The Proximal Portion of the COOH Terminus of the Oxytocin Receptor Is Required for Coupling to G_{q}, but Not G_{i}

INDEPENDENT MECHANISMS FOR ELEVATING INTRACELLULAR CALCIUM CONCENTRATIONS FROM INTRACELLULAR STORES

As the oxytocin receptor plays a key role in parturition and lactation, there is considerable interest in defining its structure/functional relationships. We previously showed that the rat oxytocin receptor transfected into Chinese hamster ovary cells was coupled to both G_{q,11} and G_{i,0}, and that oxytocin stimulated ERK-2 phosphorylation and prostaglandin E_{2} synthesis via protein kinase C activity. In this study, we show that deletion of 51 amino acid residues from the carboxyl terminus resulted in reduced affinity for oxytocin and a corresponding rightward shift in the dose-response curve for oxytocin-stimulated [Ca^{2+}]_{i} transients. However, oxytocin-stimulated ERK-2 phosphorylation and prostaglandin E_{2} synthesis did not occur in cells expressing the truncated receptor. Oxytocin also failed to increase phospholipase A activity or activate protein kinase C, indicating that the mutant receptor is uncoupled from G_{q}-mediated pathways. The Δ51 receptor is coupled to G_{i}, as oxytocin-stimulated Ca^{2+} transients were inhibited by pertussis toxin, and a G_{i}/β_{γ} sequestrant. Preincubation of Δ51 cells with the tyrosine kinase inhibitor, genistein, also blocked the oxytocin effect. A α39 mutant had all the activities of the wild type oxytocin receptor. These results show that the portion between 39 and 51 residues from the COOH terminus of the rat oxytocin receptor is required for interaction with G_{q,11}, but not G_{i,0}. Furthermore, an increase in intracellular calcium was generated via a G_{i}/β_{γ}-tyrosine kinase pathway from intracellular stores that are distinct from G_{q}-mediated inositol trisphosphate-regulated stores.

Oxytocin (OT)\(^1\) is a nine-amino acid peptide that stimulates uterine smooth muscle and mammary myoepithelial cell contraction, and prostaglandin production by uterine endometrial and amnion cells. Nucleic acid sequencing of cDNA clones of the oxytocin receptor (OTR) indicated that it is a member of the G protein-coupled receptor (GPCR) superfamily (1). As OT plays a pivotal role in parturition and lactation, there is considerable interest in defining the structure of the OTR. It has been shown for a number of GPCRs that several regions in the cytoplasmic domains contribute directly or indirectly to G protein coupling (see Ref. 2 for a review). The juxtamembrane portions of cytoplasmic loop 3 and cytoplasmic loop 2 of several family members have been implicated in receptor-G protein interactions. In addition, the COOH-terminal region of adrenergic receptors (3, 4) and other receptor types (5–7) is also required for G protein interactions, but this domain does not appear to be important for receptor function of all GPCR family members (8–10).

The COOH-terminal domain of some GPCRs plays an important role in G protein isotype selectivity (11, 12). At least four isoforms of the prostaglandin EP3 receptor, differing only at their COOH-terminal tails (produced by alternative splicing), couple to different G proteins to activate different second messenger systems (13, 14). The COOH terminus of the human parathyroid hormone receptor directs the receptor toward an interaction with G_{α}, whereas a core region composed of the first, second, and third intracellular loops can interact promiscuously with different G proteins (11). A truncated human AT1 receptor mutant lacking the carboxyl-terminal 50 residues is deficient in coupling to G_{i}, but it retains full ability to bind to G_{q} (12).

The COOH terminus of some GPCRs has also been shown to be important for desensitization, which is manifested as a diminution in responsiveness for some period of time following agonist stimulation. COOH-terminal truncation of the β_{2}-adrenergic, α_{1B}-adrenergic, lutropin/choriogonadotropin, platelet-activating factor, and neurokinin-2 receptors has been shown to impair homologous desensitization (15–20). Work on the β-adrenergic receptor, in particular, has indicated that one mechanism of desensitization involves the rapid internalization of membrane-bound receptors following agonist stimulation (15). Removal of the COOH-terminal tail of some GPCRs has been shown to greatly reduce agonist-induced internalization of the receptor while having little or no effect on signal transduction (21–25).

Most GPCRs have a conserved cysteine in the COOH-terminal cytoplasmic tail near the seventh transmembrane-spanning region. This cysteine is known to be palmitoylated in rhodopsin (26), the β_{2}-adrenergic receptor (27), and the α_{2A}-adrenergic receptor (28). In addition to the three intracellular loops delimited by transmembrane domains, a putative fourth cytoplasmic loop is formed in many GPCRs by insertion of palmitoylcysteines into the membrane lipid bilayer (29). It has...
been suggested that the fourth intracellular loop is important for G protein coupling (29), and mutation of Cys-341 in the carboxyl tail of the human β2-adrenergic receptor leads to an uncoupled, nonpalmitoylated form of the receptor (27). Studies with m2 receptors indicate that palmitoylation is not an absolute requirement for receptor interaction with G proteins, but it enhances the ability of the receptors to interact with G proteins (30). In other cases, elimination of palmitoylation sites does not affect receptor-G protein interactions (31–35). However, downregulation of the receptor number after prolonged agonist exposure was completely abolished by this mutation (32). De- palmitoylation also has been shown to increase the rate of the luteinizing hormone/human chorionic gonadotropin receptor internalization (33). In other instances, replacement of Cys results in the specific loss of coupling to one G protein isotype but not another, namely mutation of the human endothelin receptor A resulted in no effect on the ability of endothelin to activate adenylyl cyclase, but inhibited activation of PLC (36).

Previous work from our laboratory, using a CHO cell line that was stably transfected with the rat OTR, showed that OT stimulated rapid increases in intracellular Ca2+ concentration ([Ca2+]i), extracellular signal-related kinase-2 (ERK-2) phosphorylation, and PGE2 synthesis (37, 38). Furthermore, the OTR was coupled to both Gq11 and Gi in transfected CHO cells (CHO-OTR cells) (38), and in pregnant rat myometrium (39). In the present studies, we have systematically analyzed the importance of the COOH-terminal domain of the OTR on several receptor-associated processes: ligand affinity, G protein coupling via specific signal pathways, receptor desensitization, and selectivity of G-protein coupling. With the exception of a few amino acid residues, the COOH-terminal domain of the OTR is highly conserved between species (Fig. 1). This observation argues in favor of the functional importance of the COOH-terminal domain of the OTR. Previous work with the rat V1a vasopressin receptor indicated that the COOH-terminal region of the receptor is inaccessible to antibodies directed against a COOH-terminal peptide when the receptor is coupled to G proteins, but is accessible when receptor-G protein complexes are dissociated (40). As the V1a vasopressin receptor is closely related to the OTR, we have examined the importance of the COOH-terminal domain of the OTR in G protein coupling by creating COOH-terminal deletion mutants. Our approach has been to create COOH-terminal truncations of 22, 39, and 51 residues. The OTR has two adjacent Cys residues at positions 351 and 352, which are potential palmitoylation sites (Fig. 2). To determine the importance of these sites in OT action in the present studies, both these residues were replaced by Ser in one of the mutant OTRs analyzed.

**Results**

**Expression of Wild Type and Deletion Mutant Constructs**

The COOH-terminal truncation and replacement sites are shown in Fig. 2. All of the cDNAs were expressed in CHO cells, as shown by 125I-OTA binding to cell surface OTR on intact cells (Table I). The apparent Kd values of binding to the mutant receptors were comparable, but the number of receptors sites expressed per cell varied between mutants. The concentration of binding sites for the Δ51 mutant was about 10% that of the wild type. Efforts to obtain Δ51 clones with a greater number of binding sites were unsuccessful. We also determined the 125I-OTA binding properties of CHO cells (Table I). The apparent Kd values for the Δ22, Δ39, and Δ51 mutants were comparable to that of the wild type, while the Δ51 mutant had an IC50 value that was about 3–7 times greater than the others (Fig. 3).

**Effect of Truncation on the Ca2+ Response to OT**—We have shown previously that stimulation of CHO-OTR cells with OT DNA Constructs and Transfections—Rat OTR cDNA was provided by Dr. Stephen J. Lollait. Full-length and carboxyl-terminal mutants, lacking 22, 39, and 51 residues (Fig. 2), were generated from rat OTR cDNA by polymerase chain reaction, with primer sets containing an EcoRI and Xhol end. The amplified DNA fragments were ligated into the expression vector pcDNA3.1 Myc/His A (Invitrogen, San Diego, CA) in frame with the Myc/His epitopes at the 3′-terminus. Mutation of two Cys residues at positions 343 and 344 to Ser was carried out by the method of Higuchi et al. (41). The sequences of all the DNA constructs were verified by DNA sequence analysis. The primer pairs for the full-length cDNA were primer 1 (5′-GAATTCTGAGTCGGTGGCGTCG-3′) and primer 2 (5′-CTCGAGTTGACTGGTCTGGC-3′) and primer 2 (5′-CTCGAGAAGCTCTGAGACG-3′). Each truncation mutant was generated with primer 1 and a unique primer 2. The primers for Δ22, Δ39, and Δ51 were 5′-CTCGAGAGCTGGTCTGGC-3′, 5′-CTCGAGAAGCTCTGAGACG-3′, and 5′-CTCGAGGTTGAGGTGACCTGTGA-3′, respectively. For construction of the Cys for Ser replacement mutants, full-length primers 1 and 2 were used along with 5′-GTCGACGCTCTGAGACG-3′ and 5′-GTCGACGCTCTGAGACG-3′.

CHO-K1 cells were grown in α-minimal essential medium containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The expression plasmids were introduced into these cells by calcium phosphate-mediated transfection, and clonal cell lines that stably expressed the cDNAs were obtained by Geneticin (400 µg/ml) selection. The cells were maintained under an atmosphere of 5% CO2.

**Receptor Binding Assay**—Determination of the apparent Kd and Bmax values of each of the cell lines was carried out with cells in six-well plates as described previously (42), using increasing concentrations of 125I-OTA (0.14 to 100 pM) in 1 ml of Tyrode’s solution, pH 7.5, containing 1.0% bovine serum albumin. The binding data were examined by nonlinear regression analysis (GraphPad Software Inc., San Diego, CA), and binding constants were determined by assuming a single class of independent binding sites.

Oxytocin competition studies were carried out using a fixed concentration of 125I-OTA and increasing concentrations of OT (0.1–100 nM) in 24-well plates (0.3 ml/plate). The cells were then rinsed (3 × 1 ml) in assay buffer and solubilized in 1 N NaOH; radioactivity was determined with a β counter.

**Measurement of Intracellular Free Calcium Concentrations, Inositol Phosphates, and PKC Activation**—Real-time recordings of intracellular calcium concentrations (Ca2+1) were performed on single cells, as described previously (37). Each point in the figures represents the mean ± S.E. values from 35 cells. The concentration of OT-stimulated synthesis of inositol phosphates was measured as described previously (37). Translocation of PKC was determined by immunoblot analysis of the cytosol and Triton X-100-solubilized cell fractions that were prepared according to Ogihara et al. (43).

**PGE2 Synthesis and MAP Kinase Phosphorylation**—PGE2 synthesis was determined using a PGE2 enzyme immunoassay kit from Amersham Pharmacia Biotech, as described previously (37). The phosphorylation of ERK-2 MAP kinase and the effects of pertussis toxin were determined by a phospho-ERK-2 MAP kinase assay (40). For analysis of phosphorylated p38 MAP kinase, immunobots were incubated with antibody directed against dually phosphorylated p38 (New England Biolabs), followed by stripping of the blots and reprobing with an antibody to p38 (Santa Cruz Laboratories).
results in a rapid, transient increase in intracellular Ca\(^{2+}\) concentrations. Both intra and extracellular sources of Ca\(^{2+}\) were involved (37). In the present studies, OT stimulated intracellular Ca\(^{2+}\) in all of the mutant lines (Fig. 4A), while the other mutants and wild type CHO-OTR cells responded to 10 nM OT or less (data not shown). The reduced sensitivity of this mutant to OT (Fig. 4B). Neither the wild type nor any of the mutants exhibited a significantly diminished response to OT after the second dose of OT, indicating that the COOH-terminal region of the rat OTR is not involved in desensitization (Fig. 4A). The lack of desensitization was observed over a 10-fold range in OTR concentration (cells expressing wild type OTR versus those expressing the Δ51 mutation; Table 1). Cells expressing wild type OTR in concentrations that were comparable to that of the Δ51 mutant also showed no evidence of homologous desensitization (data not shown).

**OT-stimulated PGE\(_2\) Synthesis**—The addition of increasing concentrations of OT to CHO cells expressing wild type, and the Δ22, Δ39 mutant OTRs resulted in the release of PGE\(_2\) in a dose-dependent fashion, showing further that these receptors are functionally coupled to signal transduction pathways (Fig. 5). However, cells expressing the Δ51 mutant did not release PGE\(_2\) in response to OT (Fig. 5). Increasing the concentration of OT to 1 μM had no effect (data not shown). It would appear from these results that the region between 39 and 51 residues from the COOH terminus is involved in the ultimate generation of a PGE\(_2\) response. Within this region are two adjacent Cys residues (positions 351 and 352), which have been implicated in G protein interactions via palmitoylation (26–28). However, mutants (351, 352) were converted to serines.

The number of \(\text{[^{125}I]-OTA}\) binding sites per cell and apparent \(K_d\) values of the constructs shown in Fig. 1, stably expressed in CHO cells.

| Construct | Binding sites per cell (± S.E.) | \(K_d\) ± S.E. | \(IC_{50}\) (95% CL) |
|-----------|---------------------------------|----------------|---------------------|
| Wild type | 280,000 ± 81,000 | 213 ± 160 | 7.8 nM (6.2-10.7) |
| C351S,C352S | 244,410 ± 61,000 | 270 ± 270 | 10.1 nM (7.6-15) |
| Δ22 | 70,050 ± 12,560 | 32 ± 15 | 17.4 nM (11-42) |
| Δ39 | 130,100 ± 28,200 | 190 ± 175 | 6.7 nM (4.5-13) |
| Δ51 | 27,450 ± 11,300 | 110 ± 180 | 48.4 nM (26.7-260) |

**Fig. 4.** A, oxytocin-stimulated increases in intracellular Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}\]\)) in cells expressing wild type and mutant OTRs. To determine whether prior exposure of the cells to OT caused desensitization to a subsequent challenge, the cells were treated at 37 °C with a high dose of OT (200 nM) to stimulate an intracellular Ca\(^{2+}\) transient, and, after decay of the signal, the cells were rinsed at 37 °C for 10 min to dissociate bound OT. The cells were then exposed to 20 nM OT (except in the case of the Δ51 mutant, which required 200 nM), and \([\text{Ca}^{2+}\], was measured. There was no evidence of desensitization with either the wild type or mutant cells. Each point is the mean ± S.E. of 35 determinations. B, oxytocin dose-response curve using Δ51 cells. The lowest dose of OT stimulating an increase in \([\text{Ca}^{2+}\]) was about 175 nM, as opposed to the other cell lines, which responded to 10 nM OT (data not shown). Each point is the mean ± S.E. of 35 determinations.

**Fig. 3.** Competition by increasing concentrations of OT for \(\text{[^{125}I]-OTA}\) binding sites on wild type and mutant OTRs expressed in CHO cells. \(IC_{50}\) values and 95% confidence limits are shown in the inset.
As shown previously (38), OT (50 nM) causes the rapid (2 min) membrane fractions (see Ref. 38 for references). Because OT stimulated an increase in \([Ca^{2+}]_i\) in \(\Delta 51\) cells, we determined whether the inability of OT to stimulate \(PGE_2\) synthesis in these cells was the result of deficient ERK-2 phosphorylation. As shown previously (38), OT (50 nM) causes the rapid (2 min) phosphorylation of ERK-2 in CHO-OTR cells, as evidenced by the electrophoretic mobility shift of a fraction of total ERK-2 on immunoblots (pp42, Fig. 6A). Comparable results were obtained with each of the mutant OTRs after stimulation with 50 nM OT, with the exception of the \(\Delta 51\) mutant, which demonstrated only base-line phosphorylation (like OTR-negative CHO cells, Fig. 6A). To take into account the lower affinity of \(\Delta 51\) cells for OT, we used increased concentrations of OT and a more sensitive antibody assay that measures dually phosphorylated (S/T and Y) ERK-2. Concentrations of OT up to 1 \(\mu\)M had no effect on ERK-2 phosphorylation in \(\Delta 51\) mutant cells after 5 min of treatment (Fig. 6B). In contrast, 100 nM phorbol 12-myristate 13-acetate (PMA) was effective in stimulating ERK-2 phosphorylation in these cells (Fig. 6B). Treatment of the wild type and mutant cell lines and CHO cells with basic fibroblast growth factor (bFGF), 100 ng/ml for 5 min, resulted in ERK-2 phosphorylation in all of the cell lines (Fig. 6C), showing (along with the PMA results in Fig. 7B) that there is no impairment in ERK-2 phosphorylation in \(\Delta 51\) cells. bFGF stimulates ERK-2 phosphorylation by a G protein-independent mechanism (44).

**Evidence for OT-stimulated \([Ca^{2+}]_i\) Transients That Are Gq-independent—** \(G_q\)-mediated PLC activation in \(\Delta 51\) cells is not consistent with the ability of OT to elicit increases in \([Ca^{2+}]_i\), from intracellular stores on the one hand, and the inability of OT to stimulate \(PGE_2\) synthesis and ERK-2 phosphorylation on the other (38). To determine whether OT activates PLC in \(\Delta 51\) cells via \(G_q\) mediation, we measured two sequelae of \(G_q/PLC\) activity: inositol phosphates production and PKC activation. In these and subsequent experiments, a wild type clonal cell line expressing about the same number of OTRs as the \(\Delta 51\) was used. Treatment of cells expressing the full-length receptor with 500 nM OT for 30 min resulted in about a 5-fold increase in inositol phosphate (InsP) production (Fig. 7). In contrast, InsP production by \(\Delta 51\) cells was unchanged after OT treatment (Fig. 7). However, these cells were capable of being stimulated because sodium fluoroaluminate, a nonspecific G-protein activator, induced a significant increase in InsP production. Treatment of cells expressing full-length OTR with OT for 5 and 15 min resulted in the activation of PKCa, as measured by an increase in the amount of PKC associated with the membrane fraction (Fig. 8A). There was a barely detectable level of PKCa associated with the membrane fraction in \(\Delta 51\) cells in the basal state, but no increase following OT treatment (Fig. 8A). PKC was translocated from the cytosol to membrane fractions in \(\Delta 51\) cells after treatment with 100 nM PMA, indicating that the lack of effects of OT were not due to impaired PKC activation (Fig. 8B). The lack of OT activation of PKC in the \(\Delta 51\) cells is also consistent with the lack of effect of OT on ERK-2 phosphorylation and \(PGE_2\) synthesis, both of which are mediated by PKC (38).

The Presence of an Intracellular, InsP-independent Pathway in OT-stimulated \([Ca^{2+}]_i\) Release That Is Mediated by Pertussis toxin-sensitive \(G_{bg}\) and Tyrosine Kinase Activity—Treatment of \(\Delta 51\) and wild type OTR expressing cells with thapsigargin (250 nM) depleted intracellular stores of \(Ca^{2+}\) in the absence of...
extracellular Ca$^{2+}$, and inhibited the effects of OT on further increases in [Ca$^{2+}$], (Fig. 9A). The absence of InaP formation in the $\Delta S1$ cells indicates that InaP$_{\gamma}$-independent stores of Ca$^{2+}$ must be responsible for the OT-induced rise in [Ca$^{2+}$]. Cells expressing $\Delta S1$ and wild type OTRs were pretreated with selective inhibitors to determine whether the mutant G$_{i}\beta\gamma$-mediated pathways effect an intracellular Ca$^{2+}$ response to OT stimulation, and whether this process is mediated by tyrosine kinase activation. Pertussis toxin treatment (500 ng/ml for 16–20 h) completely obliterated the Ca$^{2+}$ response to OT in $\Delta S1$ expressing cells and reduced the OT-stimulated increase in wild type cells by more than 60% (Fig. 9B). Transfection of the cells with a plasmid expressing the G$_{\beta\gamma}$ sequestrant $\beta$ARK1ct (10 $\mu$g of DNA/6-cm dish, 24 h before addition of OT), completely blocked the effects of OT in $\Delta S1$ cells, as compared with cells transfected with the empty vector (Fig. 9C). However, the G$_{\beta\gamma}$ sequestrant had no effect on OT-stimulated [Ca$^{2+}$], in wild type cells (Fig. 9C). Preincubation of cells with increasing concentrations (1, 10, 100 $\mu$m) of the tyrosine kinase inhibitor, genistein, for 1 h before stimulation with OT resulted in the complete inhibition of the OT-induced [Ca$^{2+}$], transient in $\Delta S1$ cells, even at the lowest concentration (Fig. 9D). The OT response in wild type cells was reduced by more than 35% by treatment with 1 $\mu$m genistein). The inhibition was progressively greater with 10 and 100 $\mu$m genistein (Fig. 9D).

Phosphorylation of p38 MAP Kinase—To further analyze G protein-coupled pathways present in the $\Delta S1$ mutant, we examined p38 MAP kinase phosphorylation. Treatment of both wild type and $\Delta S1$ cells with 250 $\mu$m OT for 5 and 10 min resulted in increased phosphorylation of p38, as measured by immunoblotting with an antibody to dually phosphorylated p38 (Fig. 10). Although basal levels of p38 phosphorylation were elevated, the addition of OT further stimulated phosphorylation after 5 min. OT also caused an increase in p38 phosphorylation in cells expressing the wild type OTR after both 5 and 10 min (Fig. 10). Pretreatment of both mutant and wild type cell lines with oxytocin antagonist or pertussis toxin inhibited p38 phosphorylation (Fig. 10). Treatment of either cell type with PMA resulted in increased p38 phosphorylation (Fig. 10), indicating that both G$_{i}$ (pertussis toxin-sensitive) and G$_{q}$ (PKC-mediated) pathways are involved in activation of p38. G$_{q}$ is likely dissociated from the $\Delta S1$ OTR, but direct activation of PKC by PMA occurs downstream from OTR-G$_{i}$ coupling.

**DISCUSSION**

The COOH-terminal portion of GPCRs has been shown to be involved in a number of essential activities. Depending on the particular GPCR, these activities include G protein coupling (3–7), selectivity of G protein isotype (11, 11–14, 45), and homologous desensitization or internalization (15–25). Whereas truncation of some GPCRs has been shown to modify any one or more of its functions, shortening of the COOH terminus of other family members has no apparent effects (8–10). Thus, no uniform hypothesis regarding the importance of the COOH-terminal region can be applied a priori to the OTR, which has not been previously examined in detail (46, 47). In view of the high degree of homology of the COOH-terminal domain of OTRs from several species, we reasoned that conservation would be associated with some important function(s). However,
truncation of 22 and 39 residues from the COOH terminus or replacement of the Cys residues, which have been thought to be palmitoylation sites for the formation of a fourth intracellular loop, had no effect on OTR functions. These include ligand affinity, OT stimulation of increases in intracellular Ca\(^{2+}\), ERK-2 phosphorylation, and PGE\(_2\) synthesis. The mutants were also indistinguishable from the wild type OTR with respect to homologous desensitization.

Treatment of Δ51 cells with OT caused a Ca\(^{2+}\), transient, although higher concentrations of OT were required than with the other mutants or wild type receptor. This lower sensitivity of Δ51 cells is consistent with the lower affinity of the Δ51 receptor for OT. In contrast, no dose of OT (up to 1 \(\mu M\)) stimulated ERK-2 phosphorylation or PGE\(_2\) synthesis in the Δ51 cells. As we have shown previously, OT-stimulated ERK-2 phosphorylation and PGE\(_2\) synthesis are mediated by PKC (38). PKC activation occurs as a result of increased diacylglycerol synthesis, which is a product of the G\(_q\)/PLC pathway. Upon activation of cell surface receptors, PKCs translocate from the cytoplasm to membrane surfaces (48). We found that OT failed to activate PKC-\(\alpha\) translocation in the Δ51 mutant cells, but not in cells expressing the wild type OTR. The lack of PKC activation in Δ51 cells would account for the absence of OT-stimulated ERK-2 phosphorylation and PGE\(_2\) synthesis. The absence of PLC activation in the Δ51 cells accounts for the inability of OT to increase total InsP synthesis. Thus, the Ca\(^{2+}\) mobilizing effect of OT in Δ51 cells occurred independently of OTR-mediated G\(_i\)/PLC stimulation.

Our previous work, showing that wild type OTRs transfect into CHO cells are coupled to G\(_i\) as well as G\(_s\) (38), led us to consider whether the OT-stimulated increase in [Ca\(^{2+}\)]\(_i\) in Δ51 cells is mediated by G\(_i\) instead of G\(_s\). In Rat-1 and COS-7 cells, GPCRs coupled to pertussis toxin-sensitive G proteins mediate ERK-1/2 activation via a G\(_\beta\gamma\) subunit complex signal pathway that is dependent upon tyrosine phosphorylation and p21\(^{ras}\) activation (49, 50). Cellular expression of a specific G\(_\beta\gamma\) subunit sequester peptide derived from the carboxyl-terminal G\(_\beta\gamma\) subunit binding domain of the \(\beta\)-adrenergic receptor kinase 1, βARK1ct, was shown to inhibit LPA and \(\alpha_2\)-adrenergic receptor-mediated Shc phosphorylation in COS-7 cells (49). Δ51 cells were pretreated with either a G\(_i\) inhibitor (pertussis toxin), the G\(_i\) sequester (βARK1ct), or a tyrosine kinase inhibitor (genistein). All three agents inhibited OT-stimulated increases in [Ca\(^{2+}\)]\(_i\) in Δ51 expressing cells; pertussis toxin and genistein also reduced the effects of OT in wild type cells. Therefore, based on our findings, it would appear that the Δ51 mutant OTR is functionally coupled to G\(_i\) but lacks the coupling to G\(_s\). Both G\(_i\) and G\(_s\) coupling to the OTR occurs in cells expressing the wild type OTR. We would extrapolate from the results that truncation of the COOH-terminal tail by 22 or 39 residues, and mutating residues 351 and 352 (Cys → Ser) do not affect G\(_i\) coupling.

Because OT-stimulated ERK-2 phosphorylation in CHO-OTR cells is largely mediated by G\(_i\), we examined pertussis toxin-sensitive p38 MAP kinase phosphorylation as an indicator of a G\(_i\)-mediated process. This pathway has been shown to be activated upon stimulation of both G\(_\alpha\)Q11-coupled m1 and G\(_i\)-coupled m2 muscarinic-acetylcholine receptors (51). Overexpression of G\(_\beta\gamma\) or a constitutively activated mutant of G\(_\alpha\)Q11, but not G\(_\alpha\)S, also stimulated p38 kinase activity (51). p38 kinase phosphorylation was stimulated by OT in Δ51-expressing cells by a process that was completely inhibited by pertussis toxin. Because PMA also stimulated p38 phosphorylation in these cells, it is apparent that both G\(_i\) and G\(_s\) mediate p38 kinase activity. Nagao et al. (52) showed that G\(_i\)Q11 stimulates p38 MAP kinase activity through PKC and Src family kinase-dependent pathways. These findings suggest that PMA stimulation of p38 phosphorylation in Δ51 cells occurs through PKC activation of tyrosine kinase-regulated steps. Parenthetically, the present findings are the first observations of an effect of OT on p38 phosphorylation.

Pathways connecting G\(_i\) activation and intracellular Ca\(^{2+}\) transients in Δ51 cells are not currently known. Depletion of intracellular Ca\(^{2+}\) stores with thapsigargin, an inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPase activity, resulted in the loss of the Ca\(^{2+}\)- transient following OT stimulation of Δ51 cells. G\(_i\)Q11-Adenoreceptors utilize two different G\(_\alpha\) subunits to increase [Ca\(^{2+}\)]\(_i\) in rat myocytes (53). G\(_\alpha\)Q11 appears to activate InsP production and induce the release of Ca\(^{2+}\) from intracellular stores, while G\(_\alpha\)Q11 may enhance the Ca\(^{2+}\)-activated Ca\(^{2+}\) influx that replenishes intracellular Ca\(^{2+}\) stores (53). This mechanism does not appear to occur with the OTR, as both the pertussin toxin-sensitive and insensitive pathways involved intracellular Ca\(^{2+}\) stores. If G\(_\beta\gamma\)-stimulated tyrosine kinases resulted in activation of PLC-\(\gamma\), we would have expected to find a rise in inositol phosphates in the Δ51 cells after OT treatment. G\(_\beta\gamma\) activation of PLC-\(\beta\) isoforms to stimulate PtdInsP2 hydrolysis independent of protein tyrosine kinases (54) also appears to be absent in Δ51 cells. Previous work from this laboratory demonstrated that OT inhibited (Ca\(^{2+}\) + Mg\(^{2+}\)) ATPase activity in sarcolemmal membranes from the rat uterine myometrium (55). It was postulated that inhibition of the calcium pump would hinder the extrusion of intracellular Ca\(^{2+}\), thus allowing OT-stimulated elevations in [Ca\(^{2+}\)]\(_i\) to be maintained. Although mechanisms coupling the OTR and (Ca\(^{2+}\) + Mg\(^{2+}\))ATPase have not been described, it is unlikely that this pathway accounts for [Ca\(^{2+}\)]\(_i\) increases in Δ51 cells because the source of Ca\(^{2+}\) in Δ51 cells was thapsigargin-sensitive.

Mobilization of Ca\(^{2+}\) from intracellular stores is mediated by three major receptors on the endoplasmic reticulum, the InsP\(_3\), ryanodine, and sphingosine-1-phosphate systems. Although we measured total InsPs to ensure that transient increases in InsP\(_3\) would not go undetected; we were unable to detect any increase after OT treatment of Δ51 cells. These results indicate that the increase in Ca\(^{2+}\) in Δ51 cells caused by OT is not mediated by the InsP3 receptor. We did not study the effects of ryanodine on [Ca\(^{2+}\)], release, as the ryanodine receptor/Ca\(^{2+}\) release channel, which is an essential component of excitation-contraction coupling in striated muscle cells, is not found in any significant concentration in CHO cells (56, 57). Sphingosine-1-phosphate has been shown to release Ca\(^{2+}\) from the endoplasmic reticulum in several cell types in conjunction with occupancy of surface IgG receptors (58–61). Sphingosine-1-phosphate biosynthesis is catalyzed by sphingosine kinase (62, 63), a ubiquitous enzyme found in the cytosol (64, 65) and the endoplasmic reticulum (58). However, addition of the sphingosine analogue, \(\beta\)-three-dihydrosphingosine, which is a competitive inhibitor of sphingosine kinase activity (60), had no effect on OT-induced Ca\(^{2+}\)-transients (data not shown). Thus, the signals mediating the release of Ca\(^{2+}\) from intracellular stores are not known at the present time.

Residues in the N-terminal part of the COOH terminus of the human V\(_2\) vasopressin receptor have been shown to be necessary for correct folding; the COOH-terminal residues are also important for efficient cell surface expression (66). Although the reduced affinity of the Δ51 mutant for OT could be a result of its dissociation from G\(_q\), it might be due instead to modified protein folding as has been indicated for the V\(_2\) vasopressin receptor. The distinction is important because if G\(_i\)-coupled OTR has a higher affinity for OT than the G\(_s\)-coupled form, the preferred pathway for OT-stimulated increases in [Ca\(^{2+}\)]\(_i\), with low concentrations of OT would be through PLC.
activation and InsP₃-mediated stimulation of Ca²⁺ release from intracellular stores. However, because pertussis toxin and genistein substantially inhibited the OT-stimulated increase in [Ca²⁺]ᵢ in wild type cells, the OTR-associated Gi/γ protein tyrosine kinase pathway appears to be as important as the Gₛ-PLC pathway for increasing [Ca²⁺]ᵢ. Of 30 stable clones of Δ51 examined, only the one used in these studies had 125I-OTA binding activity. These results suggest that processing of the Δ51 mutant protein is impaired.

The results of our studies are summarized in Fig. 11. The model postulates that, in addition to elements of the third intracellular loop, the proximal portion of the COOH-terminal domain of the OTR is required for coupling to Gi. In contrast, coupling to Gi occurs without participation of the COOH-terminal domain of the OTR is coupled to Gi, but not Gₛ. As a result, Δ51 cells respond to OT with an increase in [Ca²⁺]ᵢ and p38 phosphorylation, but lack InsP formation, PKC translocation, ERK-2 phosphorylation, and PGE₂ synthesis.
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