Endocrine (1995) 3, 55-59

Physiological concentrations of oxytocin powerfully stimulate insulin secretion *in vitro*

E. Bobbioni-Harsch[1], S. Frütiger[2], G. Hughes[2], M. Panico[3], A. Etienne[3], F. Zappacosta[3], H.R. Morris[3] & B. Jeanrenaud[1]

[1]*Laboratories de Recherches Métaboliques of the Geneva Faculty and Department of Medicine, Geneva;* [2]*Département de Biochimie Médicale, Centre Médical Universitaire, Geneva;* [3]*Wolfson Laboratories Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK*

The effect of natural oxytocin on insulin secretion was investigated by using isolated, perfused rat pancreases. Oxytocin produced a dose-dependent stimulation of insulin secretion starting with a concentration as low as 2.3 pM and with a maximal effect obtained at 66 pM. Specific oxytocin antagonist, desGly-NH_2^9, $d(CH_2)_5$ [Tyr (Me)^2, Thr^4] OVT, reduced by 70% the stimulatory effect of 66 pM natural oxytocin. A specific oxytocin receptor agonist OH(Thr^4, Gly^7)OT showed an insulinotropic action similar to equivalent amounts of oxytocin. Replacement or modifications of Q_4, L_8 or the NH_2 terminal group in the oxytocin molecule reduced or abolished the biological activity. This study demonstrated that: (1) in normal rat pancreas, oxytocin stimulates insulin secretion at concentrations similar to those present in the plasma; (2) oxytocin exerts this secretagogue action in presence of basal physiological glucose levels (5 mM); (3) oxytocin stimulates insulin secretion by interacting with its own receptor. A potential role for oxytocin as an insulin-releasing hormone is thus conceivable.

Keywords: oxytocin; insulin; rat; pancreas

Introduction

Several laboratories (Idahl & Martin, 1971; Beloff-Chain *et al.*, 1983; Bobbioni-Harsch & Jeanrenaud, 1989) have suggested that a part of the control exerced by the central nervous system on insulin secretion is mediated by the release, from the hypothalamus or the hypophysis, of an insulin secretagogue reaching the pancreas through the blood stream. In previous studies (Bobbioni & Jeanrenaud, 1982, 1983), we demonstrated that partially purified hypothalamic extracts could stimulate insulin secretion both *in vivo* and *in vitro*. The complete purification of the extracts showed that oxytocin (OT) was responsible for this insulinotropic effect (data not shown). This result was in agreement with recent studies that clearly demonstrated an insulin stimulatory action of both OT and Vasopressin (AVP). In fact, isolated mouse islets (Gao *et al.*, 1990, 1991) perifused in presence of 15 mM glucose concentration, showed a dose-dependent increase in insulin output when challenged with OT or AVP (from 1 to 100 nM). An SV 40-transformed beta-cell line (HIT) was also demonstrated to increase insulin secretion in response to OT or AVP (Richardson *et al.*, 1990). In this case, however, OT was 100 times less powerful than AVP and the minimal OT concentration required to produce a significant effect on insulin secretion was 10 nM. As the concentrations of OT and AVP used in the studies just mentioned were largely (between 100 and 10 000 times) higher than the physiological plasma levels, the authors estimated unlikely that the β-cells of the islets of Langerhans could be influenced by circulating OT or AVP and, therefore, proposed a paracrine role for these neuropeptides in the control of insulin secretion.

In order to assign to OT a potential role as insulin-releasing hormone, it remained to demonstrate: (1) the existence of an insulin stimulating effect of the natural OT at concentrations similar to the ones physiologically measured in the plasma; (2) the specific mediation of the OT insulinotropic action by its own receptor. These were the aims of the present study in which isolated, perfused pancreases from normal rats were used because this *in vitro* model best preserves the vascular structures and the anatomical organization of the islets, and therefore allows for experimental conditions as close as possible to the physiological ones.

Correspondence: E. Bobbioni-Harsch
Received 5 July 1994; accepted 20 September 1994

Results

Separation of natural Oxytocin and evaluation of its purity

Natural OT was obtained from hypophysial extract eluted on the gradient system described in Materials and methods section. The content and the purity of the product were verified by amino acid composition, sequence and mass analysis. Both amino acid composition and sequence were consistent with the one of OT and mass analysis showed a pure spectrum with an intense quasimolecular ion $(M + H)^+$ at M/Z 1007 corresponding to the normal mass of OT. Mass analysis is, of course, not diagnostic for optical isomerism, but no covalent modifications were observed. Finally, the amount of natural OT obtained after purification was evaluated by RIA measurement.

Characterization of natural and synthetic oxytocin insulin secretion stimulating activity

As shown in Figure 1 (full circles) and Table 1, natural oxytocin administration produced a dose-related stimulation of insulin secretion from isolated, perfused rat pancreases, in the presence of a low (5 mM) glucose concentration in the perfusion medium. The lowest

natural oxytocin concentration able to evoke a significant increase in insulin output (i.e. from 12.6 ± 1.4 to 13.7 ± 1.5 ng/5 min, $n = 5$, $P < 0.05$) was 2.3 pM. The maximal effect on insulin secretion (i.e. a 871 ± 67% increase in insulin secretion, $n = 8$) was observed at 66 pM natural oxytocin concentration; higher amounts of peptide (i.e. 160 and 320 pM) did not significantly further increase the effect on insulin output. The half maximally effective concentration was evaluated to be at 32 pM.

As shown in Figure 1 (open circles) and Table 1, synthetic oxytocin, as provided by the manufacturer without further purification, also evoked insulin secretion, when infused into isolated, perfused rat pancreas. However, the overall dose-effect curve obtained with the synthetic peptide was shifted to the right when compared to that obtained with the natural peptide. In fact, the lowest synthetic oxytocin concentration able to significantly stimulate insulin secretion (i.e. from 11.4 ± 2.3 to 14.9 ± 2.4 ng/5 min, $n = 4$, $P < 0.05$) was 6.6 pM, the half maximally effective concentration was evaluated to be at 58 pM and the maximal effect (i.e. a 918 ± 126% increase in insulin secretion, $n = 12$) was obtained at 280 pM oxytocin concentration.

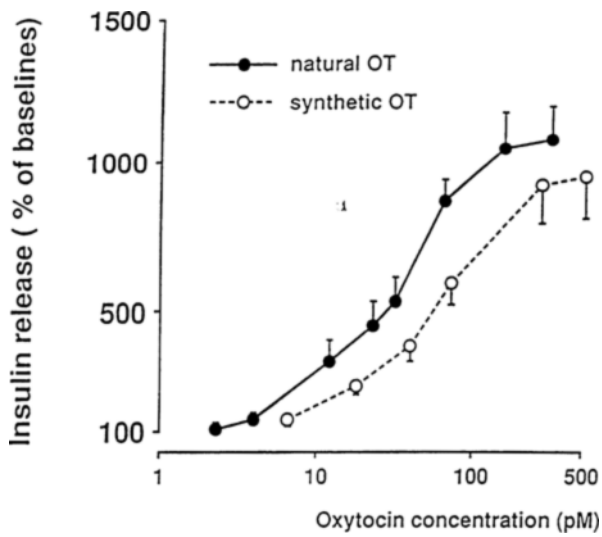

Figure 1 Natural (●) and synthetic (○) oxytocin dose-response curve on insulin output. Insulin release, measured during the 5 min infusion of natural or synthetic oxytocin at different concentrations, is expressed as a percent of basal insulin output, measured during a 5 min period preceding each infusion. (Mean of 4–12 exp. ± SEM)

Effect of oxytocin antagonist, agonist and analogs

Oxytocin antagonist, desGlyNH$_2^9$,d(CH$_2$)$_5$,[Tyr(Me)^2 Thr^4]OVT, was infused to a final concentration of 6.6 nM. The antagonist administration started 10 min before the beginning of the test. After this period, a 5 min basal sample was collected: no effect of antagonist infusion on basal insulin release was observed. Natural oxytocin was then infused to a final concentration of 66 pM. As shown in Table 2, when oxytocin (66 pM) was infused, in the presence of the antagonist (6.6 nM), insulin secretion was increased to only 240 ± 40% of baseline while oxytocin alone increased insulin output to 870 ± 70%, indicating a significant inhibitory effect of oxytocin antagonist on oxytocin-induced insulin output.

Oxytocin agonist, OH(Thr^4,Gly^7)OT infused at 66 pM concentration produced an increase in insulin secretion similar to the one measured after administration of equivalent dose of OT (Table 2). Oxytocin free acid (i.e. desamidated oxytocin) infused at 66 pM concentration, was without effect on insulin secretion (Table 2), as was 66 pM Isotocin (i.e. oxytocin analog in which Q_4 is replaced by S and L_8 by I). Mesotocin (where L_8 is replaced by I), when infused at 66 pM concentration, showed a significantly lower capacity to stimulate insulin secretion, when compared to that of equimolar amounts of oxytocin (Table 2).

Discussion

Oxytocin has been studied for its effect on insulin secretion by many investigators. When administered *in vivo*, OT enhances both glucose and glucagon plasma

Table 1 Natural and synthetic oxytocin (OT) dose-response curves on insulin output (absolute values)

Natural oxytocin	*Insulin secretion (ng/5 min)*		*Synthetic oxytocin*	*Insulin secretion (ng/5 min)*	
OT concentration	*Basal*	*Stimulation*	*OT concentration*	*Basal*	*Stimulation*
2.3 pM ($n = 5$)	12.6 ± 1.4	13.7 ± 1.5			
4 pM ($n = 9$)	11.4 ± 1.7	16.2 ± 2.6			
12 pM ($n = 4$)	10.7 ± 2.4	31.9 ± 5.1	6.6 pM ($n = 4$)	11.4 ± 2.3	14.9 ± 2.4
23 pM ($n = 5$)	11.4 ± 1.1	48.5 ± 5.2	18 pM ($n = 11$)	10.5 ± 1.1	26.8 ± 5.0
32 pM ($n = 7$)	11.6 ± 2.0	53.9 ± 5.3	40 pM ($n = 10$)	12.1 ± 1.2	41.5 ± 4.0
66 pM ($n = 8$)	11.8 ± 1.8	97.7 ± 10.8	73 pM ($n = 8$)	12.6 ± 1.4	72.6 ± 9.1
160 pM ($n = 5$)	12.9 ± 1.9	129.5 ± 14.9	280 pM ($n = 12$)	12.3 ± 1.5	97.5 ± 6.8
320 pM ($n = 10$)	11.6 ± 1.6	117.8 ± 15.4	530 pM ($n = 9$)	11.4 ± 1.4	97.1 ± 11.2

Friedman two-way ANOVA: at all the concentrations tested, oxytocin (either natural and synthetic) induced a significant (at least $P < 0.05$) increase in insulin output

Table 2 Effects of oxytocin antagonist, agonist and analogs on insulin secretion

		Insulin secretion (ng/5 min)		
Peptide	*Concentration*	*Basal*	*Infusion*	*% of baseline*
Oxytocin ($n=8$)	66 pM	11.8±1.8	97.7±10.8†	870±70
Oxytocin antagonist + Oxytocin ($n=7$)	6.6 nM 66 pM	14.4±1.2	34.7±5.0†	240±40**
Oxytocin agonist ($n=5$)	66 pM	14.4±1.8	104.0±8.3†	767±101 ns
Oxytocin free acid ($n=5$)	66 pM	13.0±0.2	12.9±0.5 ns	99±5**
Isotocin ($n=4$)	66 pM	12.5±1.3	14.1±1.0 ns	116±17**
Mesotocin ($n=4$)	66 pM	12.9±0.9	39.1±3.6‡	305±21*

*Indicates the comparison between the effect of OT 66 pM on insulin secretion and the one of OT antagonist or analogs. Statistical significance was assessed by ANOVA followed by a Turkey test: $*P<0.05$, $**P<0.001$. †Indicate the comparison made between insulin output in basal condition and during the infusion of different peptides; statistical significance was assessed by Friedman two-way ANOVA: $†P<0.05$, $‡P<0.01$

levels (Altszuler & Hampshire, 1981). An *in vivo* model does not, therefore, seem adequate to establish a direct (i.e. not mediated by hyperglycemia or hyperglucagonemia) effect of this hormone on insulin secretion. When tested *in vitro*, either on mouse isolated islets (Gao *et al.*, 1991) or on HIT cells (Richardson *et al.*, 1990), pharmacological concentrations of oxytocin were demonstrated to stimulate insulin secretion. Our results, that completely agree with these studies, showed that oxytocin could increase insulin output already at a concentration of 2.3 pM which corresponds to the basal physiological plasma levels of this hormone (Bruhn *et al.*, 1986). To our knowledge, this is the first report demonstrating that physiological plasma concentrations of oxytocin are able to stimulate insulin secretion. Furthermore, our results demonstrate this secretagogue activity of oxytocin in presence of a glucose concentration of 5 mM, i.e. a basal physiological glucose level, three times lower than that used by other authors (Gao *et al.*, 1991). Finally, in the present experiments, the amplitude of the oxytocin-evoked insulin secretion was remarkable by its extent: for instance, at 32 pM (a concentration which is still in the physiological range), oxytocin induced a fivefold increase in insulin output. A similar effect was observed, in isolated perfused rat pancreas, when increasing glucose concentration from 5 mM to 10 mM (Gerich *et al.*, 1974).

These quantitative and qualitative differences between our and other results, whether considering the effective oxytocin concentrations, the amplitude of the stimulatory effect of oxytocin on the insulin secretory response, the stimulatory effect obtained at low (5 mM) glucose concentration, are probably at least in part due to the different experimental models used. Isolated perfused pancreas, by preserving the structures of the intact organ, allows to investigate the action of oxytocin, and that of other peptides, under conditions that are close to the physiological ones.

In our study, the administration of synthetic Oxytocin could also induce insulin secretion, although with a lower potency, when compared to the natural peptide. As indicated by the manufacturer, the synthetic product contains at least 15% of non-peptide impurity. Furthermore, after HPLC purification, mass analysis and RIA measurement, we could also demonstrate the presence of a peptidic contaminant able to cross-react with the oxytocin antibody but devoided of biological activity. It is therefore reasonable to assume that some of these peptidic or non-peptidic substances may be the cause of the quantitative differences observed in the bioassay.

DesGly-NH_2^9, $d(CH_2)_5$ [Tyr(Me)^2,Thr^4]OVT has been characterized as one of the most powerful and selective oxytocin antagonists showing a very low affinity for AVP receptors (Manning *et al.*, 1989). *In vitro*, 10 nM is the concentration (pA_2) at which this substance is able to reduce the effect of a given dose of oxytocin to the one that could be obtained by giving 50% of this dose, in the absence of antagonist (Manning *et al.*, 1989). In our experiments, the antagonist was used at a concentration of 6.6 nM (a value close to its pA_2) in presence of 66 pM natural oxytocin. Under these conditions, 66 pM oxytocin induced a 240% increase of insulin output instead of the 870% observed when infusing the same concentration of oxytocin in the absence of antagonist. On the basis of the natural oxytocin dose response curve, one could extrapolate 7.5 pM as the oxytocin concentration able to produce a 250% increase in insulin output. In the present study, therefore, the antagonist showed a capacity to compete with oxytocin that was similar or even higher than that previously described (Manning *et al.*, 1989). This strongly suggests that oxytocin stimulated insulin secretion from normal rat pancreas by interacting with its own receptor, having characteristics similar to those described in other tissues such as myometrial cells (Manning *et al.*, 1989). Gao & Henquin (1993), upon using different OT and AVP antagonists, suggested two possibilities: the beta-cell could carry either two types of receptors, i.e. OT and V_1 or an unique, new type of receptor having characteristics intermediate between the 'classical' OT and V_1 receptors. Our results do not give any indications about the presence or the nature of vasopressin receptor in the beta-cell of normal rats, but they clearly demonstrated the presence of a 'clas-

sical' OT receptor through which oxytocin acted to stimulate insulin secretion. Our results do not exclude, of course, the possible presence of AVP receptors on the beta-cells, but they indicate that these receptors are not involved in the OT-stimulated insulin secretion. This conclusion is in contrast with the results obtained by Richardson *et al.* (1993) in HIT cells. In these clonal beta-cells, these authors demonstrated that oxytocin-induced insulin secretion was mediated by V_1 vasopressin receptors. It should be noted, however, that the same authors previously demonstrated that in HIT cells oxytocin was 100 times less powerful than vasopressin in stimulating insulin secretion (Richardson *et al.*, 1990). This suggests the possibility that this model of modified beta-cells does not express the OT receptor unlike normal rat or mouse beta-cells. Therefore, as indicated by these authors (Richardson *et al.*, 1993), extrapolating physiological relevance from data obtained with HIT cells is potentially dangerous.

The OT agonist, OH(Thr^4, Gly^7)OT, has been demonstrated to possess a high specificity for OT receptors: in fact, this peptide, which has an oxytocic activity similar to OT, shows an antidiuretic effect 1000 times lower than OT and it is devoided of any vasopressor activity (Lowbridge *et al.*, 1977). When infused at 66 pM, concentration OH(Thr^4, Gly^7)OT produced a stimulation in insulin secretion similar to the one of equal amounts of OT (Table 2). This result further supports that OT-receptors specifically mediated the OT-induced insulin secretion. Furthermore, the lack of vasopressor activity showed by this peptide, proved that the insulinotropic effect of OT was not secondary, in isolated perfused pancreas, to an action on the vascular system.

Results obtained when testing oxytocin analogs indicated that Q_4 and L_8 are important for insulin secretion stimulating effect: in fact, this biological activity was significantly reduced when replacing L_8 by I (i.e. isotocin) and completely abolished when replacing Q_4 by S and L_8 by I (i.e. mesotocin). The amide group of G_9 also seems to be essential for the oxytocin insulin secretion promoting activity: in fact, the de-amidated analog (i.e. oxytocin free acid) was devoided of any biological effect, when tested at 66 pM concentration.

To sum up, this study demonstrated that: (1) oxytocin exerts an insulinotropic activity at concentrations similar to the reported physiological plasma levels of this hormone, this in presence of low (5 mM) glucose concentration; (2) the receptor mediating this effect has the characteristics of a 'classical' OT receptor. These results allow us to suggest a new potential role of oxytocin i.e. that of an insulin releasing hormone.

Whilst this study clearly established that low concentrations of oxytocin have an insulin secretion stimulating effect, the question of a physiological involvement of this neuropeptide in the humoral control of the beta-cell remains open. Some authors (Wallin *et al.*, 1989) have suggested that increased plasma levels of OT during parturition could contribute to glucose homeostasis of the foetus. It has also been demonstrated that suckling increases insulin levels in lactating dogs (Ericksson *et al.*, 1987), probably via oxytocin release. It is also well known that glucose metabolism is increased during lactation (Burnol *et al.*, 1983) and that insulin plays an important role in milk production (Bolander *et al.*, 1981). In lactating animals, oxytocin could therefore contribute to the regulation of both glucose metabolism and milk production, by stimulating insulin secretion. Oxytocin could also participate to the control of B-cell activity under conditions other than parturition and lactation: thus, another interesting indication comes from the studies investigating the anorexigenic effect of oxytocin, when acting within the brain (Olson *et al.*, 1991). In fact, it has been demonstrated that food ingestion produced an elevation of plasma oxytocin levels (from 4 pM to 12 pM), already detectable 10 min after the beginning of a meal and lasting 30 min (Verbalis *et al.*, 1986). The authors interpreted such increased oxytocin plasma levels simply as a marker of the activation of the central oxytocinergic pathways projecting within the brain and mediating the anorexigenic action of oxytocin. In the present study, we have shown that oxytocin can stimulate insulin secretion by acting as an hormone (for instance, 11 pM oxytocin concentration increase by threefold insulin secretion). On the basis of these results, it is therefore possible to give a different interpretation of the observation made by Verbalis *et al.* Meal ingestion could in fact induce a coordinated activation of the oxytocinergic pathways projecting either to the neurohypophysis and within the CNS. The stimulation of the former would lead to an increase in the plasma levels of oxytocin thereby contributing to the rapid insulin secretion physiologically observed after food ingestion, while the activation of the latter would induce satiety and therefore the spontaneous cessation of the meal.

Materials and methods

Separation of natural oxytocin and evaluation of its purity

Posterior hypophyses from donor rats were extracted in 0.1 N HCl/acetone (1:1) and purified by reversed phase HPLC on a C-18 4.6 × 220 mm column eluted with 0.1% (v/v) TFA (solvent A) and 90% acetonitrile (v/v) in 0.1% TFA (v/v) (solvent B) on a gradient of zero to 30% B over 30 min, followed by 30–50% B over 10 min, followed by 50–100% B over 5 min, at a pumping speed of 1 ml/min. One minute fractions were collected and the absorbance was detected at both 214 and 280 nm.

Amino acid analysis was performed after vapour acid hydrolysis by the method described by Hughes *et al.* (1987). Amino-terminal sequences was carried out using a gas pulsed-liquid phase sequencer (Applied Biosystems, model 477A). Mass spectrometry was performed by Fast Atom Bombardment using a cesium ion gun operating at 30 KV on a ZAB2SE 2FPD instrument.

Biological tests for insulin secretion stimulating activity

Pancreases from fed *ad libitum* female Wistar rats (BW 180–200 g) were isolated as described by Grodsky *et al.* (1967) and modified by Assan *et al.* (1977). The organs were perfused at 37°C with a Krebs-Ringer bicarbonate buffer (flow rate 3.0 ml/min) containing 5.0 mM glucose, 4.0 g/l human albumin (Swiss Red Cross, Bern, Switzerland) and 2000 UIP protease inhibitor, aprotinine (Iniprol, Choay Lab., Paris, France).

After a 20 min equilibration period, tests were performed as follows: the effluent was collected during 5 min for measurements of basal insulin secretion, then the natural or synthetic peptides (reconstituted with 0.5 ml KRB) were infused during 5 min at a rate of 0.1 ml/min. During the infusion, a second 5 min sample was collected to measure the effect of the different substances on insulin secretion. A

10 min rest period was allowed before beginning another test. The duration of the rest period (i.e. 10 min) was selected on the bases of preliminary results that indicated that insulin secretion returned to basal values within 2 min after the cessation of oxytocin infusion and that β-cell responsiveness was restored within 10 min after the end of a stimulation, that pattern being independent of the amount of oxytocin used. For the same reason, six to eight tests were performed on each pancreas which received different amounts of natural and/or synthetic peptides in randomized order. The results are expressed either as absolute values (i.e. insulin output ng/5 min) and/or as per cent increase of baseline. During the experiments, the concentrations of K^+, amilase and lactico-dehydrogenase were measured in the effluent to evaluate the viability of the organ. The constancy of either O_2 consumption and CO_2 production, was also monitored. At the end of the experiments, the homogeneous diffusion of tripan blue injected into the pancreas was carefully verified.

Radioimmunoassay

Insulin was measured according to Herbert *et al.* (1965) using rat insulin (Novo Nordisk Biolabs, DK) as standard; Guinea Pig anti rat insulin serum as antibody (Linco Research Inc., St Louis, MO) and coated charcoal as separation system. The inter and intra variation coefficients were respectively 9.6 and 7.1%.

Chemicals, synthetic oxytocin, analogs, antagonist and agonist

Chemicals (analytical grade) were purchased from Fluka AG (Buchs, Switzerland), synthetic oxytocin and oxytocin analogs from Bachem (Feinchemikalien A.G., Bubendorf, Switzerland); oxytocin antagonist and agonist were kindly given by Professor J.-J. Dreifuss (Dept. of Physiology, Geneva Medical School).

Statistics

Friedman two-way ANOVA was used to evaluate the effect of each concentration of either natural or synthetic peptide infused (i.e. when comparing basal *vs* stimulated insulin values within a group). Analysis of variance followed by a Turkey test, was used when evaluating the effect on insulin secretion produced by the infusion of different peptides (i.e. comparison of % increase between groups).

Acknowledgements
This work was supported by Grants 32-26405.89 and 31-33658.92 of the Swiss National Science Foundation (Berne, Switzerland). The authors thank Mrs C. Barras, N. Paquet and Mr P. Germann for their technical assistance, Mrs T.-M. Besson and F. Touabi for their secretarial work. The authors are grateful to Professor J.J. Dreifuss for supplying oxytocin antagonist and agonist, and for his precious advice and comments.

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