Characterization of receptors mediating AVP- and OT-induced glucagon release from the rat pancreas

Yibchok-Anun, Sirintorn, Henrique Cheng, Patricia A. Heine, and Walter H. Hsu. Characterization of receptors mediating AVP- and OT-induced glucagon release from the rat pancreas. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E56–E62, 1999.—We characterized the receptors that mediate arginine vasopressin (AVP)- and oxytocin (OT)-induced glucagon release by use of a number of antagonists in the perfused rat pancreas and the fluorescence imaging of the receptors. AVP and OT (3 pM-3 nM) increased glucagon release in a concentration-dependent manner. The antagonist with potent V1b receptor-blocking activity, CL-4–84 (10 nM), abolished AVP (30 pM)-induced glucagon release but did not alter OT (30 pM)-induced glucagon release. d(CH2)5[Tyr(Me)2]AVP (10 nM), a V1a receptor antagonist, and L-366,948 (10 nM), a highly specific OT-receptor antagonist, failed to inhibit AVP-induced glucagon release. In contrast, L-366,948 (10 nM) abolished OT (30 pM)-induced glucagon release but did not change the effect of AVP. Fluorescent microscopy of rat pancreatic sections showed that fluorescence-labeled AVP and OT bound to their receptors in the islets of Langerhans and that the bindings were inhibited by 1 μM of CI-4–84 and L-366,948, respectively. Because AVP and OT at physiological concentrations (3–30 pM) increased glucagon release, we conclude that AVP and OT increase glucagon release under the physiological condition through the activation of V1b and OT receptors, respectively.

L-366,948; V1b receptor; oxytocin receptor; perfusion; fluorescence

ARGININE VASOPRESSIN (AVP) and oxytocin (OT) are synthesized in the hypothalamus and secreted from the posterior pituitary gland. AVP and OT are also found in various tissues, including ovary, oviduct, follicular fluid (22), adrenal (3), testis (14), thymus (11), and pancreas (2). In addition to the regulation of fluid homeostasis, AVP induces glycogenolysis (15), proliferation of the pituitary gland (20) and vascular smooth muscle cells (24), vasoconstriction (8), and the secretion of catecholamines (10), glucagon, and insulin (6). The major physiological functions of OT are to regulate milk ejection and uterine contractions, but it also increases adrenocorticotropic hormone (ACTH) (23), glucagon, and insulin release (6). A small concentration of 20 pg/ml of AVP and OT increased glucagon release, but not insulin release, from the perfused rat pancreas (6). Moreover, both AVP and OT elicited a concentration-dependent stimulation of glucagon release but failed to influence insulin release from rat islets (5). A high density of [3H]oxytocin binding was present in the periphery of the islets of Langerhans that corresponded to the location of pancreatic a-cells (25). Together, these findings suggest that AVP and OT may play a physiological role in increasing glucagon release.

AVP receptors have been classified into V1a, V1b, and V2 receptors. V1a receptors mediate glycogenolysis (15) and vasoconstriction (8); V1b receptors mediate the release of ACTH (4), catecholamines (10), insulin (17), and glucagon (29); and V2 receptors mediate antidiuresis (13).

A number of receptor antagonists have been used to pharmacologically characterize the receptors that mediate the effects of AVP and OT in many cells and tissues (18), including insulin and glucagon-secreting cells. AVP and OT can cross-react with each other’s receptors; for example, both AVP and OT induce insulin release through V1b receptors in the perfused rat pancreas and the clonal beta cell line RINm5F (17). In addition, both hormones induce ACTH release through V1b receptors in the rat adenohypophysis (4, 23), and lysine vasopressin stimulates porcine myometrial contractions through OT receptors (30). Similarly, in our previous study, AVP and OT induced glucagon release by activating V1b receptors in clonal alpha-cells InR1-G9 (29).

In this study, we characterized the receptors that mediate AVP- and OT-induced glucagon release by using the antagonists that block V1a, V1b, and OT receptors, respectively, from the perfused rat pancreas. In addition, we used fluorescence-labeled vasopressin (VP) and OT as ligands to detect AVP and OT receptors in the rat islets. Fluorescence-labeled peptides have been used to study AVP receptors; for example, fluorescence-labeled AVP analogs have been used to study V1a receptors (12) and V2 receptors (21). From the results of the present study, we conclude that AVP and OT play a physiological role in increasing glucagon release through V1b and OT receptors, respectively.

MATERIALS AND METHODS

Test agents. AVP, OT, d(CH2)5[Tyr(Me)2]AVP, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical (St. Louis, MO). Phenylacetyl-o-Tyr(Me)3Arg9,Lys9-amide-vasopressin (Fluo-VP) and fluo-Lys5-oxytocin (Fluo-OT) were purchased from Advanced Bioconcept (Quebec, Canada). Pentobarbital sodium was purchased from Fort Dodge Laboratories (Fort Dodge, IA). 4-OH-phenacyl-o-Tyr(Me)-Phe-Gln-Asn-Pro-Arg-NH2 (CL-4–84) was donated by Dr. Maurice Manning of Medical College of Ohio (Toledo, OH). Cyclo-(L-Pro-D-2-naphthyl-Ala-Ile-d-pipecolic acid-L-pipecolic acid-d-His) (L-366,948) was donated by Merck Research Laboratories (West Point, PA). 125I-labeled glucagon...
was purchased from Linco Research (St. Charles, MO). Glucagon antibody was donated by Dr. Joseph Dunbar of Wayne State University (Detroit, MI), and glucagon standard was donated by Eli Lilly Laboratories (Indianapolis, IN).

Pancreatic perfusion. Male Sprague-Dawley rats weighing 500–650 g were used in the dose-response experiments, and rats weighing 220–350 g were used in the receptor antagonism experiments. All the rats were born and grown in our facilities (Laboratory Animal Resource). They were maintained at 22°C, 40–60% humidity, and a 12:12-h light-dark cycle. The rats were fed ad libitum with Purina chow. The in situ rat pancreatic perfusion with an open system was performed during the daytime as previously described (27). Briefly, the rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and were maintained at 37°C on a hot plate during the experiment. The celiac arteries were cannulated with polyvinyl tubing (0.625 mm ID); then the pancreata were immediately perfused with the Krebs-Ringer bicarbonate buffer (KRB) supplemented with 20 mM HEPES, 5.5 mM glucose, 1% dextran, and 0.2% BSA as a basal medium. The KRB was continuously aerated with 95% O2-5% CO2 at pH 7.4. The perfusion rate was 1 ml/min, and the effluent fluid from the portal vein, which was cannulated with a vinyl tubing (1.12 mm ID), was −1 ml/min. The rats were euthanized immediately after the placement of cannulas and the beginning of the flow. After an equilibration period of 20 min, the effluent fluid was collected every minute. For the dose-response experiments, after the baseline period of 10 min, the perfusate containing AVP or OT (3 pM–3 nM) was administered for 10 min followed by a washout period with the basal medium for 10 min. For the antagonism experiments, after the baseline period of 5 min, the pancreas was pretreated for 10 min with the medium containing one of the three antagonists: CL-4–84 (1, 3, or 10 nM), an antagonist with potent V1b blocking activity (26); d(CH2)5[Tyr(Me)2]AVP (10 nM), a V1a receptor antagonist (19); and L-366,948 (1, 3, or 10 nM), a highly selective OT receptor antagonist (27). This was followed by the medium containing AVP or OT (30 pM) and an antagonist for 10 min, and the basal medium for another 10 min for the washout period. The perfusate containing arginine (1 mM) was administered as a positive control for 5 min at the beginning of all experiments. The effluent fractions were kept at 4°C and subsequently assayed for glucagon by use of radioimmunoassay, following the procedures provided by Linco Research.

Fluorescence imaging of AVP and OT receptors in pancreatic islets. The rat pancreas was perfused with KRB, as described in Pancreatic perfusion, for 5 min to eliminate the blood inside the pancreas. The perfusion rate was set at 3 ml/min. The pancreas was then collected and cut into small pieces (3 × 3 mm2) and frozen in −80°C isopentane. The frozen tissue was sliced into 17- to 20-μM thickness, mounted on poly-L-lysine-coated slides, and kept at −20°C until use. The tissue sections were processed following a protocol provided by the manufacturer (Advanced Bioconcept). Briefly, the frozen tissue sections were preincubated in an incubation buffer (50 mM Tris·HCl, 10 mM MgCl2, 1% BSA, 1 mg/ml bacitracin, and 0.5 mM PMSF, pH 7.4) containing CL-4–84 (1 or 10 μM), L-366,948 (1 or 10 μM), d(CH2)5[Tyr(Me)2]AVP (10 μM), and AVP (10 μM) or OT (10 μM) at 4°C for overnight and incubated with the incubation buffer containing 30 nM of Fluo-VP or Fluo-OT in the absence or presence of an antagonist or unlabeled AVP or OT, as indicated in Pancreatic perfusion, at room temperature for 1 h. After incubation, the sections were washed 4 times for 60 s in a cold rinsing buffer (50 mM Tris·HCl and 10 mM MgCl2, pH 7.4) at 4°C and air-dried in the dark under a cool stream of air. The fluorescence bindings were visualized using a fluorescent microscope (Leica DMLB; Leica Microsystems, Heerbrugg, Switzerland), and photographs were taken with a 20X lens using the Leica MPS 60-MPS 30 photographic system.

Data expression and statistical analysis. The effluent concentrations of glucagon were expressed as a percentage of the baseline level (mean of last 5 baseline values) in means ± SE. The area under the curve (AUC) for the 10-min treatment period was calculated using Transforms and Regressions (SigmaPlot 4.0; SPSS, Chicago, IL). In dose-response experiments, the AUC was expressed as a percentage of the area of the basal control group. In antagonism experiments, the AUC was expressed as a percentage of the area of the AVP or OT control group. Data were analyzed using analysis of variance (ANOVA) to determine the effect of treatment. Fisher’s least significant difference test was used to determine the difference between means for which the ANOVA indicated a significant (P < 0.05) F ratio.

RESULTS

The results in Figs. 1 and 2 show the profile of glucagon release to AVP and OT (3 pM–3 nM), respectively, together with the basal control profile, which was obtained by perfusion with KRB alone for 40 min. AVP and OT (3 pM–3 nM) increased glucagon release from the perfused rat pancreas in a concentration-dependent manner. Both peptides increased glucagon release in a biphasic pattern: a peak followed by a sustained phase or a second peak (for 3 nM AVP and OT), in which the peak was initiated in <1 min and reached the maximum within 2 min. AVP (3 pM–3 nM) induced a maximum increase in glucagon release by 2.5, 8, 12, and 10-fold, respectively, over the basal control group. The sustained glucagon release induced by AVP (3–300 pM) was ~2- to 3-fold that of the basal control group, and the second peak of glucagon release induced by 3 nM AVP was 9-fold over that of the basal control group (Fig. 1). At the highest concentration of AVP studied (3 nM), the flow rate in the portal vein was 2- to 3-fold that of the basal control group (Fig. 2). At the highest concentration of OT studied (3 nM), the flow rate in the portal vein was increased by 10.2 ± 0.3 fold.

![Figure 1](http://ajpendo.physiology.org/Downloadedfrom/) (1 of 3)

**Fig. 1.** Effect of arginine vasopressin (AVP, 3 pM–3 nM) on glucagon release to AVP and OT (3 pM–3 nM), respectively, together with the basal control profile, which was obtained by perfusion with KRB alone for 40 min. AVP and OT (3 pM–3 nM) increased glucagon release from the perfused rat pancreas in a concentration-dependent manner. Both peptides increased glucagon release in a biphasic pattern: a peak followed by a sustained phase or a second peak (for 3 nM AVP and OT), in which the peak was initiated in <1 min and reached the maximum within 2 min. AVP (3 pM–3 nM) induced a maximum increase in glucagon release by 2.5, 8, 12, and 10-fold, respectively, over the basal control group. The sustained glucagon release induced by AVP (3–300 pM) was ~2- to 3-fold that of the basal control group, and the second peak of glucagon release induced by 3 nM AVP was 9-fold over that of the basal control group (Fig. 1). At the highest concentration of AVP studied (3 nM), the flow rate in the portal vein was increased by 10.2 ± 0.3 fold.
decreased by ~20%, presumably because of vasoconstriction, but the glucagon response was not delayed or reduced to any extent. The OT (3 pM-3 nM)-induced maximum increases in glucagon release were 3, 7, 14, and 11-fold, respectively, over that of the basal control group. The sustained glucagon release by 3–300 pM OT was 2-fold, and the second peak induced by 3 nM OT was 4-fold that of the basal control group (Fig. 2). The effluent glucagon concentrations returned to the baseline on removal of AVP and OT (during the washing period) and increased to ~5- to 14-fold of the baseline value on administration of 1 mM arginine. By comparison of the AUCs, there were no significant differences between AVP and OT (3–300 pM)-induced glucagon release. At 3 nM, AVP-induced glucagon release was significantly different from that of OT. However, the difference was only in the sustained phase (Fig. 3). The EC50 of OT was $8.9 \pm 2.9$ pM, and the EC50 of AVP was estimated to be $25.1 \pm 11.3$ pM, because the maximum glucagon release was not acquired in the AVP dose-response experiment.

AVP and OT at 30 pM were used in the antagonism experiments because of the submaximal increase in glucagon release by the two peptides. At 30 pM, AVP and OT induced about four- and twofold increases in the peak and the sustained phases, respectively, compared with the basal control group. CL-4–84 (1, 3, and 10 nM), an antagonist with V1a/V1b blocking activity, inhibited AVP (30 pM)-induced glucagon release in a concentration-dependent manner (Fig. 4). By comparison of the AUCs, CL-4–84 (3 and 10 nM) significantly reduced AVP-induced glucagon release with an IC50 of 2.2 ± 0.1 nM. Pretreatment with CL-4–84 (10 nM) abolished AVP-induced glucagon release and even lowered glucagon to the levels below the baseline. However, d(CH2)5[Tyr(Me)2]AVP (10 nM), a V1a receptor antagonist, and L-366,948 (10 nM), a highly specific OT receptor antagonist, failed to inhibit AVP-induced glucagon release (Fig. 5). In contrast, L-366,948 (1, 3, and 10 nM) inhibited OT (30 pM)-induced glucagon release in a concentration-dependent manner (Fig. 6). By comparison of the AUCs, 3 nM L-366,948 significantly lowered, and 10 nM L-366,948 abolished OT-induced glucagon release. The IC50 of L-366,948 was $3 \pm 0.3$ nM. CL-4–84 (10 nM), the receptor antagonist with V1a/V1b blocking activity, did not significantly reduce OT (30 pM)-induced glucagon release (Fig. 7). None of the receptor antagonists alone significantly changed glucagon release.

The results in Figs. 8 and 9 show the fluorescence imaging of AVP and OT receptors in the rat islets. Fluorescent microscopic examination of the pancreatic
sections incubated with either Fluo-VP or Fluo-OT revealed selective fluorescence labeling of AVP and OT receptors expressed in the rat pancreatic islets (Figs. 8B and 9B) compared with the negative control (Figs. 8A and 9A). The binding was specific because the fluorescence was no longer detectable when the incubation was performed in the presence of 10 µM VP or OT (Figs. 8C and 9C). The fluorescence labeling of Fluo-VP was selective for V1b receptors because it was blocked by preincubation of the tissue sections with 1 µM CL-4–84 (Fig. 8D), but not by 10 µM L-366,948 (Fig. 8E) or 10 µM d(CH₂)₅[Tyr(Me)²]AVP (Fig. 8F). The fluorescence labeling of Fluo-OT was selective for OT receptors expressed in the rat pancreatic islets, because it was blocked by preincubation of the tissue sections with 1 µM L-366,948 (Fig. 9D) but not by 10 µM CL-4–84 (Fig. 9E).

DISCUSSION

In the present study, AVP and OT (3 pM-3 nM) evoked glucagon release from the perfused rat pancreas in a concentration-dependent manner, in which AVP and OT at 3 and 30 pM increased glucagon release by about three- and eightfold, respectively. These findings indicated that AVP and OT may physiologically have increased glucagon release, because the concentrations of AVP and OT studied (3 and 30 pM) are similar to the plasma concentrations of AVP (3–20 pM) (9) and OT (8–25 pM) in the rat (16). This statement is supported by the findings that a neural lobe extract evoked glucagon release (5) and a rise in plasma glucagon concentrations of the rats subjected to hemorrhage that was found to be mediated by an increase in the release of AVP and OT (7). In addition, AVP and OT are present in human and rat pancreatic extracts, suggesting that both peptides are synthesized in the pancreas and thus could exert a paracrine function on pancreatic hormone release (2).

The increase in glucagon release mediated by AVP and OT (30 pM) was higher in the larger rats (500–650 g, ~1 yr old, used in the dose-response experiments) than in the smaller rats (220–350 g, 2–3 mo old, used in the antagonism experiments). We speculate that the pancreata of the larger (or older) rats express more V1b and OT receptors or have a more active signal transduction system for these receptors than the smaller (or younger) rats. More work is needed to find out why
these peptides evoke more glucagon release in larger (older) rats than in smaller (younger) rats.

CL-4–84 is an antagonist with high affinity for both $V_{1a}$ ($K_i = 0.45 \pm 0.04 \text{ nM}$) and $V_{1b}$ ($K_i = 2.2 \pm 0.1 \text{ nM}$) receptors (26). It is also a weak OT antagonist [$\text{agonic affinity (pA}_2 = 7.38 \pm 0.06$] (19). $d\text{(CH}_2\text{)}_5\text{Tyr(Me)}_2\text{AVP}$ is a potent and selective $V_{1a}$ receptor antagonist ($\text{pA}_2 = 8.62$) (18). L-366,948 is a highly selective OT receptor antagonist, which is >400 times more selective for OT receptors than for $V_{1a}$ and $V_{2}$ receptors (27). We also confirmed the results from pancreatic perfusion by detecting these receptors by use of fluorescence labeling with VP and OT. We found that Fluo-VP and Fluo-OT selectively bound to $V_{1b}$ and OT receptors, respectively, in the rat islets. The labels of Fluo-VP and Fluo-OT were seen in the entire islets, an observation suggesting that both $V_{1b}$ and OT receptors are expressed in pancreatic $\alpha$- and $\beta$-cells, among others. In addition, in perfused rat pancreata, we found that 0.3 nM OT increased insulin release about threefold over the basal insulin level and that this increase was antagonized by 3 nM L-366,948 (unpublished data). The present finding is different from the previous one from our laboratory, in which 100 nM OT induced insulin release from rat perfused pancreas by activating $V_{1b}$ receptors (17). Moreover, in an autoradiographic binding study of the rat pancreas, a high density of [H]oxytocin binding was found in the periphery of the islets, which corresponded to the localization of $\alpha$-cells (25). Thus the lower concentration of OT (0.3 nM) may induce insulin release by activating OT receptors, whereas the higher concentration of OT (100 nM) may induce insulin release by activating $V_{1b}$ receptors (17).

Our present findings suggest that AVP evokes glucagon release by activating $V_{1b}$ receptors in $\alpha$-cells of the rat pancreas, which is similar to AVP-induced ACTH release from the rat adenohypophysis (4), catecholamine release from the rat adrenal medulla (10), and glucagon release from the hamster glucagonoma IN-R1-G9 cells (29). However, in the perfused rat pancreas, OT evoked glucagon release by activating OT but not AVP receptors. In the dog, OT has been shown to increase plasma levels of glucose, insulin, and glucagon and to increase the rate of glucose production and uptake by activating OT receptors (1). These results differ from those of ours in IN-R1-G9 cells, in which OT increased glucagon release through $V_{1b}$ receptors (29). In addition, AVP and OT increased ACTH release through $V_{1b}$ receptors (4, 23). Although AVP and OT
induced glucagon release by activating different receptors, our preliminary data showed that there was no synergism between these two peptides (unpublished data). The action of AVP on glucagon release exerted an inverse relationship with glucose concentrations; in the presence of 1.4 mM glucose, AVP (3 pM)-induced glucagon release was significantly higher than that in the presence of 5.5 mM glucose (unpublished data).

By comparison of the responses at the same concentration of AVP and OT, the potencies of both peptides were similar in the perfused rat pancreas, with the exception that 3 nM of AVP evoked a significantly higher increase in glucagon release than 3 nM of OT. This finding differs from that of our previous study, in which OT-induced glucagon release in In-R1-G9 cells was ~30-fold less potent than AVP (29). Apparently, OT receptors are not expressed in clonal In-R1-G9 α-cells. We also confirmed these findings by detecting AVP and OT receptors in In-R1-G9 cells by use of fluorescence-labeled VP and OT. We found that 30 nM of both Fluo-VP and Fluo-OT bound to V1b receptors on the cell membrane because the bindings were blocked by 30 nM CL-4–84 but not by 300 nM L-366,948 or d(CH2)5[Tyr(Me)2]AVP (unpublished data). OT, therefore, increases glucagon release from In-R1-G9 cells by activating V1b receptors (29). Based on these findings, we conclude that In-R1-G9 cells are not an adequate model for the study of OT-induced glucagon release.

Our present findings suggest that AVP and OT increase glucagon release under the physiological condition by activating V1b and OT receptors, respectively. Because specific V1b receptor antagonists are currently unavailable for the characterization of these receptors, further studies utilizing molecular approaches are warranted to confirm our present findings.

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REFERENCES


