cheng & Prusoff ($K_i = IC_{50}/(1 + C/K_d)$) using data from competition experiments. Values are means ± s.e.mean obtained from 3–6 independent experiments performed in duplicate. Corresponding values for human oxytocin and AVP receptors are taken from previously reported data. Key to references: [a]Jasper *et al.*, 1995, [b]Serradeil-Le Gal *et al.*, 1993, [c]Serradeil-Le Gal *et al.*, 1996 and [d]Williams *et al.*, 1995.

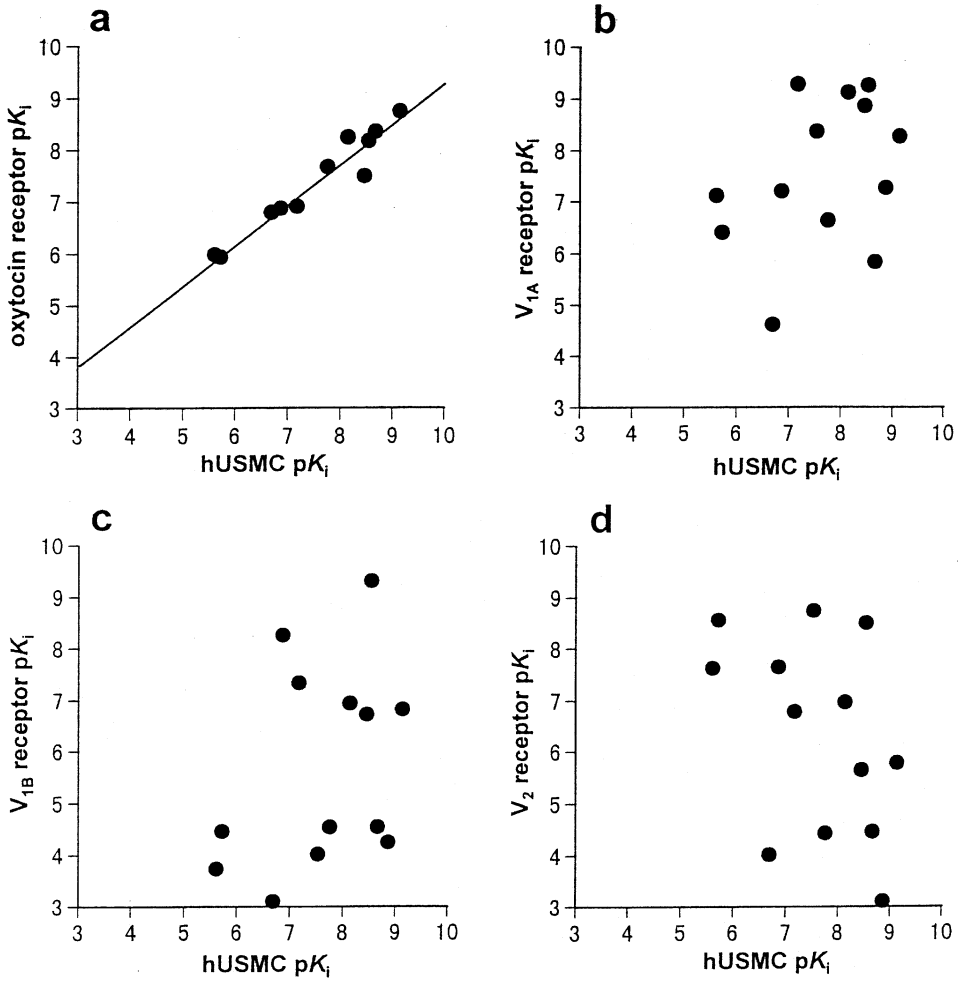

Figure 3 Ligand selectivity of human USMC receptors assessed using (a) human oxytocin, (b) V_{1A}, (c) V_{1B} and (d) V_2 receptors (constructed from data given in Table 1 and previously reported data, Tahara *et al*., 1998). pK_i ($-\log K_i$) values for binding of oxytocin and AVP receptor agonists and antagonists tested to human USMC membranes are plotted against corresponding values determined for both human myometrium, and AVP receptor subtypes (V_{1A}, V_{1B} and V_2) expressed on CHO cell plasma membranes.

of the stimulus. On the contrary, dDAVP did not cause an increase in $[Ca^{2+}]_i$ at concentrations up to 1 μM (data not shown). The oxytocin receptor antagonists, atosiban and L-371257, potently inhibited an increase in $[Ca^{2+}]_i$ stimulated by 100 nM oxytocin in a concentration-dependent manner with K_i values of 0.49 (0.28–0.86) nM and 0.46 (0.29–0.73) nM, respectively (Figure 4b, Table 2). In contrast, the inhibitory potency of the V_{1A} receptor selective antagonists, SR 49059 and OPC-21268, and the V_{1A}/V_2 receptor antagonist, YM087, was 18–20 times lower than that of oxytocin receptor antagonists (Table 2). Furthermore, the V_2 receptor selective antagonists, SR 121463A and OPC-31260, did not potently inhibit oxytocin-induced $[Ca^{2+}]_i$ increase. These K_i values correspond well with the K_i values obtained from the [^{3}H]-oxytocin displacement experiments.

Oxytocin-induced hyperplasia

Addition of oxytocin and AVP significantly and concentration-dependently increased cell number in USMC. The EC_{50} values of oxytocin and AVP were 5.47 (4.34–6.91) nM and 1190 (239–5950) nM, respectively (Figure 5a). The oxytocin receptor antagonists, atosiban and L-371257, potently and concentration-dependently inhibited hyperplasia stimulated by 100 nM oxytocin with K_i values of 0.47 (0.02–1.35) nM and 1.42 (0.67–3.00) nM, respectively (Figure 5b, Table 2). In

Table 2 Effect of oxytocin and AVP receptor agonists and antagonists on $[Ca^{2+}]_i$ response and hyperplasia in human uterine smooth muscle cells

Compounds	EC_{50}/K_i (nM) $[Ca^{2+}]_i$ *response*	*Hyperplasia*
Agonists		
Oxytocin	2.09 (1.19–3.65)	5.47 (4.34–6.91)
AVP	114 (52–251)	1190 (239–5950)
[Asu^{1,6}]-oxytocin	7.04 (3.44–14.4)	
[Thr^4, Gly^7]-oxytocin	71.4 (29.7–172)	
Antagonists		
Atosiban	0.49 (0.28–0.86)	0.47 (0.02–1.35)
L-371257	0.46 (0.29–0.73)	1.42 (0.67–3.00)
d(CH$_2$)$_5$Tyr(Me)AVP	1.39 (0.67–2.90)	3.70 (1.06–12.9)
dDAVP	20.5 (9.2–45.4)	14.4 (4.2–50.0)
SR 49059	13.4 (8.1–22.0)	16.2 (10.4–25.1)
OPC-21268	13.0 (8.5–19.9)	88.4 (38.8–201)
SR 121463A	398 (267–596)	512 (235–1120)
OPC-31260	400 (185–865)	821 (306–2200)
YM087	8.59 (5.90–12.5)	45.0 (26.4–76.7)

The half-maximal effective concentration (EC_{50}) was calculated for the concentration-response curve obtained for each agonist. The half-maximal concentration inhibiting 100 nM oxytocin-induced responses (IC_{50}) is given for each antagonist. Apparent K_i values were calculated from Cheng & Prusoff (1973) relationship, $K_i = IC_{50}/(1+[L]/EC_{50})$. Values are means with 95% confidence limits obtained from four independent experiments.

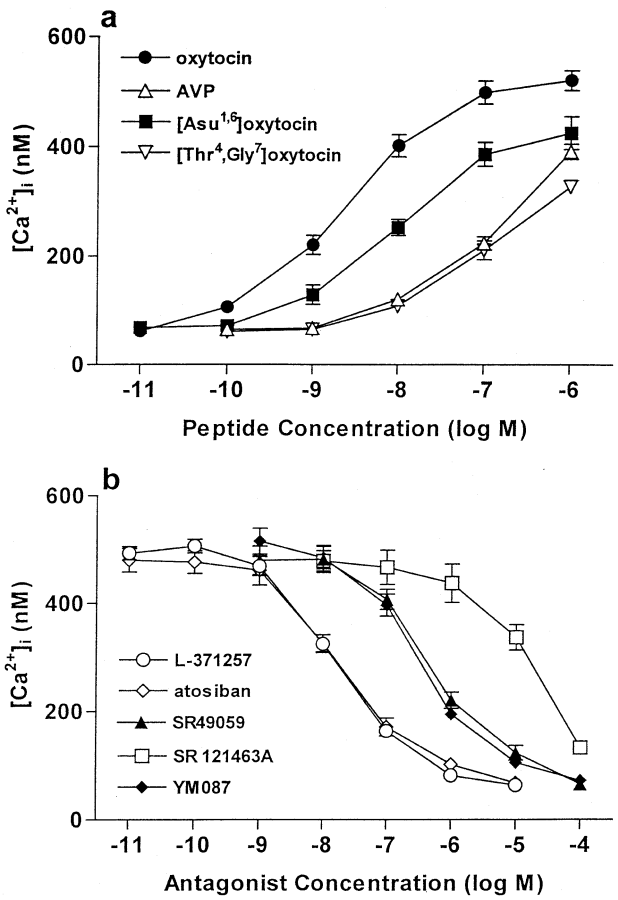

Figure 4 (a) Effect of oxytocin, AVP, [Asu^{1,6}]-oxytocin and [Thr^4,Gly^7]-oxytocin on $[Ca^{2+}]_i$ increase in human USMC. (b) Inhibitory effect of oxytocin and AVP receptor antagonists (preincubation of 3 min) on the response to a submaximal concentration of oxytocin (100 nM). Values are means ± s.e.mean of four independent experiments.

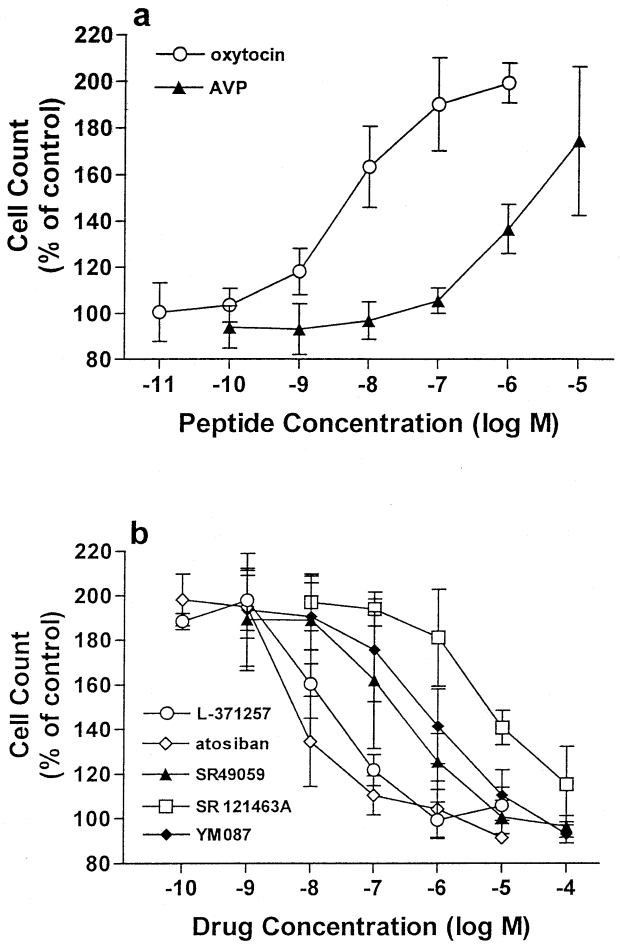

Figure 5 (a) Effect of oxytocin and AVP on hyperplasia of cultured human USMC. (b) Inhibitory effect of oxytocin and AVP receptor antagonists on the response to a submaximal concentration of oxytocin (100 nM). Values are means ± s.e.mean of four independent experiments.

contrast, the inhibitory potency of the V_{1A} receptor selective antagonists, SR 49059 and OPC-21268, and the V_{1A}/V_2 receptor antagonist, YM087, was 30–200 times lower than that of oxytocin receptor antagonists (Table 2). Furthermore, the V_2 receptor selective antagonists, SR 121463A and OPC-31260, did not potently inhibit oxytocin-induced $[Ca^{2+}]_i$ increase.

Discussion

The data generated during this study clearly demonstrate the existence of specific, high-affinity binding sites for oxytocin in human USMC. Binding of [^{3}H]-oxytocin to USMC membranes is time-, temperature- and protein concentration-dependent. The population of receptor sites is homogeneous, as indicated by the linear Scatchard plot of the binding experiments shown in Figure 1. The K_d of the oxytocin receptors in USMC is the nanomolar range, in agreement with K_d values obtained for oxytocin receptors from other tissues and species (Kimura *et al.*, 1994; Soloff *et al.*, 1977; Bonne & Cohen, 1975). Furthermore, the maximal oxytocin binding capacity of USMC is also in the same order of magnitude as the maximal oxytocin binding capacities found in rat uterus and mammary gland (Soloff *et al.*, 1977).

Radioligand binding experiments using a variety of oxytocin and AVP receptor agonists and antagonists indicate pharmacologic similarity between human USMC and uterine tissue oxytocin binding sites which is distinct from the known AVP receptor subtypes (Tahara *et al.*, 1998). The oxytocin receptor antagonist, L-371257, exhibits much higher affinity for the oxytocin receptors in USMC than for the AVP receptor subtypes V_{1A}, V_{1B} and V_2, which is consistent with earlier results (Williams *et al.*, 1995). In addition, the selective oxytocin receptor agonists [Asu^{1,6}]-oxytocin and [Thr^4,Gly^7]-oxytocin possess similar high affinities for USMC. In contrast, the V_{1A} receptor selective antagonist SR 49059, and the V_2 receptor selective antagonist SR 121463A show appropriate selectivity for their AVP receptor subtypes but had measurably lower affinities for oxytocin binding sites in USMC. For the entire series of oxytocin and AVP receptor agonists and antagonists tested, there was a highly significant correlation between pK_i values determined with USMC and the corresponding values measured for oxytocin receptors in human myometrium ($r=0.932$, $P<0.0001$; $y=0.77x+1.58$). No such correlation was found when comparing the ligand specificity of oxytocin receptors in USMC with those of V_{1A}, V_{1B} and V_2 receptors (r value of 0.138, $P=0.21$; 0.121, $P=0.24$; and 0.188, $P=0.14$, respectively).

Following the characterization of oxytocin receptor in USMC, the signal transduction pathway of USMC was investigated. Oxytocin activates phospholipase C-mediated hydrolysis of polyphosphoinositides *via* the oxytocin receptors to generate second messenger, IP_3, which induces an increase in

free intracellular calcium. Oxytocin, AVP, [Asu^{1,6}]-oxytocin and [Thr^4,Gly^7]-oxytocin administered to USMC increased $[Ca^{2+}]_i$ in a concentration-dependent manner and the relative order of potency was oxytocin > [Asu^{1,6}]-oxytocin > AVP = [Thr^4,Gly^7]-oxytocin. These agonistic potencies are consistent with published affinities for oxytocin receptors and biological oxytocic activities (Lowbridge *et al*., 1977; Yamanaka *et al*., 1970). Nevertheless, for AVP in particular, an approximately 10 fold difference was obtained between affinity and agonistic potency. It has been reported that an approximately 100 fold concentration of AVP is required to induce the same physiological response as that induced by oxytocin in oocytes expressing oxytocin receptors, and this was not consistent with the observation in uterus strips (Kimura *et al*., 1994). Such a difference remains unclear and has to be studied thoroughly, however, AVP may act as a partial agonist at human oxytocin receptors. In contrast, oxytocin-induced $[Ca^{2+}]_i$ increases were effectively blocked by oxytocin and AVP receptor antagonists. The relative order of potency was L-371257 = atosiban > d(CH$_2$)$_5$Tyr(Me)AVP > YM087 > OPC-21268 = SR49059 > dDAVP > SR121463A = OPC-31260. These antagonistic potencies are consistent with their affinities for oxytocin receptors in USMC. L-371257 and atosiban, potent oxytocin receptor antagonists, blocked the effect of oxytocin on $[Ca^{2+}]_i$ increase in USMC at concentrations well below the micromolar levels needed to antagonize the actions of AVP *via* the V_{1A} and V_2 receptors *in vitro* (Pettiborne *et al*., 1989). This suggests that the high-affinity [^{3}H]-oxytocin binding sites found in USMC are functional oxytocin receptors coupled to a $[Ca^{2+}]_i$ increasing mechanism. Recent studies have reported that oxytocin induces $[Ca^{2+}]_i$ mobilization in human uterine smooth muscle (Schrey *et al*., 1988) and myometrium (Szal *et al*., 1994). The present results provide further support for a general role of this system to mediate the contractile effects of oxytocin on smooth muscle, as previously suggested for the uterine tissues (Phillippe, 1994; Miyauchi & Uchida, 1994; Nohara *et al*., 1996).

The important function of oxytocin in parturition is based on its contractile effects on uterine myometrium at term. It has been reported that in addition to the maintenance of labour, oxytocin is a key initiator of parturition, at term and in premature labours (Soloff *et al*., 1979; Fuchs *et al*., 1982; Chan *et al*., 1982). However, the exact role of oxytocin remains unclear because the precise location of oxytocin in the complex chain of events leading to labour and delivery remains unknown. It is clear that parturition is highly regulated and likely involves the interplay of many additional factors, including prostaglandins, oestrogen, progesterone, cytokines and possibly bacterial endotoxins (Fuchs *et al*., 1984; Novy & Liggins, 1980; Romero *et al*., 1989). Unfortunately the experiments in this study provide no information of the regulation or production of these factors in USMC; to determine this, additional studies are required to identify the cellular mechanisms leading to labour. From the present data, however, it is clear that oxytocin could play an important role in the regulation of human uterine smooth muscle cell contraction *via* the specific oxytocin receptors.

Oxytocin has mitogenic properties in mouse mammary gland, rat cortical and hypothalamic astroglia and human vascular endothelial cells mediated by stimulation of oxytocin receptors (Sapino *et al*., 1993; Lucas *et al*., 1995; Thibonnier *et al*., 1999). Furthermore, it has been reported that oxytocin stimulates mitogen-activated protein (MAP) kinase activity in human uterine myometrial cells (Nohara *et al*., 1996; Ohmichi *et al*., 1995). From these observations, it can be inferred that the oxytocin receptors in the human myometrium may contribute to uterine growth associated with diverse physiologic and pathophysiologic situations. However, no data is available concerning a potential growth-promoting role in human myometrial cells. The present results demonstrate that oxytocin can induce cell proliferation in cultured human USMC. It has been reported that AVP causes a mitogenic effect on cultured human vascular smooth muscle cells and oxytocin also exhibited weak mitogenic activity through the V_{1A} receptors (Serradeil-Le Gal *et al*., 1995). However, in this study, oxytocin induced potent mitogenic effect than that of AVP in human USMC. Furthermore, oxytocin-induced hyperplasic effect was more effectively inhibited by oxytocin receptor antagonist, atosiban and L-371257, than V_{1A} receptor antagonist, SR 49059, suggesting that oxytocin induces mitogenic effect through the oxytocin receptors in human USMC. To our knowledge, this is the first demonstration of oxytocin receptor involvement in human myometrial cell proliferation. Although the physiological and pathophysiological function of oxytocin-induced proliferation in human USMC remains unclear, the present study suggests that oxytocin may contribute to the process of hyperplasia which occur in the uterus throughout pregnancy and variety of disease states such as endometriosis and endometrial cancer.

In conclusion, using receptor binding, second messenger and proliferation assays, the oxytocin receptor in human USMC was pharmacologically characterized. USMC should prove useful in studying the binding and biochemical function of oxytocin and its analogues, as well as aid in the evaluation of future human oxytocin receptor antagonists.

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