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Research Article

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Cholinergic Modification of Glucose-Induced Biphasic Insulin Release in Vitro

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ABSTRACT An in vitro system for perfusion of rat pancreatic islets has been utilized to define the effects of cholinergic agents on the dynamics of insulin release. In the absence of glucose the effects of either acetylcholine or acetyl- β -methylcholine were minimal at concentrations up to 10^{-5} mM. In the presence of low glucose concentration (2.4 mM), both of the muscarinic agents produced dose-dependent biphasic insulin release. Under these conditions significant insulin release was observed over both phases at concentrations of the muscarinic agents as low as 10^{-8} mM. Further, the dose response curves relating muscarinic concentration to the total amount of insulin released in each of the two phases showed lack of parallelism between the curves. Nicotinic acid in concentrations up to 10^{-5} mM had no effect on insulin release in the presence of 2.4 mM glucose. When the glucose concentration was increased to 16.4 mM, the effects of the muscarinic agents were significantly less than those observed in the presence of 2.4 mM glucose. This held true whether the effect was defined as absolute increment due to the muscarinic agent or as percentage of enhancement. Atropine inhibited insulin release induced by both acetylcholine and by 16.4 mM glucose. These data indicate that cholinergic stimulation can play a significant role in modifying insulin release patterns.

INTRODUCTION

Since the mid-1800's considerable interest has centered on the role of the central and autonomic nervous systems in modifying glucose homeostasis (1-10). Pertinent to this study is the evidence that has accumulated in support of a role for the autonomic nervous system in modifying insulin release from the pancreas. This has included the demonstration that epinephrine can inhibit

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insulin release in vitro (11, 12) and in vivo (13, 14), an effect ascribed to α -adrenergic receptor activation (12, 15, 16), possibly mediated through modification in calcium flux (15). In contrast to the α -adrenergic effect, a stimulatory effect of β -adrenergic agents on insulin release has been demonstrated (12, 17, 18). Further, prior exposure of pancreatic pieces to the adrenergic agents epinephrine, norepinephrine (12), and diazoxide (19) has resulted in enhancement of the insulin response to subsequent glucose challenge, an effect apparently dependent on the integrity of both α - and β -adrenergic receptors.

Data have also accumulated to support a role for the parasympathetic autonomic nervous system in modifying insulin release. Acetylcholine stimulates insulin release in vitro (11, 15), as defined in a static system, and cholinergic agents (20, 21) including nicotinic acid (22) stimulate insulin release in vivo. Further, both vagal stimulation (23, 24) and pancreatic nerve stimulation (25) result in an increase in insulin release. Finally, atropine has been shown to inhibit conditioned insulin release (26), insulin release secondary to vagal stimulation (20, 21), and to inhibit insulin release in response to oral glucose but not that induced by intravenous glucose (27).

This study was designed to determine the effects of cholinergic agents on the two phases of glucose-induced insulin release as defined in an in vitro dynamic system, to define any dose dependency of these effects, and to determine the effect of the glucose concentration on these responses.

METHODS

This study utilized a modification of the perfusion system previously described (12, 19, 28, 29). In the present system pancreatic islets were used in lieu of pancreatic pieces: although this is more complicated, some advantages have accrued. First, the basal insulin release is reduced. Second, the requirement for Trasylol in the system is obviated. And third, the reproducibility appears to be enhanced. Islets were obtained by a modification of the methods of Lacy

TABLE I
Effect of AcCh, MCh, and Nicotinic Acid on Insulin Release in the Absence of Glucose

Agent	Concentration	First phase	P vs. C	Second phase	P vs. C
		$\bar{x} \pm \text{SEM}$		$\bar{x} \pm \text{SEM}$	
	mM	ng		ng	
Control (C)		1.5 \pm 0.2		18.7 \pm 2.2	
AcCh	2.7 \times 10 ⁻⁶	1.2 \pm 0.1	NS	23.2 \pm 1.4	NS
	5.4 \times 10 ⁻⁵	2.4 \pm 0.3	NS	37.7 \pm 2.0	<0.01
MCh	2.7 \times 10 ⁻⁶	1.32 \pm 0.2	NS	20.4 \pm 1.4	NS
	5.4 \times 10 ⁻⁵	1.2 \pm 0.1	NS	25.4 \pm 2.1	NS
Nic A.	2.7 \times 10 ⁻⁶	1.4 \pm 0.3	NS	20.4 \pm 1.4	NS
	5.4 \times 10 ⁻⁵	1.5 \pm 0.4	NS	17.5 \pm 1.8	NS

A minimum of six experiments were performed for each study. In those experiments in which a flat response was obtained, the phases were arbitrarily defined by reference to corresponding experiments performed in the presence of 50% glucose. Nic A, nicotinic acid.

and Kostianovsky (30) and of Montague and Cook (31). In brief, pancreases were removed from fasting (16 h) male Wistar rats weighing 200–250 g, under pentothal anaesthesia. The abdomen was opened and the common bile duct isolated and tied at its entrance to the duodenum. The duct was then cannulated with a 23 G scalp vein needle through which 5–10 ml of Hanks' solution was injected to distend the pancreas. The pancreas was then dissected free, in toto, minced with scissors into pieces of approximately 1–2 mm in diameter, and washed. Three such minced pancreases were incubated at 37°C for 16–20 min in a metabolic shaker with Krebs-Ringer bicarbonate (KRB)¹ to which collagenase was added (5 mg/20 ml). During incubation the pancreatic pieces were gassed with 95% O₂ and 5% CO₂.

Islets were recovered from the digested material with Pasteur pipettes and a binocular dissecting microscope. 40 islets were placed in each of two perfusion chambers; the time lapse from removal of the pancreases to this point was 25–30 min.

Buffer flow through the chambers was maintained at 2.0–2.2 ml/min by means of a double-channel continuous infusion pump. Buffers and perfusion chambers were kept at 37°C by means of a closed water jacket system under thermostatic control. Buffers were continuously gassed with 95% O₂ and 5% CO₂, and the pH was maintained at 7.4. The KRB buffer used contained 0.5 g/100 ml bovine serum albumin.

In each of these experiments, a prestimulation period of 25 min perfusion with KRB plus glucose, 50 mg/100 ml preceded the experimental periods of 60 min. The anatomical integrity of the islets after 2 h perfusion was confirmed by electron microscopy, and physiological responsiveness remained intact for up to 4 h of perfusion.

Effluent samples were collected continuously with a fraction collector mounted in a cold box. Samples were collected representing (a) successive 1-min periods of flow over the latter 5 min of the prestimulation period (b) successive 30-s periods of flow for the first 6 min of the test period and (c) successive 1-min periods of flow for the latter 54 min of the test period. The immunoreactive insulin

content of the effluent samples was measured by a modification of a double antibody radioimmunoassay technique (32).

Experiments were designed to determine the dynamics of insulin release in the presence of (a) glucose, 0, 2.4, and 16.4 mM; (b) acetylcholine (AcCh), over the range 2.7 \times 10⁻⁶–2.7 \times 10⁻⁵ mM, in the absence of glucose and in the presence of 2.4 or 16.4 mM glucose; (c) acetyl- β -methylcholine (methacholine, MCh) over the range 2.7 \times 10⁻⁶–5.4 \times 10⁻⁴ mM, also in the presence and absence of glucose, and (d) nicotinic acid (2.7 \times 10⁻⁶–5.4 \times 10⁻⁴ mM), similarly in the presence and absence of glucose. Finally, the effect of atropine, 1.1 \times 10⁻⁴ mM, was determined on insulin released by 16.4 mM glucose and on insulin release induced by AcCh, 1.1 \times 10⁻⁴ mM, plus 2.4 mM glucose. In these experiments control and test experiments were run in parallel, where appropriate.

The first and second phases of the biphasic responses were defined by dynamic studies and the total amounts of insulin released during these phases were calculated by simple addition for the first phase (over which all samples were assayed) and by assessment of the area under the insulin release rate: time plot for the second phase (not all samples were assayed— all for the first 5 min, then every fifth sample to 60 min). This method provides an accurate assessment of the total amount of insulin released in the second phase, as confirmed in this and in previous studies, by measuring the insulin content of all the perfusate collected over the appropriate period (12, 19). For those studies that exhibited flat responses, phases were arbitrarily defined by reference to appropriate matching experiments (12, 19). Statistics used either the Wilcoxon rank test and/or the Student t test.

RESULTS

As indicated in Table I, in the absence of glucose, none of the agents used (in concentrations up to 5.4 \times 10⁻⁵ mM) produced any significant acute (first phase) insulin release above that seen with buffer alone. Further, neither MC nor nicotinic acid produced enhancement of insulin release above basal during the latter portions of the perfusion. AC, 5.5 \times 10⁻⁵ mM, did enhance this

¹ Abbreviations used in this paper: AcCh, acetylcholine; KRB, Krebs-Ringer bicarbonate; MCh, acetyl- β -methylcholine (methacholine).

TABLE II
Effect of AcCh, MCh, and Nicotinic Acid on the Two Phases of Insulin Release
in the Presence of 2.4 mM Glucose

Agent	Concentration	First phase	P vs. C	Second phase	P vs. C
		$\bar{x} \pm \text{SEM}$		$\bar{x} \pm \text{SEM}$	
	<i>mM</i>	<i>ng</i>		<i>ng</i>	
Control (C)	—	0.9 ± 0.2		4 ± 0.8	
AcCh	2.7 × 10 ⁻⁸	5.2 ± 0.2	<0.01	19 ± 0.9	<0.01
	2.7 × 10 ⁻⁷	7.7 ± 0.2	<0.01	35 ± 1.3	<0.01
	2.7 × 10 ⁻⁶	9.6 ± 0.3	<0.01	58 ± 1.7	<0.01
	5.4 × 10 ⁻⁶	11.4 ± 0.6	<0.01	133 ± 2.6	<0.01
	2.7 × 10 ⁻⁵	14.0 ± 0.6	<0.01	165 ± 7.1	<0.01
	5.4 × 10 ⁻⁵	14.5 ± 0.3	<0.01	173 ± 3.2	<0.01
	1.1 × 10 ⁻⁴	24.5 ± 1.2	<0.01	182 ± 0.4	<0.01
	2.7 × 10 ⁻⁴	48.2 ± 0.6	<0.01	278 ± 11.5	<0.01
MCh	2.7 × 10 ⁻⁶	130.0 ± 1.2	<0.01	407 ± 3.2	<0.01
	2.7 × 10 ⁻⁶	5.1 ± 0.4	<0.01	129 ± 2.2	<0.01
Nic A	5.4 × 10 ⁻⁵	23.5 ± 0.3	<0.01	202 ± 6.1	<0.01
	2.7 × 10 ⁻⁶	0.7 ± 0.2	NS	5.2 ± 0.6	NS
	5.4 × 10 ⁻⁵	0.6 ± 0.1	NS	6.5 ± 0.5	NS

A minimum of six experiments were performed for each study.
Nic A, nicotinic acid.

secondary phase of insulin release (latter 50 min of perfusion). It is of note that insulin release in the absence of glucose was observed to be greater than in the presence of 2.4 mM glucose (Tables I and II). This is associated with morphological evidence of bursa-derived cell damage when cells are perfused for longer than 2 h in the absence of glucose.

In the presence of 2.4 mM glucose both AC and MC induced biphasic patterns of insulin release (Figs. 1 and 2) in concentrations as low as 2.7 × 10⁻⁸ mM (Table II). These effects were significant for both phases of insulin release (Table II). Further, the effects were dose-dependent for both agents. In contrast, nicotinic acid did not affect either the first or second phase of

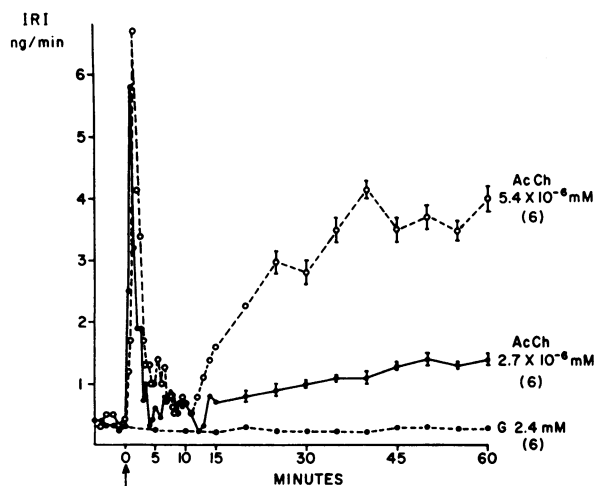


FIGURE 1 Effect of AcCh on the dynamics of insulin release (IRI) in the presence of 2.4 mM glucose (G). Numbers in parentheses refer to the number of experiments, points and bars represent the means and SEM's. In these experiments a preliminary wash period of 25 min preceded the experimental period of 60 min of continuous perfusion with AcCh in the concentrations indicated.

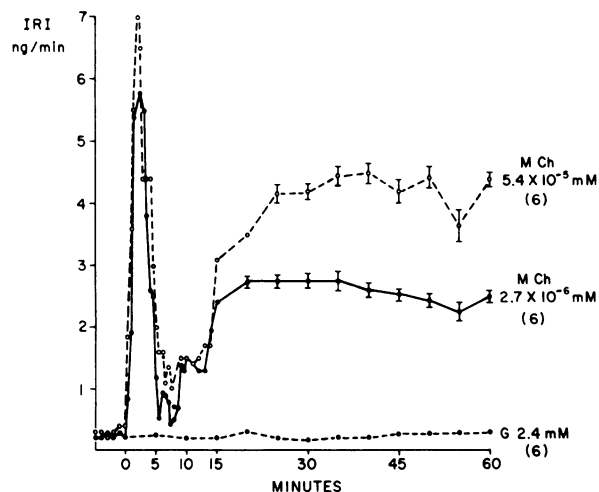


FIGURE 2 Effect of MCh on the dynamics of insulin release (IRI) in the presence of 2.4 mM glucose (G). Numbers in parentheses refer to the number of experiments, points and bars represent the means and SEM's. In these experiments a preliminary wash period of 25 min preceded the experimental period of 60 min of continuous perfusion with MCh in the concentrations indicated.

TABLE III
Effects of AcCh, MCh, and Nicotinic Acid (Nic. A) on the two phases of
Insulin Release in the Presence of 16.4 mM Glucose

Agent	Concentration	First phase $\bar{x} \pm \text{SEM}$	P vs. C	Second phase $\bar{x} \pm \text{SEM}$	P vs. C
	mM	ng		ng	
Control (C)	—	13.8 ± 0.9		203 ± 7.5	
AcCh	2.7×10^{-6}	13.6 ± 0.2	NS	216 ± 4.3	NS
	5.4×10^{-5}	16.3 ± 0.8	NS	290 ± 3.0	<0.01
	2.7×10^{-3}	138.0 ± 5.0	<0.01	503 ± 15.0	<0.01
MCh	2.7×10^{-6}	17.9 ± 0.8	<0.05	216 ± 1.5	NS
	5.4×10^{-5}	17.8 ± 0.8	<0.05	237 ± 1.3	<0.01
Nic A	2.7×10^{-6}	13.4 ± 0.6	NS	206 ± 2.3	NS
	5.4×10^{-5}	12.6 ± 0.2	NS	205 ± 1.9	NS

A minimum of six experiments were performed for each study.

insulin release in the presence of 2.4 mM glucose (Table II).

Comparison of the dose response curves relating log [AcCh] to total amount of insulin released during the first phase of insulin release with that relating log (AcCh) to the total amount of insulin released during the second phase of insulin release reveals lack of parallelism of the two curves, with an acute increase in responsiveness of the second phase occurring one to two orders of AcCh concentration lower than the concentration at which a similar increase in responsiveness of the first phase is observed (Fig. 3).

In contrast to the marked effects of muscarinic agents in the presence of 2.4 mM glucose, either AcCh or MCh had much less effect when added to 16.4 mM glucose (Table III). Thus, AcCh 2.7×10^{-6} mM had no effect on either the first or second phases of insulin release induced by 16.4 mM glucose, whereas 5.4×10^{-5} mM AcCh produced significant enhancement of only the secondary phase. Mc had minimal effect on both phases of glucose-induced insulin release. This was statistically significant for the first phase at both 2.7×10^{-6} and 5.4×10^{-5} mM, and for the second phase only at 5.4×10^{-5} mM. Nicotinic acid had no effect on insulin release induced by 16.4 mM glucose in concentrations up to 5.4×10^{-5} mM. The marked differences noted in the response to muscarinic agents due to changes in glucose concentration are illustrated by comparison of Tables I, II, and III. The increments observed in both phases of insulin release induced by both concentrations (2.7×10^{-6} mM and 5.4×10^{-5} mM) of the muscarinic agents were greater in the presence of 2.4 mM glucose than those observed in either the absence of glucose or in the presence of 16.4 mM glucose (Fig. 4). It was not possible to analyze this statistically.

Atropine, 1.1×10^{-4} mM, inhibited both phases of insulin release in response to 1.1×10^{-4} mM AcCh in the

presence of 2.4 mM glucose and also inhibited both phases of the insulin release in response to 16.4 mM glucose (Table IV). The insulin release in response to 1.1×10^{-4} mM AcCh plus 2.4 mM glucose was comparable to that induced by 16.4 mM glucose. Addition of the same concentration of atropine (equimolar with AcCh) produced similar inhibition of the second phase of insulin in both situations (56.3% for AcCh + mM 2.4 glucose and 57.4% for mM 16.4 glucose); however, the atropine-induced inhibition of cholinergically induced first phase exceeded that of the glucose-induced first phase (90.6% vs. 76.1%).

Finally, comparison of the effects on insulin release of increasing the glucose concentration from 2.4 to 16.4 mM

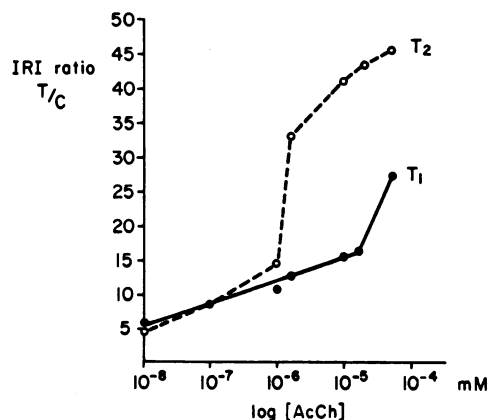


FIGURE 3 Effect of increasing the AcCh concentration on the total amount of insulin released (IRI) during the first and second phases of insulin release in the presence of 2.4 mM glucose. Phases were defined by reference to Fig. 1. Vertical axis represents the ratios of insulin released in first phase (T_1) or second phase (T_2) in response to AcCh to that released over comparable periods in the presence of 2.4 mM glucose alone (C). The horizontal axis represents the log of the molar AcCh concentrations.

TABLE IV
Effects of Atropine on the Two Phases of Insulin Release Induced by 2.4 mM Glucose plus 1.1×10^{-4} AcC, and by 16.4 mM Glucose

G	Agents		First phase $\bar{x} \pm \text{SEM}$	P values	Second phase $\bar{x} \pm \text{SEM}$	P values
	AcCh	Atr				
	<i>mM</i>		<i>ng</i>		<i>ng</i>	
2.4	—	—	0.9 ± 0.2		4.0 ± 0.8	
2.4	1.1×10^{-4}	—	24.5 ± 1.2	<0.01	182 ± 0.4	<0.01
2.4	1.1×10^{-4}	1.1×10^{-4}	2.3 ± 0.03		79.6 ± 2.3	
16.4	—	—	13.8 ± 0.09	<0.01	203 ± 7.5	<0.01
16.4	—	1.1×10^{-4}	3.3 ± 0.15		86.4 ± 1.8	

A minimum of six experiments were performed for each study.
 G, glucose; Atr, atropine.

with the effects of adding AcCh reveal that the insulin released in the first phase by 16.4 mM glucose is approximately equivalent to that induced by 2.7×10^{-5} mM

AcCh (first phase 13.8 ± 0.9 cf. 14.0 ± 0.6 ng insulin; second phase 203 ± 7.5 cf. 165 ± 7.1 ng insulin). That is, acute insulin release in the presence of glucose is more sensitive to AcCh addition than to increases in glucose concentrations, by some orders of magnitude.

DISCUSSION

These results confirm the results of previously reported *in vitro* studies in which insulin release was stimulated by cholinergic agents (11, 15). Furthermore, they demonstrate that the direct effects of cholinergic agents are confined to those with muscarinic activity, nicotinic acid being ineffective in stimulating insulin release in either the presence or absence of glucose. This suggests that the enhancement in insulin release observed *in vivo* with nicotinic agents (22) may be related to ganglion stimulation.

Although these results may have been anticipated, the following observations are of particular interest. For this system and in the concentrations used, muscarinic agents required the presence of glucose to produce significant insulin responses. Further, muscarinic agents in very low concentration (e.g. 2.7×10^{-11} mM AcCh) could produce a biphasic insulin response in the presence of nonstimulating concentrations of glucose. That is, the insulin release mechanism, at least in the presence of low glucose concentrations, is particularly sensitive to cholinergic agents. Furthermore, it is evident that the islets were considerably more responsive, in terms of acute response, to the effect of muscarinic agents than to the effects of high glucose concentration, the acute response to either AcCh or MCh at concentrations of the order of 10^{-4} mM being considerably greater than that seen with the glucose concentration increased to the order of 16 mM. This type of effect could have some significance in light of *in vivo* studies demonstrating enhanced insulin release when glucose is given intraduodenally as compared with intravenously (33, 34), and the effect of cholinergic antagonists on centrally in-

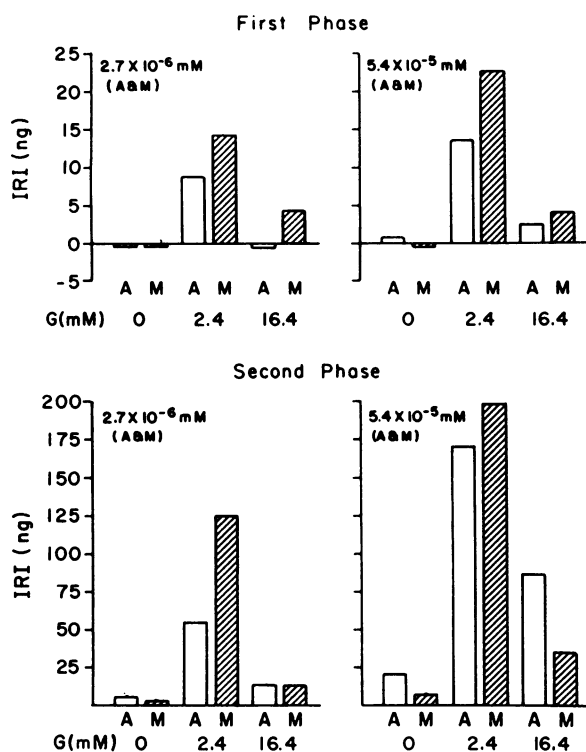


FIGURE 4 Increments induced in the first and second phases of insulin release (IRI) by AcCh (A) and MCh (M) in the absence of glucose and in the presence of 2.4 and 16.4 mM glucose (G). The vertical axis represents the mean increment in insulin release (IRI) (in nanograms) induced by the addition of AcCh (A) or MCh (M) to buffer containing, 0, 2.4, or 16.4 mM glucose. The upper two panels represent first phase increments, the lower two panels, second phase increments. Glucose concentrations are indicated on the horizontal axis. In all instances the maximum increments were observed in the presence of 2.4 mM glucose.

duced insulin release (26). That is, it is possible that reflex functions in response to food, local or central, may influence insulin release. Furthermore, the marked sensitivity of the islet to cholinergic agents, particularly the acute response, is such as would be expected if "anticipation" were necessary to elicit normal insulin responses. In this context it has been demonstrated that insulin release may be provoked both hypnotically (35) and by sham feeding (36, 37). These observations, about acute responsiveness of the bursa-derived cell to AcCh and the apparent presence of reflex pathways, have potential significance in that there appears to be a "delay" in insulin release in diabetics (38-40).

These results may also be considered in light of previous data suggesting that while both phases of glucose induced insulin release may be influenced by similar agents, there are differences, at least in emphasis, in the major mechanisms through which these phases are generated (12, 19, 29, 41, 42). Thus, it is observed that while muscarinic agents can stimulate biphasic insulin release in the presence of glucose, it is evident that the dose-response relationships are different for the two phases. The agents used appear to be more effective at lower concentrations in generating the second versus the first phase. Further, atropine at equimolar concentration with AcCh produced differing degrees of inhibition of the two phases of AcCh-induced insulin release (90% inhibition of the first versus 56% inhibition of the second). In the presence of near-maximal stimulation of insulin release by glucose, neither AcCh nor MCh provided very much increment in either phase of insulin release except at very high concentrations (2.7×10^{-8} mM). That is, these agents were less effective insulinogues in the presence of high glucose concentrations. This type of response may be expected if the major role of parasympathetically mediated insulin release was in the acute or reflex stage, but it is a response that would allow for further modification by parasympathetic mechanisms should the need arise.

Finally, atropine in the concentration used (equimolar with that concentration of AcCh that produces comparable insulin release to that obtained with 16.4 mM glucose) not only inhibited insulin release induced by AcCh but also inhibited glucose-induced insulin release. While atropine was less effective in inhibiting the first phase of insulin release induced by glucose than by AcCh, it was equally effective in inhibiting the secondary phases of insulin release induced by either agent. These observations raise questions regarding the specificity of the effect of atropine on B-cell function and hence on the validity of the assumption that inhibition of insulin release by atropine is solely due to inhibition of a cholinergically mediated process. Furthermore, the observations suggest that AcCh and glucose share, at least in part, a common primary pathway in initiating

insulin release and/or that their stimulatory effect is dependent on a common "permissive" event that is blocked by atropine. These latter possibilities are supported by the observation that AcCh produced a lesser increment in insulin release at higher glucose concentrations than at lower glucose concentrations.

In summary. Insulin release *in vitro* appears to be particularly sensitive to cholinergic stimulation; the two phases of insulin release exhibit different orders of sensitivity to cholinergic agents and to the inhibitory effect of atropine; the effects of cholinergic stimulation are dependent on the glucose concentration; and that the inhibitory effects of atropine on insulin release are not limited to inhibition of cholinergic insulinogogue activity.

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