0026-895X/88/020218-07$02.00/0

MOLECULAR PHARMACOLOGY, 33:218-224

Oxytocin Induces a Transient Increase in Cytosolic Free [Ca^{2+}] in Renal Tubular Epithelial Cells: Evidence for Oxytocin Receptors on LLC-PK1 Cells

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Received July 7, 1987; Accepted November 18, 1987

SUMMARY

We examined the effects of oxytocin on renal tubular epithelial LLC-PK1 cells. In cells loaded with Fura 2, we found that 1 μM oxytocin induced a rapid increase in cytosolic free $[Ca^{2+}]_i$ from 120 nM to 250 nM within 12 sec. $[Ca^{2+}]_i$ then decreased and leveled at 148 nM. Calcium was mobilized from intra- and extracellular sources. Oxytocin-induced calcium mobilization was dose dependent (EC_{50} between 5 and 30 nM). Oxytocin also stimulated calcium efflux which was blocked by the selective oxytocin antagonist KB-5-21. Calcium mobilization was a likely consequence of enhanced phosphatidylinositol turnover, because oxytocin rapidly increased the formation of inositol phosphates including $Ins_{1,4,5}P_3$. Calcium transients were induced by oxytocin and the oxytocin selective analog AM-2-40 and blocked by the oxytocin-selective antagonist KB-5-21. Lysine vasopressin, the selective V2 agonist dDAVP, and the V1-selective agonist SK&F 105349 were at least 10- to 100-fold less potent than oxytocin and exhibited only partial agonist activity. Using peptide analogs, a poor correlation was found between antagonism of oxytocin-induced calcium transients of LLC-PK1 cells and pig kidney V2 and rat liver V1 receptor affinity. These data indicate that oxytocin-induced calcium transients in LLC-PK1 cells were not mediated by V1 or V2 vasopressin receptors, but by oxytocin receptors. However, the poor correlation between antagonism at the LLC-PK1 receptors and the rat uterus oxytocin receptors suggests marked differences in antagonist recognition. We have also identified specific, saturable, high affinity oxytocin-binding sites of low density on intact LLC-PK1 cells (K_D = 1.9 nM; B_{max} = 3.2 fmol/10^6 cells). The relative analog affinities for these binding sites correlated well with their effects on oxytocin-induced calcium transients. We conclude that in LLC-PK1 cells, oxytocin stimulates a transient rise in cytosolic free $[Ca^{2+}]_i$ and the formation of inositol phosphates, including $Ins_{1,4,5}P_3$. The effects on $[Ca^{2+}]_i$ probably are not mediated by V1 and V2 vasopressin receptors, but by putative oxytocin receptors.

Stimulation of uterine contraction and milk ejection are the best known actions of oxytocin. Since oxytocin is found in both the female and male hypophysis, other functions of oxytocin have been sought. Particularly, the effects of oxytocin on renal function have been extensively studied. Oxytocin was reported to enhance glomerular filtration rate and to be natriuretic (1). In the absence of vasopressin, oxytocin promotes antidiuresis and negative free water clearance, actions likely mediated by V2 receptors. A synergistic effect of oxytocin and vasopressin on sodium excretion was found in neurohypophysectomized rats (2). In these animals oxytocin potentiated the natriuretic response of vasopressin but reduced the antidiuretic activity of vasopressin. The molecular basis for these actions is not known, but could possibly involve modulation of cytosolic free $[Ca^{2+}]_i$. Teitelbaum and Berl (3) found that increased cytosolic $[Ca^{2+}]_i$ markedly decreased vasopressin-induced cAMP formation in cultured rat medullary collecting tubule cells. Dillingham *et al.* (4) showed that increased cytosolic $[Ca^{2+}]_i$ enhanced the hydroosmotic response to vasopressin and cAMP in rabbit cortical collecting tubules, indicating a post-cAMP effect. These findings suggest that oxytocin could modulate the antidiuretic activity of vasopressin by altering $[Ca^{2+}]_i$. Here we report that the pig kidney-derived LLC-PK1 cells responded to oxytocin with a transient increase in cytosolic $[Ca^{2+}]_i$ and an increased

ABBREVIATIONS: AVP, arginine vasopressin; LVP, lysine vasopressin; dDAVP, desamino[1]D-Arg[8]AVP; SK&F 101485, d$(CH_2)_5^1$D-Ile[2]Val[4]AVP; SK&F 101498, d$(CH_2)_5$D-Tyr(Et)[2]Val[4]AVP; SK&F 103561, des(ProGly)dPen[1]D-Tyr(Et)[2]AVP; SK&F 104222, desGlyd$(CH_2)_5^1$amino-L-suberic acid[1,6]D-Tyr(Et)[2]Val[4]AVP; SK&F 105349, Phe[2]Ile[3]Orn[8]vasopressin; SK&F 102793, des(ArgGlyNH$_2$)d$(CH_2)_5$D-Tyr(Et)[2]Val[4]guanidinoputrescine[8]vasopressin; AM-2-40, vasopressin]-[4]Gly[7]oxytocin; DES, diethylstilbestrol; KB-5-21, desGlyd$(CH_2)_5^1$Tyr(Me)[2]Thr[4]arginine vasotocin; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene bis(oxyethylene nitrilo) tetraacetic acid; PIP_2, phosphatidylinositol bisphosphate; IP_1, inositol monophosphate; $Ins_{3,4}P_2$, inositol 3,4-bisphosphate; $Ins_{1,4}P_2$, inositol 1,4-bisphosphate; $Ins_{1,3,4}P_3$, inositol 1,3,4-trisphosphate; $Ins_{1,4,5}P_3$, inositol 1,4,5-trisphosphate; IP_4, inositol tetrakisphosphate; HPLC, high performance liquid chromatography; Fura 2/AM, {1-[2-(5-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, pentaacetoxymethyl ester}.

formation of inositol phosphates. We present evidence that this calcium response was not likely mediated by V1 or V2 vasopressin receptors, but by oxytocin receptors. We also identified and characterized specific oxytocin-binding sites which likely represent the putative oxytocin receptors of LLC-PK1 cells.

Experimental Procedures

Materials. $^{45}CaCl_2$ (16.16 mCi/mg), [^{3}H]oxytocin (33.7 Ci/mmol), and [^{3}H]AVP (40 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). *myo*-[2-^{3}H]Inositol (110 Ci/mmol) was from Amersham Corp. (Chicago, IL). [^{3}H]LVP (22.8 Ci/mmol) was prepared at SK&F Laboratories (Philadelphia, PA). Dowex AG 1-X8, 100–200 mesh, formate form, was obtained from Bio-Rad Laboratories (Richmond, CA). FCS was from KC Biological (Lenexa, KS). DMEM, trypsin and DPBS with 0.5 mM $MgCl_2$ and 0.7 mM $CaCl_2$ were from Gibco (Grand Island, NY). Oxytocin, dDAVP, and LVP were purchased from Bachem (Torrance, CA). Samples of AM-2-40 and KB-5-21 were kindly given to us by Prof. M. Manning, Medical College of Ohio (Toledo, OH). Other peptide analogs used were SK&F 105349, SK&F 101485, SK&F 101498, SK&F 103561, SK&F 104222, and SK&F 102793. These analogs were synthesized at SK&F Laboratories.

Cell culture. LLC-PK1 cells (ATCC CL 101) and rat thoracic aorta-derived smooth muscle cells (A10; ATCC, CRL 1476) were obtained from the American Type Culture Collection (Bethesda, MD). LLC-PK1 cells were cultured in monolayer in DMEM with 10% FCS. A-10 cells were cultured with 20% FCS. All experiments were performed with cells in logarithmic growth. For the determination of cAMP formation, calcium efflux, formation of inositol phosphates, and oxytocin-binding sites, 35-mm 6-well Linbro plates were inoculated with 7.5×10^4 cells/well. Calcium transients were determined on cells in suspension obtained by harvesting cells grown in monolayer in T-150 culture flasks inoculated with 1.5×10^6 cells. The experiments were performed after 3 days in culture. At that time about 10^6 and 15×10^6 cells were present per 35-mm culture well and T-150 culture flask, respectively.

Calcium transients. The culture medium was removed and the cells were washed in DPBS. The cells were trypsinized for 3 min at 37° and collected in DMEM with 10% FCS. The cells were washed with Krebs buffer (118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 11.1 mM glucose, 1.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5 mM HEPES buffer, pH 7.4, and 0.1% BSA). The cells were suspended at 2×10^6 cells/ml and incubated for 30 min with 2 μM Fura 2/AM at 37°. The cells were washed with Krebs buffer and incubated for another 20 min at 37° without Fura 2/AM. The cells were stored at 10^7 cells/ml in ice until used. Calcium transients were measured using a temperature-controlled, 1-cm quartz cuvette with a stirred cell suspension at 10^6 cells/ml of Krebs buffer (2 ml total) in a spectrofluorometer (Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia) equipped with a 75-W xenon lamp. The cells were excited at 340 nm and the light emitted at 510 nm was measured. At the end of each transient, extracellular Fura 2 was determined with 25 mM Tris and 2.5 mM EGTA, F_{min} was determined with 0.05% Triton X-100, and F_{max} was determined with 5 mM $CaCl_2$. $[Ca^{2+}]_i$ was calculated using a calcium binding constant of 224 nM as indicated by Grynkiewicz *et al.* (5).

The antagonist potencies of vasopressin and oxytocin analogs were determined by incubating the cells for 2 min at 37° with the analogs at the indicated concentrations, followed by stimulation with 30 nM oxytocin. K_i values were calculated according to the method of Cheng and Prusoff (6), employing 10 nM as the EC_{50} value of oxytocin.

Calcium efflux. $^{45}Ca^{2+}$ efflux was measured according to a modification of the procedure by Masters *et al.* (7). Cells were incubated with $^{45}CaCl_2$ (5 μCi/ml of culture medium) for 20 hr. The medium was removed and the cells were washed three times with HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, 0.06% BSA, and 25 mM HEPES, pH adjusted to 7.4 with Tris base). After a 20-min incubation at 37° in HEPES buffer, the cells were incubated with or without oxytocin in fresh HEPES buffer. After 10 min the reaction was stopped by rapidly replacing the medium with HEPES buffer with 10 mM $LaCl_3$ without Na_2HPO_4. After washing the cells in this buffer four times, the cells were scraped in a solution containing 1 mM HEPES, 5 mM EGTA, and 3% (w/v) Triton X-100. Cell-associated ^{45}Ca was measured in a liquid scintillation counter.

cAMP determination. Cells were washed three times with $DPBS^{2+}$ (DPBS plus 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose, and 0.2% BSA). The cells were then incubated with $DPBS^{2+}$ containing 0.5 mM isobutylmethylxanthine in the presence of oxytocin or LVP for 10 min at 37°. The reaction was stopped by adding ice-cold trichloroacetic acid to a final concentration of 10%. After extraction of trichloroacetic acid with ether, cAMP was determined using radioimmunoassay kits purchased from New England Nuclear.

[^{3}H]Oxytocin binding to LLC-PK1 cells in monolayer. The culture medium was removed, the cells were washed three times with $DPBS^{2+}$, and the binding was initiated with 1 ml of $DPBS^{2+}$ containing [^{3}H]oxytocin, with or without 10 μM oxytocin, for nonspecific and total binding, respectively. The cells were incubated at 0° for 4 hr. The incubation medium was removed, the cells were washed with $DPBS^{2+}$, and the cells were removed by scraping. The cell suspension was rapidly filtered through Amicon filters (0.45 μm), and the cell-associated radioactivity was measured.

The affinity of vasopressin and oxytocin analogs for the oxytocin-binding sites was determined in competition binding experiments by incubating the cells for 4 hr at 0° with 3.6 nM [^{3}H]oxytocin in the presence of different concentrations of the analogs.

[^{3}H]LVP binding to pig kidney V2 receptors. The affinity of vasopressin analogs for pig kidney V2 receptors was determined in competition binding experiments using renomedullary membranes as described previously (8). The affinity was expressed as a K_{Bind} value calculated with the IC_{50} value, the radioligand concentration, and the K_D of [^{3}H]LVP (3.6 nM; Ref. 8) according to the method of Cheng and Prusoff (6).

[^{3}H]AVP binding to rat liver V1 receptors. The affinity of vasopressin analogs for rat liver V1 receptors was determined in competition binding experiments using rat liver membranes as described previously (9). The affinity was expressed as a K_{Bind} value calculated with the IC_{50} value, the radioligand concentration, and the K_D of [^{3}H]AVP (0.38 nM; Ref. 9) according to the method of Cheng and Prusoff (6).

Formation of inositol phosphates. LLC-PK1 cells were cultured in 35-mm 6-well Linbro plates for 3 days in inositol-free culture medium. During the third day, 2 μCi of [^{3}H] inositol/ml were present. The culture medium was removed and the cell monolayer was washed with HEPES buffer. The cells were then incubated for 20 min at 37° with 1 ml of HEPES buffer per well and washed three times with warm (37°) HEPES buffer. The cells were stimulated with oxytocin and LVP as follows. The incubation medium was removed and the cells were immediately incubated for 15 sec at 37° with 1 ml of HEPES buffer, with or without 100 nM oxytocin or 100 nM LVP. The reaction was stopped with ice-cold trichloroacetic acid (15% final), and the inositol phosphates were extracted and subsequently separated by HPLC on a Whatman Partisil 10-SAX column (25 × 0.46 cm), as previously described (10, 11). The radioactivity of the 0.4-ml fractions was determined in a Beckman liquid scintillation counter.

Inhibition of oxytocin-induced contraction of rat uterus. Uteri of virgin female rats (Sprague-Dawley; 160–180 g) were used 18 hr after an intramuscular injection of 50 μg of DES/rat in glyceryl tripelargonate. Inhibition of oxytocin-induced contraction was determined as described by Krejci *et al.* (12). Full oxytocin dose response curves were developed in the presence of different concentrations of antagonists.

Results

Oxytocin-induced calcium transients. A typical transient of cytosolic free $[Ca^{2+}]_i$ induced by 1 μM oxytocin is shown

in Fig. 1. Within 12 sec, $[Ca^{2+}]_i$ increased from 120 nM to a peak concentration of 250 nM, followed by a decrease to a new, higher basal $[Ca^{2+}]_i$ level of 148 nM within 60 sec. The basal $[Ca^{2+}]_i$ in cells incubated for 2 min in calcium-free Krebs buffer with 1 mM EGTA was 110 nM. In these cells 1 μM oxytocin increased $[Ca^{2+}]_i$ to 160 nM; $[Ca^{2+}]_i$ then declined to 100 nM. Whereas peak $[Ca^{2+}]_i$ attained in calcium-free medium was reduced by 60%, the rate at which peak $[Ca^{2+}]_i$ was obtained did not change. These findings indicate that oxytocin induced release of calcium from intracellular stores and calcium influx. Furthermore, the increased basal $[Ca^{2+}]_i$ is probably dependent on calcium influx.

Calcium efflux. Oxytocin also stimulated calcium efflux. LLC-KP1 cells were loaded with $^{45}Ca^{2+}$ for 20 hr, washed, and subsequently incubated for 10 min at 37° with or without 100 nM oxytocin. Oxytocin decreased cell-associated $^{45}Ca^{2+}$ by 21.5% (Table 1). At 1 μM, the oxytocin-selective antagonist KB-5-21 (anti-oxytocin pA_2 = 7.69, anti-vasopressor pA_2 = 6.48)[1] did not affect calcium efflux but inhibited the oxytocin-induced efflux by 62% (Table 1).

Formation of inositol phosphates. LLC-PK1 cells rapidly responded to the addition of oxytocin with an increase in inositol phosphate formation. Phosphatidylinositol hydrolysis of cells labeled with [^{3}H]inositol was stimulated for 15 sec with 100 nM oxytocin or 100 nM LVP. The inositol phosphates were extracted and separated by HPLC. Typical chromatograms of extracts of control and oxytocin-stimulated cells are shown in Fig. 2. Oxytocin increased $Ins_{1,4,5}P_3$, $Ins_{1,4}P_2$, and IP_1 (82, 109, and 20%, respectively; Table 2). LVP increased IP_1 by 19% but did not affect $Ins_{1,4,5}P_3$ or $Ins_{1,4}P_2$.

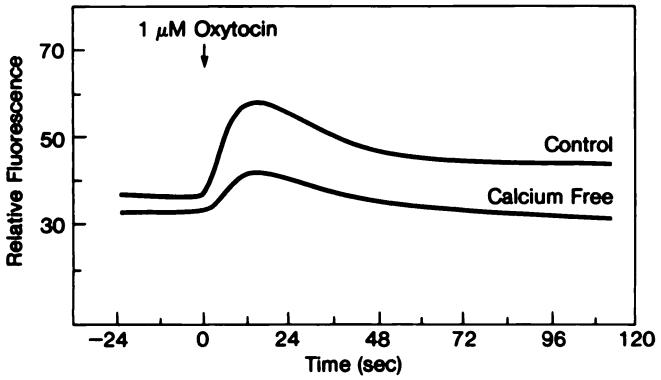

Fig. 1. Oxytocin-induced calcium transients in LLC-PK1 cells in the presence or absence of extracellular calcium. LLC-PK1 cells were trypsinized and loaded with Fura 2 as described under Experimental Procedures. Subsequently, the cells were incubated for 20 min at 37° in Krebs buffer or Krebs buffer without $CaCl_2$. One mM EGTA was added to the cells in calcium-free Krebs buffer 2 min before the cells were stimulated with 1 μM oxytocin. In the absence of calcium the peak $[Ca^{2+}]_i$ was reduced by about 60% and $[Ca^{2+}]_i$ returned to the basal concentration (about 100 nM).

TABLE 1

Calcium efflux from LLC-PK1 cells induced by oxytocin

LLC-PK1 cells were incubated for 20 hr in culture medium with 5 μCi of $^{45}Ca^{2+}$/ml. Cell-associated $^{45}Ca^{2+}$ was measured after incubating the cells for 10 min at 37°. Mean values ± standard errors of triplicate determinations are presented. The experiment was repeated with similar results.

Additions	Cell-associated $^{45}Ca^{2+}$
	dpm × 10^{-3}
Control	180.8 ± 9.1
100 nM Oxytocin	141.9 ± 5.2[a]
1 μM KB-5-21	180.5 ± 7.1
100 nM Oxytocin plus 1 μM KB-5-21	166.1 ± 4.7[b]

[a] Significantly different from control, $p < 0.05$.
[b] Significantly different from oxytocin, $p < 0.05$.

[1] Prof. M. Manning, personal communication.

Pharmacological characterization of the receptors that mediate oxytocin-induced calcium transients. LLC-PK1 cells express vasopressin V2 receptors that stimulate cAMP formation (13, 14). Fig. 3A shows that LVP and the V2-selective analog dDAVP were less potent agonists (EC_{50} = 250 and 750 nM, respectively) than oxytocin (EC_{50} ≃ 20 nM). Moreover, LVP and dDAVP were partial agonists. Assuming that the maximal $[Ca^{2+}]_i$ induced by oxytocin was 100%, LVP and dDAVP stimulated $[Ca^{2+}]_i$ maximally to 50 and 10%, respectively. This is consistent with the low potency of LVP for $Ins_{1,4,5}P_3$ formation. At 100 nM, LVP did not significantly increase $Ins_{1,4,5}P_3$ formation (Table 2). The stimulation of $[Ca^{2+}]_i$ contrasted with the stimulation of cAMP formation where LVP and oxytocin were full agonists (Fig. 3B) and LVP was 30-fold more potent (EC_{50} = 0.3 nM) than oxytocin (EC_{50} = 11 nM). These agonist data suggest that the oxytocin-induced increase in $[Ca^{2+}]_i$ was not mediated by V2 receptors. This was further supported by studies with antagonists in competitive inhibition experiments as illustrated in Fig. 4. The analog rank orders for antagonism of oxytocin-increased $[Ca^{2+}]_i$ and affinity for pig kidney V2 receptors were different (Table 3).

The potential role of V1 receptors in effecting oxytocin-induced calcium transients was studied with SK&F 105349, a V1-selective agonist (15). Fig. 5A shows that in LLC-PK1 cells oxytocin was >100-fold more potent than SK&F 105349 in eliciting a calcium response. In the vascular smooth muscle-derived A10 cells, which express V1 receptors (16), oxytocin was a weak partial agonist, whereas SK&F 105349 was a potent full agonist (K_{act} ≃ 10 nM). Together with the low potency of LVP, these data suggest that the oxytocin-induced increase in free $[Ca^{2+}]_i$ was not mediated by V1 receptors. Additional evidence against a role of V1 receptors is provided by the poor correlation between rat liver V1 affinity and oxytocin antagonism (Table 3).

The oxytocin-selective agonist AM-2-40 (17) also stimulated calcium transients in LLC-PK1 cells (Fig. 6). Furthermore, the oxytocin-selective antagonist KB-5-21 was a potent inhibitor of oxytocin-induced calcium transients (Fig. 4, Table 3). These findings suggest that an oxytocin receptor rather than a V1 or V2 receptor mediated the induction of calcium transients. However, the antagonist rank orders for the contraction of rat uterus and the increased free $[Ca^{2+}]_i$ in LLC-PK1 cells induced by oxytocin did not correlate (Table 3). This indicated that the antagonist recognition patterns of the rat uterus receptor and the putative oxytocin receptors of LLC-PK1 cells are quite different.

Specific oxytocin-binding sites on LLC-PK1 cells. At 0°, LLC-PK1 cells in monolayer specifically bound [^{3}H]oxytocin (Fig. 7). The specific binding was saturable (B_{max} = 3.2 fmol/10^6 cells) and of high affinity (K_D = 1.9 nM; Fig. 7, *inset*).

Specific [^{3}H]oxytocin binding could be inhibited with vasopressin and oxytocin analogs (Table 4). KB-5-21, a potent and oxytocin-selective antagonist, and SK&F 103561, a potent ox-

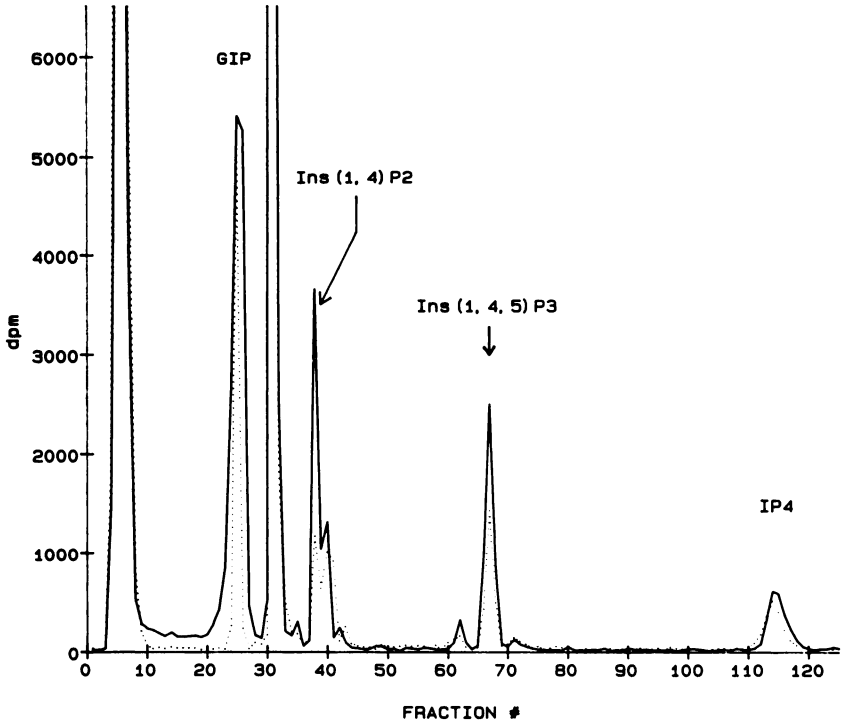

Fig. 2. Effect of oxytocin on phosphatidylinositol turnover of LLC-PK1 cells in monolayer. [^{3}H]Inositol-labeled cells were incubated for 15 sec with (——) or without (· · ·) 100 nM oxytocin at 37°. The reaction was stopped with 15% trichloroacetic acid, and the inositol phosphates were extracted and subsequently separated by HPLC on a Whatman Partisil 10-SAX column. Fractions of 0.4 ml were collected and the radioactivity was measured. The chromatograms of a typical experiment are presented (dpm/3 × 10^5 cells). The peaks were identified with radioactive standards. Inositol and IP_1 eluted at fractions 5 and 32, respectively. The experiment was repeated twice with similar results.

TABLE 2

Effect of oxytocin and LVP on phosphatidylinositol turnover of LLC-PK1 cells in monolayer

[^{3}H]Inositol-labeled cells were incubated for 15 sec at 37° in HEPES buffer (control), with 100 nM oxytocin, or 100 nM LVP. The reaction was stopped with 15% ice-cold trichloroacetic acid. Inositol phosphates were extracted and chromatographed on a Whatman Partisil 10-SAX HPLC column as described under Experimental Procedures. The radioactivity of each inositol phosphate was totaled and mean values ± standard errors of three determinations are presented.

Inositol phosphates	Control	Oxytocin	LVP
		dpm/3 × 10^5 cells	
IP_1	31,505 ± 100	37,722 ± 1301*	37,520 ± 470*
$Ins_{1,4}P_2$	1,990 ± 444	4,155 ± 90*	1,605 ± 100
$Ins_{3,4}P_2$	2,231 ± 275	2,126 ± 115	2,110 ± 50
$Ins_{1,3,4}P_3$	259 ± 76	384 ± 74	286 ± 31
$Ins_{1,4,5}P_3$	2,155 ± 176	3,931 ± 474*	1,863 ± 164
IP_4	1,780 ± 71	1,906 ± 344	1,399 ± 41

* Significantly different from corresponding control values, $p < 0.05$.

ytocin and V1 antagonist, were also potent inhibitors of [^{3}H] oxytocin binding. Inhibition of [^{3}H]oxytocin binding by the oxytocin-selective agonist AM-2-40 and LVP required higher analog concentrations (Table 4) as would be predicted from the calcium transient data (Figs. 6 and 3A, respectively). These data suggest that the [^{3}H]oxytocin-binding sites may represent the putative oxytocin receptors that mediate calcium transients.

Discussion

In this study we showed that oxytocin induced a transient rise in cytosolic free $[Ca^{2+}]_i$ of renal tubular epithelial LLC-PK1 cells (Fig. 1) with a profile typical of vasopressin-induced calcium transients in hepatocytes (18) and vascular smooth muscle cells (19, 20). The basal $[Ca^{2+}]_i$ level of the trypsinized LLC-PK1 was 120 nM, similar to the values reported by Bonventre and Cheung (21) for LLC-PK1 cells in suspension (98 ± 5 nM) and by Tang and Weinberg (22) for cells in monolayer (73 ± 3 nM). Within 12 sec after 1 μM oxytocin, $[Ca^{2+}]_i$ increased about 2.5-fold. $[Ca^{2+}]_i$ then declined rapidly but remained elevated at about 148 nM. This elevated $[Ca^{2+}]_i$ concentration was dependent on a new steady state between calcium influx, calcium efflux, and, presumably, intracellular calcium sequestration. Calcium influx occurred because $[Ca^{2+}]_i$ returned to basal levels after oxytocin treatment in calcium-free medium. Calcium efflux was increased by oxytocin (Table 1). In calcium-free medium the peak $[Ca^{2+}]_i$ was reduced by 60%, indicating that oxytocin induced calcium mobilization from intracellular stores and from the extracellular medium (Fig. 1).

Oxytocin likely induced calcium transients via activation of phospholipase C, resulting in hydrolysis of phosphatidylinositol-bisphosphate (PIP_2). The hydrolysis product of PIP_2, $Ins_{1,4,5}P_3$, has been recognized as the principal mediator of intracellular calcium release (23, 24). Oxytocin induced the formation of $Ins_{1,4,5}P_3$ (Table 2) within 15 sec, a time consistent with rapid calcium mobilization. $Ins_{1,4,5}P_3$ has been shown to release calcium from intracellular stores of LLC-PK1 cells (25, 26).

We next sought to classify the receptor type that mediated oxytocin-induced calcium transients in LLC-PK1 cells. LLC-PK1 cells express V2 vasopressin receptors coupled to cAMP formation (13, 14, 27). Tang and Weinberg (22) and Burnatowska-Hledin and Spielman (28) reported that LVP also increases cytosolic free $[Ca^{2+}]_i$ in LLC-PK1 cells. In the present study we confirmed their observations but found that, compared to oxytocin, LVP was only a weak, partial agonist (Fig. 3; EC_{50} = 250 nM, maximum $[Ca^{2+}]_i$ = 50%). This finding, together with the lack of correlation between antagonism of oxytocin-increased $[Ca^{2+}]_i$ in LLC-PK1 cells and pig kidney V2 vasopressin receptor binding (Table 3), argues against V2 receptor-mediated calcium transients.

The higher potency of oxytocin, the weak agonism of the V1-selective agonist SK&F 105349 (Fig. 5) (15), and the poor

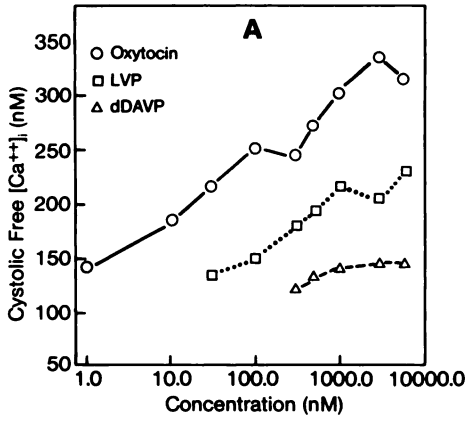

Fig. 3. A. Dose response of the induction of calcium transients in LLC-PK1 cells by oxytocin, LVP, and dDAVP, a selective V2 agonist. LLC-PK1 cells were trypsinized and loaded with Fura 2 as described under Experimental Procedures. Calcium transients of the cells in suspension at 37° were recorded. Peak $[Ca^{2+}]_i$ concentrations are presented. Oxytocin is more potent ($K_{act} \simeq 20$ nM) and induces a higher $[Ca^{2+}]_i$ than LVP ($K_{act} \simeq 250$ nM), and dDAVP ($K_{act} \simeq 750$ nM). LVP and dDAVP are weak, partial agonists. The experiment was repeated with similar results. B. Dose response of the stimulation of cAMP formation in LLC-PK1 cells by oxytocin and LVP. Intra- and extracellular cAMP of the cells in monolayer in response to a 10-min incubation with the agonists were measured by radioimmunoassay. Mean values ± standard errors (*bars*) are given ($n = 3$). LVP is a more potent stimulator of cAMP formation ($K_{act} = 0.3$ nM) than oxytocin ($K_{act} = 11$ nM).

correlation between antagonism of oxytocin-increased $[Ca^{2+}]_i$ in LLC-PK1 cells and rat liver V1 receptor affinity (Table 3) all argue against V1 receptor-mediated calcium transients.

The findings that oxytocin was a potent and effective agonist, the activity of the oxytocin-selective agonist AM-2-40 (Fig. 6) (17), and the potent antagonism of the oxytocin-selective antagonist KB-5-21 (Fig. 4, Table 3) support our hypothesis that the calcium transients in LLC-PK1 cells are mediated by oxytocin receptors. The lack of correlation between antagonism of oxytocin-increased $[Ca^{2+}]_i$ in LLC-PK1 cells and oxytocin-induced contraction of rat uterus could indicate a different oxytocin receptor subtype. However, this receptor may also represent the same oxytocin receptor subtype that exhibits marked species differences in antagonist recognition patterns. For oxytocin receptors this has been clearly shown by Åkerlund *et al.* (29, 30) and for vasopressin V1 and V2 receptors by Stassen *et al.* (9).

Evidence for the existence of specific oxytocin-binding sites

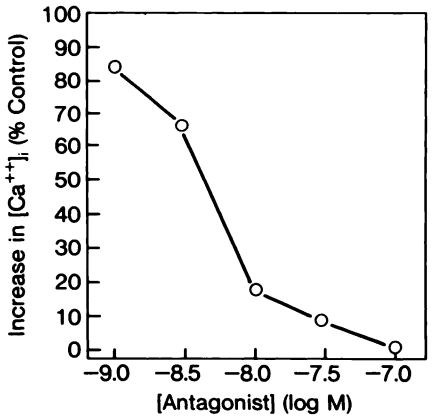

Fig. 4. Inhibition of oxytocin-induced calcium transients in LLC-PK1 cells by KB-5-21, a selective oxytocin antagonist (M. Manning, personal communication). LLC-PK1 cells were trypsinized and loaded with Fura 2 as described under Experimental Procedures. The cells were incubated for 2 min at 37° with KB-5-21 at the concentrations indicated before stimulation with 30 nM oxytocin. The maximal increase in $[Ca^{2+}]_i$ in response to 30 nM oxytocin was taken as 100%.

TABLE 3

Rank order of vasopressin analogs for antagonism of oxytocin-induced calcium transients in LLC-PK1 cells, pig kidney V2 receptor affinity, rat liver V1 receptor affinity, and antagonism of oxytocin-induced rat uterus contraction

Calcium transients were determined in response to 30 nM oxytocin after the LLC-PK1 cells were incubated for 2 min at 37° with antagonists as described under Experimental Procedures. V2 receptor affinity was determined in competitive radioligand binding experiments with [^{3}H]LVP and pig renomedullary membranes. V1 receptor affinity was determined in competitive radioligand binding experiments with [^{3}H]AVP and rat liver plasma membranes. K_B values were determined using the uterine horns of DES-treated, immature rats.

Vasopressin analogs (SK&F no.)	Oxytocin-induced $[Ca^{2+}]_i$ K_i	Pig kidney K_{displ}	Rat liver K_{displ}	Rat uterus K_B
		nM		
104222	0.1	7.1	ND[a]	52.0
102793	0.7	14.5	1.2	82.0
103561	1.2	317.0	0.15	1.9
101498	1.3	12.0	1.4	2.1
KB-5-21	1.4	>10,000.0	ND	ND
101485	>125.0	1,220.0	243.0	32.0

[a] ND, not determined.

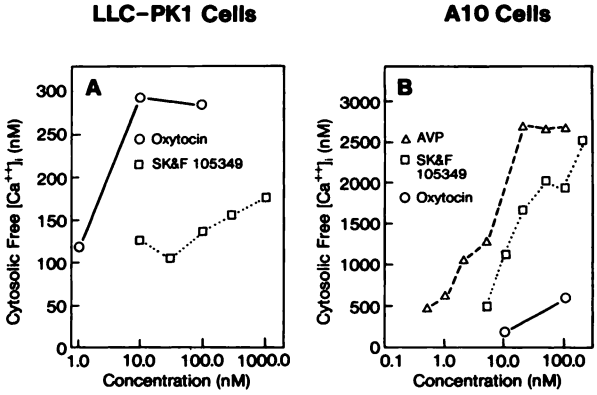

Fig. 5. Dose response of the induction of calcium transients by SK&F 105349, a V1-selective agonist (15) in LLC-PK1 cells (A) and vascular smooth muscle (A10) cells (B). LLC-PK1 and A10 cells were trypsinized and loaded with Fura 2 as described under Experimental Procedures. In LLC-PK1 cells, oxytocin was more potent than SK&F 105349; in A10 cells, SK&F 105349 was more potent ($K_{act} = 10$ nM) than oxytocin. The experiment was repeated with similar results.

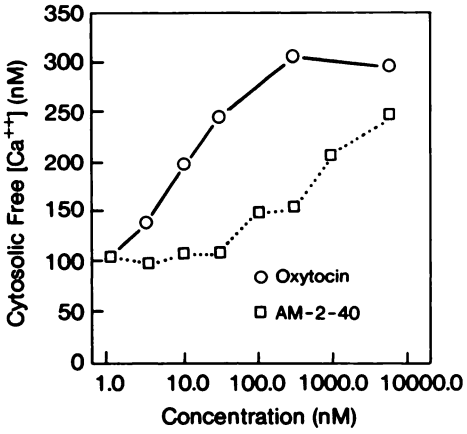

Fig. 6. Dose response of the induction of calcium transients in LLC-PK1 cells by AM-2-40, an oxytocin-selective agonist (17). LLC-PK1 cells were trypsinized and loaded with Fura 2 as described under Experimental Procedures. AM-2-40 induced calcium transients with a K_{act} of approximately 1 μM. The experiment was repeated twice with similar results.

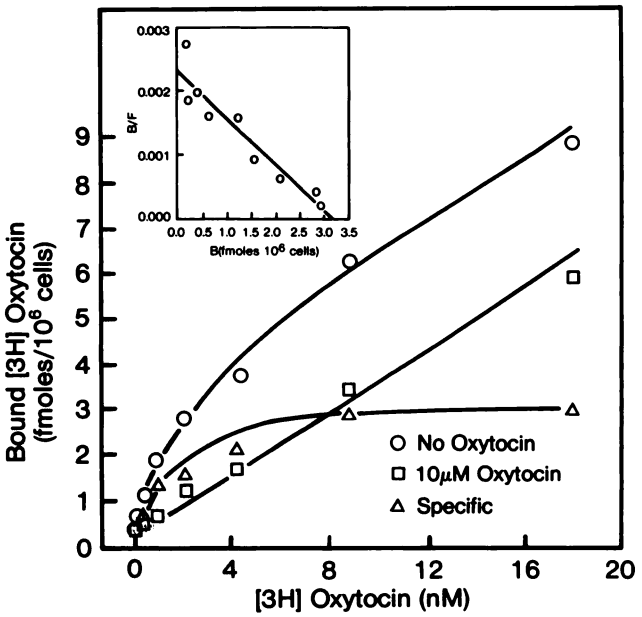

Fig. 7. Saturation equilibrium binding of [^{3}H]oxytocin to LLC-PK1 cells. LLC-PK1 cells in monolayer were incubated for 4 hr at 0° in DPBS containing 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose, and 0.2% BSA with [^{3}H]oxytocin at the concentrations indicated, with (nonspecific) or without (total) 10 μM oxytocin. *Inset.* Oxytocin binds to LLC-PK1 cells with a single high affinity (r = 0.95; K_D = 1.9 nM) and is saturable (B_{max} = 3.2 fmol/10^6 cells). Mean values of triplicate determinations are presented.

on LLC-PK1 cells was obtained with [^{3}H]oxytocin. LLC-PK1 cells expressed specific, high affinity oxytocin-binding sites (Fig. 7; B_{max} = 3.2 fmol/10^6 cells, K_D = 1.9 nM). The affinities of the oxytocin-binding sites and the V2 receptors as measured with [3H]LVP were similar, but the density of the oxytocin sites was only about 10% of that of the V2 receptors (K_D = 3.0 nM, $B_{max} \simeq$ 40 fmol/10^6 cells) (14). Specific [^{3}H]oxytocin binding was inhibited by peptide analogs with relative potencies similar to their effects on calcium transients (Table 4). Therefore, we conclude that these specific binding sites represent the functional oxytocin receptors.

In these studies we showed that oxytocin induced calcium transients ($K_{act} \simeq$ 20 nM) and calcium efflux in LLC-PK1 cells.

TABLE 4

Inhibition of [^{3}H]oxytocin binding to LLC-PK1 cells in monolayer by peptide analogs

LLC-PK1 cells in monolayer were incubated for 4 hr at 0° with 3.6 nM [^{3}H]oxytocin with or without the analogs. Nonspecific binding was determined with 10 μM oxytocin and was 21%. Mean values ± standard errors of triplicate determinations are presented.

Analogs	Inhibition of [^{3}H] oxytocin binding
	%
10 nM KB-5-21	57 ± 2
10 nM SK&F 103561	60 ± 5
1000 nM AM-2-40	77 ± 6
100 nM LVP	35 ± 6

Calcium transients were probably induced by receptor-activated phosphatidylinositol turnover. Cytosolic free calcium was derived from intracellular and extracellular sources. We presented evidence that oxytocin did not cause calcium transients in LLC-PK1 cells by activation of vasopressin V1 or V2 receptors, but by activation of oxytocin receptors. The analog recognition patterns of the putative oxytocin receptors of LLC-PK1 cells and rat uterus oxytocin receptors were different. LLC-PK1 cells expressed specific, saturable, and high affinity oxytocin-binding sites (K_D = 1.9 nM; B_{max} = 3.2 fmol/10^6 cells) which probably represent the functional oxytocin receptors. Since LLC-PK1 cells express both vasopressin V2 receptors, coupled to adenylate cyclase, and oxytocin receptors, coupled to calcium signaling, these cells may be a model to study the coordinate regulation of renal epithelial cell function by oxytocin and vasopressin.

Acknowledgments

We would like to thank Dr. G. Dubyak (Case Western Reserve University, Cleveland, OH) for his assistance in establishing the fluorescence methods, Dr. M. Moore for the synthesis of SK&F 105349, Prof. M. Manning of the Medical College of Ohio (Toledo, OH) for samples of AM-2-40 and KB-5-21 and the data on the receptor selectivity of KB-5-21, Ms. G. Sosnowski for technical support with the uterine contraction studies, and Drs. S. T. Crooke and W. F. Huffman for continued encouragement and support.

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