Contribution of neural intrapancreatic non-cholinergic non-adrenergic mechanisms to glucose-induced insulin release in the isolated rat pancreas

N. Weigert, M. Dollinger, R. Schmid and V. Schusdziarra

Department of Internal Medicine II, Technical University of Munich, FRG

Summary. In the isolated rat pancreas the effect of intrapancreatic non-adrenergic non-cholinergic nerves was examined upon insulin, glucagon and somatostatin release during perturbations of perfusate glucose. Elevation of glucose from 1.6 to 8.3 mmol/l increased insulin and somatostatin secretion and inhibited glucagon release. The first phase of insulin secretion was significantly reduced by the neurotoxin tetrodotoxin to 55 % of the controls ($p < 0.05$). The somatostatin response was attenuated by tetrodotoxin while the change of glucagon remained unaffected. In contrast the combined adrenergic and cholinergic blockade with atropine, phentolamine and propranolol (10^{-5} mol/l) did not modify the insulin, glucagon and somatostatin response. When glucose was changed from 8.3 to 1.6 mmol/l, the reduction of insulin and somatostatin release was not modified by tetrodotoxin, but stimulation of glucagon was significantly attenuated by 60–70 % ($p < 0.03$), which was similar to the effect of combined adrenergic and cholinergic blockade. Subsequently, the effect of neural blockade was examined during more

physiological perturbations of perfusate glucose levels. When glucose was changed from 3.9 to 7.2 mmol/l, tetrodotoxin also attenuated first phase insulin response by 40 % while cholinergic and adrenergic blockade had no effect. The nitric oxide synthase inhibitor N^G-Nitro-L-arginine-methyl-ester (L-NAME) did not alter the glucose-induced insulin response indicating that nitric oxide is not involved in this mechanism. It is concluded that neural non-adrenergic non-cholinergic mechanisms contribute to the first, but not second phase of glucose-induced insulin release. Non-adrenergic non-cholinergic effects do not participate in regulation of glucagon and somatostatin secretion under the conditions employed. The non-adrenergic non-cholinergic effect is most likely of peptidergic nature and remains to be examined in greater detail.

Key words: Intrinsic nerves, isolated rat pancreas, insulin, somatostatin, glucagon, glucose, adrenergic, cholinergic.

The islets of Langerhans are complex functional units composed of endocrine cells which are supplied by blood vessels and autonomic nerves [1]. The pancreas receives an abundant supply of extrinsic autonomic nerves from the vagal trunks and the splanchnic nerves [2]. Apart from this extrinsic nervous system the pancreas contains intrinsic neurons, which form periinsular, periacinar, and perivascular plexuses which are connected with one another and with intrapancreatic ganglia [3]. These intrapancreatic neurons are still observed after extrinsic denervation [4,5].

This morphological relationship makes it likely that nerves are involved in the physiological regulation of islet function. The participation of the adrenergic and cholinergic nervous system is fairly well documented and has been summarized previously [2, 6–10]. On the other hand, the contribution of the intrinsic nervous elements is largely unknown. In a classical approach Hisatomi et al. [11] have shown that adrenergic fibres of the intrinsic system

are important for regulation of A-cell function while they do not contribute to insulin and somatostatin release.

Apart from the classical neurotransmitters many different peptides have been localized in nerve terminals throughout the pancreas such as cholecystokinin (CCK) [12, 13], calcitonin gene-related peptide (CGRP) [14], met-enkephalin and leu-enkephalin [12, 15], galanin [16], gastrin-releasing peptide (GRP) and bombesin-like immunoreactivity [17], neuropeptide Y (NPY) [18], PHI [19], substance P [12, 20] and vasoactive intestinal polypeptide (VIP) [12, 19]. All these neuropeptides can influence islet hormone secretion, when they are infused in vivo or in vitro [6]. This and the morphological data raise the possibility of a functional role of these neuropeptides in islet cell hormone secretion.

For the vast majority of neuropeptides specific receptor antagonists are not available to examine their biological role. Therefore, we have employed the neurotoxin te-

trodotoxin (TTX) which blocks all neural elements that are activated by an influx of Na^+ . This comprises all the adrenergic and cholinergic neurons and presumably the majority of peptidergic neurons as well [21–23]. Cholinergic and adrenergic neurons were antagonized by the respective specific blocking agents [7]. From the difference in these effects, the potential involvement of the peptidergic system was deduced.

Accordingly, the influence of intrapancreatic non-adrenergic non-cholinergic neural elements on insulin, glucagon and somatostatin secretion during perturbations of perfusate glucose levels was examined in the isolated perfused rat pancreas.

Materials and methods

The experiments were performed in an isolated rat pancreas preparation according to the detailed description of Sussman et al. [24]. After an overnight fast, male Wistar rats (body weight 300–350 g, Charles River Wiga GmbH, Sulzfelden, FRG) were anaesthetized (pentobarbital sodium, 60 mg/kg i.p.; Rhone-Merieux, Köln, FRG). Pancreata were isolated and placed in an organ bath at 37°C and perfused through the celiac and mesenteric artery in a single pass perfusion system at a constant flow rate of 2 ml/min as described previously [25].

The perfusion medium consisted of a Krebs-Ringer buffer solution containing 4% dextran T70 (Pharmacia, Uppsala, Sweden) and 0.2% human serum albumin (Behring, Marburg, FRG). In the first series of experiments the perfusate glucose concentrations were changed between 1.6 mmol/l (30 mg/dl) and 8.3 mmol/l (150 mg/dl). In a subsequent experimental group more physiological perturbations of perfusate glucose between 3.9 and 7.2 mmol/l (70 and 130 mg/dl) were employed. The perfusate was gassed with 95% O_2 and 5% CO_2 and the pH was maintained between 7.35 and 7.45. The pancreatic venous effluent was collected via a catheter in the portal vein at 1-min intervals and frozen immediately for subsequent radioimmunoassays.

For equilibration after the end of surgery, the pancreas was perfused for 20 min with buffer containing the glucose concentration of the initial 10-min experimental basal period. Thereafter the glucose concentration was elevated or reduced for 20 min which was followed by another 20-min period with perfusate glucose levels of the basal period.

For neural blockade either a combination of 10^{-5} mol/l atropine sulphate (Sigma, München, FRG), 10^{-5} mol/l phentolamine (Ciba, Basel, Switzerland) and 10^{-5} mol/l propranolol (Rhein-Pharma, Heidelberg, FRG), or the neurotoxin TTX (10^{-6} mol/l, Sigma) was added during the entire experimental period. For blockade of nitric oxide synthase N^G -Nitro-L-arginine-methylester (L-NAME; Sigma) was added to the perfusate at 10^{-4} mol/l, a concentration which has recently been shown to be effective [26].

In controls the pancreas was perfused at a constant glucose concentration of 1.6 or 8.3 mmol/l with and without the neuronal blocking agents atropine, phentolamine and propranolol or the neurotoxin TTX, respectively. In each pancreas only one experiment was performed.

Perfusate insulin [27], glucagon [28] and somatostatin [29] were determined radioimmunologically as described elsewhere. Antibody 30K for glucagon measurements and antibody 80C for somatostatin measurements were generously provided by Dr. R.H. Unger (Dallas, Texas, USA). For insulin determination a commercially available insulin-kit (IDW, Frankfurt, FRG) was employed.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Integrated hormone secretion was calculated as the sum of the differences between each point during stimulation and the mean value of the preceding baseline

period. Minute-to-minute variations were evaluated by the use of the Friedman two-way analysis of variance, followed by the Wilcoxon matched-pairs signed rank test if the former allowed rejection of the null hypothesis. The difference of integrated values between treatment groups was analysed by analysis of variance for multiple determinations. Differences resulting in p -values of 0.05 or less were considered significant.

Results

Effect of TTX

The elevation of the perfusate glucose concentration from 1.6 to 8.3 mmol/l (Fig. 1) elicited a rise of perfusate insulin levels from 36 ± 7 pmol/l to a maximum of 427 ± 79 pmol/l within 5 min. Thereafter insulin decreased during the ensuing 5 min to 188 ± 29 pmol/l and increased again in a second phase to 297 ± 43 pmol/l. When the glucose level was restored to 1.6 mmol/l, insulin levels promptly returned to basal values.

The addition of the neurotoxin TTX (10^{-6} mol/l) to the perfusate resulted in a significant reduction of the insulin peak during the first phase of glucose-induced insulin secretion (427 ± 79 pmol in the controls vs 224 ± 43 pmol/l during TTX, $p < 0.03$) (Fig. 1). Additionally the integrated insulin response to the first 10 min of high glucose was significantly lower than in the controls (4026 ± 876 pmol/10 min in the controls vs 2041 ± 304 pmol/10 min during TTX, $p < 0.03$). The second phase insulin secretion was similar in both groups.

Perfusate somatostatin levels rose after the elevation of the glucose concentration from a mean baseline of 34 ± 7 to a maximal level of 77 ± 11 ng/l ($p < 0.01$) and returned to basal values thereafter. TTX attenuated the rise of somatostatin significantly. The integrated somatostatin level during the first 10 min of high glucose was 283 ± 46 ng/l in the controls and only 65 ± 40 ng/l ($p < 0.03$) in the presence of TTX (Fig. 1 A).

Glucagon secretion was suppressed during the phase of elevated glucose from 302 ± 42 to 90 ± 7 ng/l ($p < 0.01$) and rose again to 283 ± 58 ng/l when the perfusate glucose concentration had been reduced to 1.6 mmol/l. TTX had no effect on glucose-induced suppression of glucagon secretion. However, the rebound during the subsequent phase of low perfusate glucose was significantly attenuated by TTX (Fig. 1 A). The incremental glucagon release between 30 and 50 min was 2439 ± 650 ng/20 min in the controls and only 857 ± 123 ng/20 min with TTX ($p < 0.01$).

When the perfusate glucose concentration was first decreased from 8.3 to 1.6 mmol/l and then increased again to 8.3 mmol/l, insulin secretion was almost completely suppressed during the period of glucopenia. After elevation of the glucose concentration insulin was stimulated in a biphasic pattern with a maximum of 594 ± 50 pmol/l during the first and 391 ± 72 pmol/l during the second phase (Fig. 2).

TTX did not modify the suppression of insulin release by glucopenia (Fig. 2 A), but reduced significantly the maximal (594 ± 50 vs 268 ± 87 pmol/l, $p < 0.01$) and the integrated (3794 ± 535 vs 1919 ± 369 pmol/10 min, $p < 0.03$) insulin release during the first phase of glucose-induced

Fig. 1 A, B. Effect of 10^{-6} mol/l tetrodotoxin (A, $n = 8$, $\bullet$) or combined adrenergic/cholinergic blockade with 10^{-5} mol/l atropine, 10^{-5} mol/l phentolamine and 10^{-5} mol/l propranolol (B, $n = 9$, $\blacksquare$) on insulin, somatostatin and glucagon secretion from the isolated perfused rat pancreas during perturbations of perfusate glucose levels from 1.6 to 8.3 and back to 1.6 mmol/l. Control group with perfusion of glucose without the respective blocking agents ($n = 8$, $\circ$) (mean $\pm$ SEM)

Fig. 2 A, B. Effect of 10^{-6} mol/l tetrodotoxin (A, $n = 8$, $\bullet$) or combined adrenergic/cholinergic blockade with 10^{-5} mol/l atropine, 10^{-5} mol/l phentolamine and 10^{-5} mol/l propranolol (B, $n = 9$, $\blacksquare$) on insulin, somatostatin and glucagon secretion from the isolated perfused rat pancreas during perturbations of perfusate glucose levels from 8.3 to 1.6 and back to 8.3 mmol/l. Control group with perfusion of glucose without the respective blocking agents ($n = 8$, $\circ$) (mean $\pm$ SEM)

insulin secretion without affecting the second phase of stimulation. This effect of TTX upon the first phase of glucose-stimulated insulin release was comparable to the results shown in Figure 1 A.

Somatostatin levels decreased by 21 ± 5 ng/l ($p < 0.01$) after reduction of perfusate glucose and returned to basal values during the following hyperglycaemia. In this experimental setting somatostatin secretion was not affected by TTX (Fig. 2 A).

The decline in glucose concentration elicited a rise in mean glucagon levels from 70 ± 11 to 402 ± 92 ng/l ($p < 0.01$). Glucagon levels remained elevated throughout the glucopenic period and returned promptly to baseline levels when the perfusate glucose concentration was restored to the initial value. TTX reduced the glucagon response to low perfusate glucose from 4620 ± 695 ng/20 min in the control experiments to 1521 ± 638 ng/20 min ($p < 0.01$).

Effect of combined adrenergic and cholinergic blockade

The combined blockade of adrenergic and cholinergic mechanisms by simultaneous infusion of atropine (10^{-5} mol/l), phentolamine (10^{-5} mol/l) and propranolol (10^{-5} mol/l) through the entire perfusion period did not modify glucose-induced secretion of insulin and somato-

statin (Fig. 1 B). The integrated first phase insulin response during TTX (2041 ± 304 pmol/10 min) was also significantly lower than that during adrenergic and cholinergic blockade (3164 ± 362 pmol/10 min; $p < 0.05$). Similar to the experiments with TTX the glucopenia-induced rebound of glucagon secretion was significantly reduced to 1392 ± 178 ng/20 min ($p < 0.05$) during adrenergic and cholinergic blockade. The magnitude of glucagon suppression by TTX and the combined adrenergic and cholinergic blockade showed no significant difference.

When perfusate glucose was decreased from initially 8.3 to 1.6 mmol/l the simultaneous infusion of atropine, phentolamine and propranolol did not alter the decrease and increase of insulin and somatostatin secretion following reduction and subsequent elevation of glucose levels, respectively (Fig. 2 B). The integrated insulin release during the first 10 min after the rise of glucose in the presence of TTX was not only significantly lower compared to controls but also in comparison to the value during adrenergic and cholinergic blockade (1919 ± 369 vs 3388 ± 246 pmol/10 min; $p < 0.02$). The glucagon response to glucopenia, however, was significantly reduced to 30 % of the control values by the adrenergic and cholinergic blockade (1401 ± 389 ng/20 min vs 4620 ± 695 ng/20 min; $p < 0.01$). The reduction of the glucagon response to low perfusate glucose by atropine, phentolamine and propranolol and by TTX was nearly identical.

Fig. 3 A, B. Effect of 10^{-6} mol/l tetrodotoxin (—●—) upon insulin, somatostatin and glucagon release from the isolated perfused rat pancreas during perfusion with a constant glucose concentration of 1.6 mmol/l (**A**, $n = 8$) or 8.3 mmol/l (**B**, $n = 8$), respectively. Control group with perfusion of glucose without tetrodotoxin ($n = 7$, ○) (mean $\pm$ SEM)

Fig. 4 A, B. Effect of combined adrenergic and cholinergic blockade (—■—) with 10^{-5} mol/l atropine, 10^{-5} mol/l phentolamine and 10^{-5} mol/l propranolol upon insulin, somatostatin and glucagon release from the isolated perfused rat pancreas during perfusion with a constant glucose concentration of 1.6 mmol/l (**A**, $n = 8$) or 8.3 mmol/l (**B**, $n = 8$), respectively. Control group with perfusion of glucose without the respective blocking agents ($n = 7$, ○) (mean $\pm$ SEM)

In control experiments TTX (Fig. 3) or the combined adrenergic and cholinergic blockade (Fig. 4) infused for 30 min had no effect on insulin, somatostatin and glucagon release during a constant glucose concentration of 1.6 or 8.3 mmol/l, respectively.

To examine if similar effects of TTX can be observed at more physiological changes of perfusate glucose levels the first series of experiments was repeated with glucose levels increasing from 3.9 to 7.2 mmol/l. This elevation of perfusate glucose concentration elicited a significant rise in mean insulin levels from 43 ± 7 pmol/l to 290 ± 29 pmol/l in the first and 239 ± 14 pmol/l in the second phase of stimulation as shown in Figure 5. As in the first series of experiments TTX (Fig. 5 A) reduced significantly the maximal (290 ± 29 vs 166 ± 51 pmol/l, $p < 0.03$) and the integrated (2440 ± 138 vs 1701 ± 253 pmol/10 min, $p < 0.05$) insulin release during the first phase of glucose-induced insulin secretion without affecting the second phase of stimulation. The blockade of adrenergic and cholinergic mechanisms by simultaneous infusion of atropine, phentolamine and propranolol (Fig. 5 B) was without effect on first and second phase insulin secretion also at these more physiological perturbations of perfusate glucose levels. There was also a significant difference between first phase insulin response during TTX and adrenergic/cholinergic blockade, respectively (1701 ± 253 vs 2744 ± 210 pmol/10 min; $p < 0.03$).

The increase of somatostatin secretion from 36 ± 6 ng/l to 67 ± 21 ng/l ($p < 0.05$) following the elevation of glucose level from 3.9 to 7.2 mmol/l and the subsequent decrease of somatostatin release after reduction of glucose level to 3.9 mmol/l were attenuated neither by TTX (Fig. 5 A) nor by adrenergic and cholinergic blockade (Fig. 5 B).

Fig. 5 A, B. Effect of 10^{-6} mol/l tetrodotoxin (**A**, $n = 8$, —●—) or combined adrenergic/cholinergic blockade with 10^{-5} mol/l atropine, 10^{-5} mol/l phentolamine and 10^{-5} mol/l propranolol (**B**, $n = 9$, —■—) on insulin, somatostatin and glucagon secretion from the isolated perfused rat pancreas during more physiological perturbations of perfusate glucose from 3.9 to 7.2 mmol/l and back to 3.9 mmol/l. Control group with perfusion of glucose without the respective blocking agents ($n = 10$, ○) (mean $\pm$ SEM)

Fig. 6. Effect of the nitric oxide synthase inhibitor N^G -nitro-L-arginine-methyl-ester (L-NAME, 10^{-4} mol/l) on insulin secretion from the isolated perfused rat pancreas during a change of perfusate glucose levels from 3.9 to 7.2 mmol/l and back to 3.9 mmol/l. Control group ($n = 10$, $\circ$); L-NAME group ($n = 5$, $\bullet$) (mean $\pm$ SEM)

Glucagon secretion was suppressed from 72 ± 6 to 29 ± 3 ng/l ($p < 0.01$) by elevation of perfusate glucose concentration and it rose again to 64 ± 9 ng/l when the glucose concentration was restored to 3.9 mmol/l. This rebound secretion of 442 ± 62 pg/20 min was significantly reduced by TTX (140 ± 33 pg/20 min, $p < 0.03$; Fig. 5A) and by combined adrenergic and cholinergic blockade (156 ± 96 pg/10 min, $p < 0.05$; Fig. 5B) similar to the first series of experiments.

In order to examine whether the foregoing non-cholinergic non-adrenergic effect might be mediated by nitric oxide, we tested the effect of L-NAME on insulin release. As shown in Figure 6 the addition of L-NAME to the perfusate had no significant effect on glucose-induced insulin secretion. During the first phase insulin rose to a maximum of 301 ± 38 pmol/l in the presence of L-NAME compared to 290 ± 29 pmol/l in controls (NS). The second phase rise was 206 ± 21 pmol/l vs 239 ± 14 pmol/l (NS), respectively. The integrated insulin response during the first phase was 2676 ± 382 vs 2440 ± 138 pmol/10 min in the control group (NS).

Discussion

The isolated perfused pancreas has been separated from the extrinsic innervation and the remaining intrapancreatic neurons and ganglia are known to act independently [4, 5]. Typical cholinergic and adrenergic [2, 7, 9, 10] as well as peptide containing nerves [12–20] have been observed in the islets, which might be regulators of islet cell function.

The puffer-fish poison TTX is unique in its selective blocking action on the increase of nerve membrane so-

dium conductance, thereby preventing the propagation of action potentials. This effect is exerted at very low concentrations (10^{-6} to 10^{-8} mol/l) without any effect on other membrane parameters [21–23]. A comparison of TTX with other blocking agents specifically raises the possibility of differentiating between the action of classical neurotransmitters such as acetylcholine and catecholamines, on the one hand, and the non-cholinergic non-adrenergic system (NANC), on the other hand. The latter most likely represents the large group of peptidergic neurotransmitter candidates, although recent studies have provided evidence that nitric oxide should be considered as another candidate transmitter of NANC effects [30–32].

The unchanged insulin, somatostatin and glucagon release in control experiments (constant glucose of 1.6 or 8.3 mmol/l) demonstrates that TTX does not exert a toxic effect on the endocrine pancreas, and it suggests that neural mechanisms do not contribute to the secretion of the three islet hormones at constant glucose levels. This is in accordance with previous studies in isolated pancreatic islets [33–35]. On the other hand, phentolamine and propranolol when given separately raise basal insulin and somatostatin secretion in the rat pancreas [11], which is in contrast to the data of the present study. This difference cannot as yet be explained and requires further experimental work.

The insulin response to an increase and the glucagon response to a decrease of perfusate glucose levels was modified by TTX. The neurotoxin significantly reduces the first but not the second phase of glucose-induced insulin secretion. As TTX has no effect on glucose-induced insulin secretion from isolated islets [33–35], it appears likely that it reduces glucose-stimulated insulin release by blocking neural mechanisms. In line with this notion are the findings of Stagner and Samols [36] who demonstrated an effect of TTX of insulin oscillations in the perfused canine pancreas.

In contrast adrenergic and cholinergic antagonists had no influence on glucose-stimulated insulin secretion. The concentrations of the respective antagonists have previously been shown to be effective [37–40]. The lack of an effect of isolated or combined adrenergic and cholinergic blockade upon glucose-stimulated insulin release has been reported previously [11, 36]. The difference between the effects of TTX and the combined adrenergic and cholinergic blockers upon the first phase insulin response suggests that NANC mechanisms contribute to first phase insulin secretion. NANC effects could be mediated either by neuropeptides or by nitric oxide. The latter substance, however, exerts an inhibitory effect in isolated islets [41] and it has no effect on insulin release from the perfused pancreas [41] which is in agreement with the present data.

Accordingly, intrapancreatic glucose-sensitive peptidergic neurons can contribute to the regulation of B-cell function. From the candidate peptidergic neurotransmitters which are located in intrapancreatic nerves CCK [13], GRP [42], PHI [19] and VIP [43] can stimulate insulin secretion. NPY [18, 44], substance P [45, 46] and certain opioid peptides [47] have both stimulatory and inhibitory effects on insulin release, depending on the species exam-

ined and the experimental conditions employed. The proof for a regulatory role of the endogenously released peptides awaits the development of potent and selective neuropeptide antagonists. In non-insulin-dependent diabetes mellitus the early postprandial insulin response is attenuated and the first pulse of insulin secretion after breakfast is delayed and of reduced amplitude [48]. An alteration of peptidergic neural factors could contribute to a reduced early insulin response according to the present data. This is supported by data from Giugliano et al. [49] who demonstrated an improvement of insulin secretion in non-insulin-dependent diabetic patients after administration of naloxone, a specific antagonist of endogenous opioid peptides.

The rise of glucagon during reduction of perfusate glucose was similarly attenuated by TTX or combined adrenergic/cholinergic blockade. In view of the virtually identical effects of phentolamine described by Hisatomi et al. [11], the rise of glucagon is most likely due to α -adrenergic mechanisms without any additional cholinergic and peptidergic effects. The present data also suggest that the residual increase of glucagon which is still present during neural blockade might reflect a progressively reduced restraint of A-cell function by falling insulin levels rather than being mediated by any neural factors.

The present data confirm the previously reported direct stimulatory action of glucose upon pancreatic somatostatin secretion [50]. TTX or the combined adrenergic and cholinergic blockade had no consistent influence on somatostatin secretion. This suggests that neural mechanisms are presumably of only minor importance, if at all, for glucose-induced somatostatin release. It should be kept in mind though, that during other stimulatory conditions cholinergic and adrenergic mechanisms may very well contribute to pancreatic somatostatin release, as shown by several *in vitro* and *in vivo* studies [6–8, 37, 39, 40, 51].

In conclusion the present data suggest that not only adrenergic intrapancreatic neurons but also peptidergic neurons are sensitive to changes in glucose concentration. The peptidergic neural mechanisms contribute to first phase insulin secretion but have no effect on glucose-induced alteration of glucagon and somatostatin release. The participating neuropeptides should be characterized when more specific blocking agents are available.

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Prof. Dr. V. Schusdziarra
Department of Internal Medicine II
Technical University of Munich
Ismaninger Straße 22
W-8000 München 80
FRG