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The purinergic nerve hypothesis and insulin secretion

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With 4 tables

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The existence of non-adrenergic, non-cholinergic nerves in the vertebrate autonomic nervous system is recently recognized (7). Evidence has been presented that a purine nucleotide, probably adenosine triphosphate (ATP), is the neurotransmitter in at least some of these nerves (5) and they have therefore been called "purinergic" (6). In 1978 *Burnstock* offered direct evidence for ATP release from non-adrenergic, non cholinergic "Purinergic" nerves (10).

The high sensitivity of smooth muscle to adenine nucleotides was recognized long before it was postulated that ATP was a neurotransmitter (15). The form and time course of the response to exogenously applied ATP closely mimics that of purinergic nerve stimulation. Typically, the relaxation produced by ATP or by nerve stimulation rapidly reaches a maximum which quickly declines, this is in contrast to the inhibitory responses to noradrenaline and sympathetic nerve stimulation which reach a maximum more slowly and are maintained for a longer time (8).

In a previous study on the effect of exogenous ATP on insulin secretion we found that there was immediate and excessive production of insulin release in the portal vein after ATP injection in the carotid artery (45). Thus the effect of ATP on insulin secretion is a rapid process, therefore it should occur through nervous mechanism. It also raises the possibility that purinergic nerves regulate insulin secretion.

The present work is to discuss the possible role of purinergic nerves in insulin secretion.

Materials and methods[1])

Experimental procedures

Male albino rats, of the Wistar strain weighing from 150 to 200 g and fed stock diet, were used throughout the experiment. Food and water were allowed ad libitum. The effect of ATP after intravenous glucose administration was investigated in normal and partially alloxan diabetic groups, each group was divided into two subgroups with and without ATP.

In all groups bilateral adrenalectomy was carried out under hexobarbital anesthesia. The effect of adrenalectomy on insulin secretion was discussed in another publication (46).

[1]) The practical work was carried out in the Central Institute of Diabetes Gerhardt Katsch, Karlsburg, DDR.

Blood samples (0.3–0.5 ml) were drawn from the cannula in the external jugular vein, using heparinized Eppendorf centrifuge tubes which were stored immediately at 4 °C. Aliquots of blood (0.01 ml) were used for determining the glucose enzymatically by glucose oxidase method (22).

The blood samples were centrifuged, and the separated plasma was kept in a deep-freeze for later insulin assay.

The radioimmunologically reactive insulin (IRI) was analyzed using alcohol precipitation according to the back-titration principle of *Wright* (49). Pure rat insulin was used as reference standard. Standard dilutions as well as the rat plasma samples (dilution 1:1) were incubated at room temperature overnight with an excess of anti-pig-insulin serum. The free antibodies were titrated back by the subsequent addition of an excess of I^{125}-insulin (ZeiK Rossendorf/Dresden, 70 mCi/mg). Alcohol is used to separate free from antibody bound insulin.

Antibody bound insulin I^{125} thus precipitated was counted by a Scintillation counter and the insulin value was calculated. For all dilutions a 0.04 M phosphate buffer with 0.3% bovine serum albumin (pH 7.4) was used.

Glucose tolerance

Glucose (1 g/kg body wt) in a 20 percent solution was injected in one minute into the exposed external jugular vein, after overnight fast. Blood was withdrawn from the jugular vein before and at 2, 5, 10, 30 and 60 minutes after glucose injection. ATP (Richter) 2 mg/rat in 0.2 ml saline was injected immediately after glucose administration.

Alloxan diabetes

Alloxan (60 mg/kg) was injected into a tail vein after 24 hours fast. Alloxan was dissolved in citric acid phosphate buffer pH 4 immediately before injection. Final concentration was 24 mg/ml. The diabetic animals used were those who had mild diabetes of non-fasting blood glucose levels of about 200 mg/100 ml.

A new set of experiments were performed in which the carotid artery was cannulated for arterial inflow and the portal vein was cannulated to collect the venous effluent. Each catheter was washed by syringing with physiological saline containing heparin (1%) after each blood withdrawal. After completing both catheterizations, the rat was allowed to stabilize for at least 15 min in order to recover from the immediate effect of these procedures.

Insulin secretion in the portal vein was determined before and after injection of glucose and glucose plus ATP, at 2, 5, 10 and 30 minutes.

Results and discussion

Anatomical studies revealed that the pancreas receives a generous supply of nerve fibers via the pancreatic nerves, which enter the organ with the pancreatic arteries (48). Although the innervation of the pancreas has long been known to affect the secretion of the exocrine pancreas, less attention has been paid to possible effects on the endocrine pancreas.

Research on insulin secretion until very recently has dealt primarily with circulating factors. Possible reasons are:

a) Several early experiments indicated that denervation of the pancreas had little or no effect on blood glucose regulation (e.g. 1.).
b) Insulin secretion is observed in preparations in vitro and appears to be analogous to that observed in vivo, and
c) transplanted pancreas is reported to maintain blood glucose within the normal range (3).

This evidence, coupled with the obvious control of the endocrine pancreas by blood glucose and other circulating factors, appears to have overshadowed experiments on potential neural influences.

Nevertheless literature dealing with autonomic influences on the secretion of the endocrine pancreas pointed out that stimulation of the parasympathetic nervous system leads to insulin secretion, whether at the lateral hypothalamic nuclei, the motor nuclei of the vagus, the vagal trunks, or the mixed pancreatic nerves. Stimulation of the sympathetic nervous system inhibits insulin secretion, this occurring at the ventromedial hypothalamic nuclei, the splanchnic nerves, or the mixed pancreatic nerves (48).

Two categories of reflex secretion of insulin mediated by the nervous system are described. In one, insulin is secreted when the brain is exposed to an increase of glucose uptake, an effect that is eliminated by inactivation of the vagus nerves: In the other, secretion of insulin is elicited by food-related stimuli (sight, smell, taste, etc.) unrelated to the caloric challenge to the pancreas.Thus, insulin secretion is shown to be brought under arbitrary stimulus control through conditioning procedure (48).

Most vertebrate species have a rich supply of autonomic neurons to the islets of Langerhans. In general, both adrenergic and cholinergic fibers innervate all three types of islet secretory cells, although there are species differences in the relative amounts of these two fiber types (48).

Experiments done by *Burnstock* over the past decade have revealed a third component in the autonomic nervous system which is neither adrenergic nor cholinergic. These nerves are strongly represented in the gastrointestinal tract of a wide range of vertebrate species and have also been identified in lung, trachea, bladder, oesophagus, eye, seminal vesicle and in some parts of the cardio-vascular system and brain.

In tissues where purinergic nerves have been demonstrated experimentally, nerve profiles can be seen which are different from those of adrenergic, cholinergic or sensory neurons. These profiles contain a predominance of "large opaque vesicles" which can be distinguished from "large granular vesicles" found in small numbers in both adrenergic and cholinergic nerves. Large opaque vesicles are larger (80–200 nm) than the large granular vesicles (60–120 nm), have a less prominent halo between the granular core and the vesicle membrane and have a less granular matrix (8).

In the pancreas however, electron microscopy has also revealed the nature of the neural elements contained within the islets.

Several categories of neuron terminals have been differentiated within the islets according to the type of "synaptic" vesicles present. There appears to be two major categories exist: those mainly containing small (200–500 Å) electron-lucent (agranular) vesicles and those mainly containing small (300–500 Å) electro-lucent vesicles with electron-dense or granular cores (25, 41). The former category of vesicles is generally held to be cholinergic (36) and the latter adrenergic (4, 36).

Both categories of neuron terminals may contain additional larger (500–1000 Å) cored vesicles (48).

This last category of "larger vesicles" may be purinergic neuron terminals.

According to *Burnstock* (8) the purinergic nerve hypothesis includes the following evidences: (1) synthesis and storage of ATP in nerves; (2) release of ATP from the nerves when they are stimulated; (3) mimicry by exogenously applied ATP of the action of nerve released transmitter; (4) the presence of $Mg^{2\pm}$ activated ATPase, 5-nucleotidase and adenosine deaminase, enzymes which inactivate ATP; (5) the similar blocking and potentiating effects produced by drugs on the responses to exogenously applied ATP and nerve stimulation.

Little is known about the participation of adenosine triphosphate (ATP) in the events leading to release of insulin from the pancreatic B-cells. An indirect importance of ATP is suggested by its role as parent substance for cyclic AMP which is considered to act as an intracellular signal for insulin release (26, 39). *R. Candela* (34) provided in vivo data indicating that the intravenous injection of ATP was stimulant to insulin secretion. Also by using slices of rabbit pancreas, the same author (35) found that ATP caused insulin secretion.

Table 1.

		Intravenous glucose tolerance					
		0	2	5	10	30	60 min
Sub group (a)	mean	96	381	359	307	223	165
Control rats	SEM	±7	±29	±24	±20	±11	±17
	n	11	11	10	11	11	11
Sub group (b)	mean	86	377	284	231	153	113
Control rats	SEM	±6	±48	±23	±17	±14	±20
+ ATP	n	8	4	8	8	8	6
	p	>.05	>.05	<.01	<.01	<.01	<.02
		Insulin					
		0	2	5	10	30	60 min
Sub group (a)	mean	46	194	81	118	69	64
Control rats	SEM	±7	±40	± 8	±23	±10	± 8
	n	8	9	8	9	9	7
Sub group (b)	mean	47	161	140	143	134	121
Control rats	SEM	±3	±21	±11	± 7	±11	±14
+ ATP	n	8	7	8	8	8	8
	p	>.05	>.05	<.01	>.05	<.01	<.01
		G/I					
		0	2	5	10	30	60 min
Sub group (a)	mean	2.0	3.1	4.6	3.2	3.7	1.9
Control rats	SEM	±.14	±.79	±.66	±.57	±.60	±.14
	n	7	9	8	9	9	6
Sub group (b)	mean	1.8	2.0	2.4	1.7	1.3	0.79
Control rats	SEM	±.14	±.26	±.34	±.14	±.13	±.09
+ ATP	n	6	5	7	7	7	6
	p	> .05	> .05	< .02	< .05	< .01	< .01

These early experiments were not conclusive because insulin could not be measured directly.

In the present work the radio-immunologically reactive insulin was determined to evaluate the effect of ATP after i.v. glucose injection on insulin changes in external jugular vein in normal and partially alloxan diabetic rats.

From the present results, it is clear that the animals responded to glucose and ATP with immediate and maximal output of insulin and as the concentration of sugar in the external jugular vein reaches its peak so does the concentration of insulin (Table 1).

In ATP-treated rats, the much faster clearance of blood sugar was associated with a slight but significant increase in insulin secretion. The changes in jugular vein insulin was not a simple reflection for that of the portal vein. Since the hormone is released into the portal vein and must traverse the liver before entering the systemic circulation.

Most of the tissues in the body degrade insulin to some extent, but very little occurs in the plasma. The liver is very active in this regard (37). A large proportion of endogenous insulin is removed by the liver (37) and the rate of hepatic uptake of insulin may be markedly and rapidly affected by acute physiological processes.

Failure to demonstrate increased secretion as judged by absence of any rise in peripheral venous insulin concentration was illustrated. Within minutes of stimulating the thoracic dorsal vagus trunk of the dog, *Kaneto* et al. (21) found that insulin concentration in portal venous plasma rose from 200 to 300 uU/ml but observed no significant change in the femoral vein (14 to 17 uU/ml). The extensive dilution of insulin upon entering the systemic circulation and removal of insulin by the liver could mask actual increases in pancreatic secretion of insulin.

As a direct measurement of insulin secretion in vivo, we cannulated the carotid artery for arterial inflow and the portal vein was cannulated to collect the venous effluent. In this design of experiments ATP has a marked effect on insulin secretion, while it does not affect the blood glucose level. Thus the insulin increased from 98 ± 17 to 273 ± 80, two minutes after glucose injection. While it increased from 127 ± 30 to 775 ± 23 uU/ml after glucose and ATP (Table 4). The generally rapid effect of ATP on insulin secretion clearly supports the suggestion that ATP has its site of action restricted to a surface receptor.

Burnstock et al. (9) suggested that ATP exerts its action directly on the smooth muscle membrane through specific "purinergic" receptors.

From the present results it is also clear that ATP closely mimics the response to cholinergic nerve stimulation on insulin secretion (21). But the effect of ATP is more pronounced. It is suggested therefore that purinergic nerve stimulation is more specific for insulin secretion.

In alloxan diabetic rats, it has been found that the neuron terminals in contact with B cells are enlarged and "dystrophic" after the administration of alloxan (41).

In the present work the glucose tolerances of alloxan diabetic rats were abnormal, and despite the high blood glucose levels, the plasma insulin were low and did not increase after glucose stimulation (Table 3).

Table 2.

		Intravenous glucose tolerance				
		0	5	10	30	60 min
Alloxan diabetic rats	mean	121	303	316	282	273
	SEM	± 13	± 29	± 19	± 23	± 25
	n	9	8	9	9	9
Alloxan diabetic rats + ATP	mean	118	263	264	207	201
	SEM	± 8	± 25	± 26	± 28	± 24
	n	5	5	5	5	4
	p	>.05	>.05	>.05	>.05	>.05
		Insulin				
		0	5	10	30	60 min
Alloxan diabetic rats	mean	42	50	52	53	48
	SEM	± 6	± 4	± 5	± 4	± 6
	n	10	9	9	9	9
Alloxan diabetic rats + ATP	mean	46	142	101	124	116
	SEM	± 10	± 22	± 17	± 29	± 16
	n	5	5	5	5	5
	p	>.05	<.01	<.01	<.01	<.01
		G/I				
		0	5	10	30	60 min
Alloxan diabetic rats	mean	2.8	6.0	6.0	4.9	5.6
	SEM	± .8	± .8	± .9	± .8	± .8
	n	9	9	8	9	8
Alloxan diabetic rats + ATP	mean	2.5	1.8	2.6	1.9	1.7
	SEM	± .3	± .4	± .9	± .7	± .5
	n	5	5	5	5	5
	p	> .05	< .01	< .01	< .01	< .01

ATP can also increase insulin secretion significantly from the still functioning B-cells in the partially alloxan diabetic rats (Table 2). The effect of ATP was more pronounced on the insulinogenic index or G/I ratio of both normal and alloxan diabetic rats (Tables 1 and 2). All G/I values for the diabetic group were higher than those of the control and the diabetic group treated with ATP. This suggests an insufficient insulin secretion in the diabetic group which becomes normal by ATP.

By comparing the insulin concentrations in control and alloxan diabetic rats it could be seen that normal rats responded to massive hyperglycemia after glucose injection with immediate and maximal outpouring of insulin, in contrast to diabetic rats which have no initial response at all. This indicates that the sensitivity of the diabetic rats to glucose is decreased. Thus ATP could increase the sensitivity of the diabetic rats to glucose.

Table 3.

		Intravenous glucose tolerance					
		0	2	5	10	30	60 min
Control rats	mean	96	381	359	307	223	165
	SEM	± 7	± 29	± 24	± 20	± 11	± 17
	n	11	11	10	11	11	11
Alloxan diabetic rats	mean	121	407	303	316	282	273
	SEM	± 13	± 24	± 29	± 19	± 23	± 25
	n	9	8	8	9	9	9
	p	>.05	>.05	<.05	>.05	<.05	<.01
		Insulin					
		0	2	5	10	30	60 min
Control rats	mean	46	194	81	118	69	64
	SEM	± 7	± 40	± 8	± 23	± 10	± 8
	n	8	9	8	9	9	7
Alloxan diabetic rats	mean	42	52	50	52	53	48
	SEM	± 6	± 6	± 4	± 5	± 4	± 6
	n	10	10	9	9	9	9
	p	>.05	<.01	<.05	<.01	>.05	>.05

Table 4.

				Glucose in the portal vein				
		before narcosis	after narcosis	0	2	5	10	30 min
Control rats	mean	77	78	50	180	235	235	131
	SEM	± 3	± 4	± 5	± 43	± 42	± 35	± 72
	n	6	4	6	6	6	6	5
Control rats + ATP	mean	80	87	48	218	200	192	119
	SEM	± 3	± 6	± 2	± 32	± 12	± 12	± 16
	n	6	6	7	6	7	7	5
	p	>.05	>.05	>.05	>.05	>.05	>.05	>.05
		Insulin in the portal vein						
		0	2	5	10	30 min		
Controls rats	mean	98	273	292	330	358		
	SEM	± 17	± 80	± 96	± 103	± 122		
	n	7	7	7	7	5		
Control rats + ATP	mean	127	775	778	783	766		
	SEM	± 30	± 23	± 36	± 40	± 31		
	n	6	6	9	9	5		
	p	>.05	<.001	<.001	<.001	<.001		

Previously *Cerasi* et al. (11) suggested that the defect in the diabetic and prediabetic beta cell is probably limited to the specific glucose receptor, the sensitivity of which to glucose is decreased.

In the mechanism involved in insulin secretion, energy expenditure is obviously necessary. The insulin stimulatory effect normally produced by glucose is abolished in the presence of cyanide, 2,4-dinitrophenol or anoxia, all of which interfere with oxidative processes (27).

Malaisse et al. (27) suggested that insulin secretion could be possibly initiated by ATP. *Larkins* and *Martin* (23) suggestes that the role of ATP in the intracellular translocation of insulin may be three-folded.

a) For the translocation of newly synthesized protein from the rough surfaced endoplasmic reticulum to the Golgi complex by way of Golgi vesicles.
b) for the formation of mature storage granules and
c) for the movement of granules to the plasma membrane during exocytosis.

Glucose is the major physiological stimulus for insulin secretion. A variety of drugs and hormones can either inhibit or stimulate insulin release in the presence of glucose. Many investigations have shown that when the glucose concentration is altered in vivo or in vitro there is parallel change in insulin release from pancreatic tissue (30). In studies with the perfused pancreas, "pulse" administration of glucose leads to an almost immediate transient release of insulin (44).

The changes in blood insulin during oral glucose tolerance tests are well defined. As the concentration of sugar in the blood reaches its peak, so does the concentration of insulin, and both return to base line within 2½ hours. The peak concentration of insulin reached is proportional to the amount of sugar ingested (40). Less well defined is the pattern of insulin release that follows intravenous injection of sugar. The peak is probably reached very rapidly; *Soeldner* et al. (42) found a peak to be achieved within 1 min, while *Seltzer* (40) found the peak response within 5 min.

It has been shown that glucose absorbed through the intestine is a more potent stimulus than glucose given by intravenous infusion. *McIntyre* and co-workers (31) compared the response to continuous infusion of 90 g per hour of glucose given either into a vein or through a tube in the jejunum. The intrajejunal infusion produced a smaller elevation of the blood sugar; nevertheless, insulin production was greater. In fact, the peak concentration of insulin can be twice as high during intrajejunal infusion as during intravenous infusion of the same glucose load. *Elrick* et al. (17) also compared insulin production after oral and intravenous glucose and found the former to be more potent stimulus.

One interpretation of these findings as suggested by *Dupre* (14) is that a hormone from the gastrointestinal tract stimulates the release of insulin. However after recognition that purinergic nerves are strongly represented in the gastro-intestinal tract one may suggest that glucose evokes its insulinogenic signal through purinergic nerves.

Glucose appears to act directly on the secretory mechanism, for quantities of dinitrophenol sufficient to block insulin synthesis do not block the

initial secretory response (19). *Cerasi* et al. (11) suggested that glucose evokes its insulinogenic effect by acting on a specific receptors on the beta cell membrane.

Also agents (e.g. plorizin and 3-0-methyl glucose) that inhibit active glucose transport in other tissues have no effect on the stimulation of insulin secretion by glucose (13). This supports the suggestion that glucose has its site of action restricted to a surface receptor.

Hellma et al. (20) using obese hyper-glycemic mice found considerably more ATP in the islets than in the rest of the pancreas after stimulation with glucose. ATP could be coming from purinergic nerves.

Release of ATP or its metabolites during purinergic nerve stimulation has been demonstrated (39, 43). The amount of ATP increases 2–6fold during isometric responses to purinergic nerve stimulation. No significant release of ATP was detected during direct muscle stimulation (10).

One of the most important criteria for a substance to be established as a neurotransmitter is to show that it is released during nerve stimulation (16). The finding that glucose stimulation increases ATP in pancreatic islet (20) provide evidence for a purinergic innervation of the islet cells. Cholinergic innervation of the islets could also play a role in the regulation of insulin secretion (12, 32). Acetylcholine enhances the release of insulin (28, 29). The pancreatic B-cell has been shown to have both α and β adrenergic receptors (33).

Epinephrine inhibits the insulin secretion ordinarily stimulated by hyperglycemia (47).

It could be concluded therefore that purinergic, cholinergic and adrenergic innervation of the islets regulate insulin secretion.

Similarly microelectrode recording from intestinal single muscle cells has demonstrated that there is multiple innervation by cholinergic, adrenergic and "purinergic" nerves (2). In fact, as many as three or more nerve terminals (sometimes of different types) have been reported close to the same islet secretory cell (18).

Also it may be postulated that the insulin stimulatory effect normally produced by glucose is through purinergic innervation. And it could be possible that one of the causes of diabetes is a defect in the purinergic innervation of the islet cells thus the sensitivity of the islets to glucose is decreased. ATP is considered the principal transmitter released from purinergic nerves causing insulin secretion. The presence of enzymes (ATPase) which inactivate ATP in the insulin containing granules (24) support the suggestion that ATP is the principal transmitter released from purinergic nerves causing insulin secretion.

Summary

In adrenalectomized rats the effect of i.v. injection of glucose and ATP on insulin changes in external jugular vein was determined in normal and alloxan diabetic animals.

In another set of experiments the direct effect of ATP on insulin secretion was investigated. Glucose and ATP were injected in the carotid artery and the blood samples were withdrawn from the portal vein.

In these experiments there was immediate and excessive production of insulin release in the portal vein after ATP injection in the carotid artery.

In alloxan diabetic rats, despite the high blood glucose levels, the plasma insulin was low and did not respond to glucose stimulation.

ATP could increase the sensitivity of the diabetic rats to glucose.

The possible role of purinergic nerves in insulin secretion is discussed.

It is concluded that multiple innervation of the islets by purinergic, cholinergic and adrenergic nerves, regulate insulin secretion.

It is suggested that:

1. Purinergic nerve stimulation is more specific for insulin secretion.
2. ATP is considered the principal transmitter released from purinergic nerves causing insulin secretion.
3. The insulin stimulatory effect normally produced by glucose is through purinergic nerves.
4. It could be possible that one of the causes of diabetes is a defect in the purinergic innervation of the islet cells thus the sensitivity of the islets to glucose is decreased.

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