Glucose-, calcium- and concentration-dependence of acetylcholine stimulation of insulin release and ionic fluxes in mouse islets

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Mouse islets were used to define the glucose-dependence and extracellular Ca^{2+} requirement of muscarinic stimulation of pancreatic β -cells. In the presence of a stimulatory concentration of glucose (10 mM) and of Ca^{2+} , acetylcholine (0.1–100 μM) accelerated ^3H efflux from islets preloaded with *myo*-[^3H]inositol. It also stimulated $^{45}\text{Ca}^{2+}$ influx and efflux, $^{86}\text{Rb}^{+}$ efflux and insulin release. In the absence of Ca^{2+} , only 10–100 μM -acetylcholine mobilized enough intracellular Ca^{2+} to trigger an early but brief peak of insulin release. At a non-stimulatory concentration of glucose (3 mM), 1 μM - and 100 μM -acetylcholine increased $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^{+}$ efflux in the presence and absence of extracellular Ca^{2+} . However, only 100 μM -acetylcholine marginally increased $^{45}\text{Ca}^{2+}$ influx and caused a small, delayed, stimulation of insulin release, which was abolished by omission of Ca^{2+} . At a maximally effective concentration of glucose (30 mM), 1 μM - and 100 μM -acetylcholine increased $^{45}\text{Ca}^{2+}$ influx and efflux only slightly, but markedly amplified insulin release. Again, only 100 μM -acetylcholine mobilized enough Ca^{2+} to trigger a peak of insulin release in the absence of Ca^{2+} . The results thus show that only high concentrations of acetylcholine ($\geq 10 \mu\text{M}$) can induce release at low glucose or in a Ca^{2+} -free medium. β -Cells exhibit their highest sensitivity to acetylcholine in the presence of Ca^{2+} and stimulatory glucose. Under these physiological conditions, the large amplification of insulin release appears to be the result of combined effects of the neurotransmitter on Ca^{2+} influx, on intracellular Ca^{2+} stores and on the efficiency with which Ca^{2+} activates the releasing machinery.

INTRODUCTION

It has been known for two decades that vagal stimulation increases plasma insulin [1–3], and that cholinergic agents induce insulin release *in vitro* [4]. This stimulation results from a direct action of acetylcholine (ACh) on muscarinic receptors in β -cells. However, in spite of a better understanding of the transduction mechanisms of muscarinic signals, several facets of ACh effects in β -cells remain controversial or unclear.

Several studies using different types of preparations have shown that ACh stimulation of insulin release from normal β -cells is suppressed in the absence of extracellular Ca^{2+} [5–9] or in the presence of a blocker of Ca^{2+} channels [10]. These observations indicate that ACh-induced insulin release depends on the availability of extracellular Ca^{2+} and suggest that an increase in Ca^{2+} influx could at least contribute to the secretory effects of the neurotransmitter. This view is indirectly supported by the comparison of $^{45}\text{Ca}^{2+}$ efflux from islets perfused with or without Ca^{2+} , and challenged by ACh in the presence of stimulatory concentrations of glucose, leucine or tolbutamide [8,11]. It is also in keeping with the evidence that ACh increases a Ca^{2+} -dependent electrical activity in β -cells [11–13]. However, attempts to demonstrate that ACh stimulates Ca^{2+} influx have yielded conflicting results. It has been reported that ACh increases $^{45}\text{Ca}^{2+}$ uptake at a non-stimulatory concentration of glucose, while stimulating [10] or not affecting [7] insulin release. Moreover, ACh was reported to potentiate glucose-induced insulin release without increasing

$^{45}\text{Ca}^{2+}$ uptake in β -cells [7,10]. The picture was further complicated by the recent demonstration that carbamoylcholine (CCh) is able to induce insulin release (albeit transiently) from *ob/ob* mouse islets perfused with a Ca^{2+} -free medium [14]. It was also reported that the increase in cytoplasmic Ca^{2+} brought about by CCh persists in the absence of extracellular Ca^{2+} or when Ca^{2+} channels are blocked by verapamil [15]. The latter observations support the concept that a mobilization of intracellular Ca^{2+} , most likely mediated by inositol 1,4,5-trisphosphate [15–18], is essential for the stimulation of insulin release by muscarinic agents.

The glucose-dependency of muscarinic effects in β -cells also remains somewhat unclear. There is no dispute that muscarinic agonists do not induce insulin release in a glucose-free medium [12,19,20]. However, several groups have observed a stimulation in the presence of only 1.7–3 mM-glucose [5,15,20–22], whereas others consistently failed to observe such an effect at sub-stimulatory concentrations of glucose [7,8,11,16,23,24]. Finally, whereas there is general agreement that muscarinic agonists are very efficient in the presence of stimulatory concentrations of glucose, their effects in the presence of a maximally effective concentration of the sugar have not been characterized.

One possible reason for these discrepancies is the use of various preparations and of widely different concentrations of distinct cholinergic agents. In the present study, performed with normal mouse islets, we have tried to characterize better the Ca^{2+} - and glucose-dependence of muscarinic stimulation of β -cells. We compared the

Abbreviations used: ACh, acetylcholine; CCh, carbamoylcholine

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effects of various concentrations of ACh and CCh in islets perfused with a medium containing Ca^{2+} or not, and supplemented with different concentrations of glucose.

EXPERIMENTAL

All experiments were performed with islets isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), which had been killed by decapitation. The medium used was a bicarbonate-buffered solution which contained 120 mM-NaCl, 4.8 mM-KCl, 2.5 mM- CaCl_2 , 1.2 mM- MgCl_2 and 24 mM- NaHCO_3 . It was gassed with O_2/CO_2 (47:3) to maintain pH 7.4 and was supplemented with bovine serum albumin (1 mg/ml). Ca^{2+} -free solutions were prepared by replacing CaCl_2 by MgCl_2 , and the concentration of total residual Ca^{2+} was less than 10 μM . In certain experiments Ca^{2+} -free solutions were supplemented with 0.5 mM-EGTA.

For the experiments of $^{86}\text{Rb}^+$ efflux (used as tracer for K^+) or $^{45}\text{Ca}^{2+}$ efflux, islets were first loaded with the tracers during 90 min of incubation in a medium containing 10 mM-glucose. Batches of 30 islets ($^{86}\text{Rb}^+$ efflux) or 40 islets ($^{45}\text{Ca}^{2+}$ efflux) were then placed in parallel chambers and perfused at a flow rate of 1.3 ml/min [25]. ^3H efflux from islets preloaded with *myo*-[2- ^3H]inositol was measured in the same system by using batches of 50 islets for each experiment, after 2 h of loading with the tracer. Previous studies [26–29] have shown that the ^3H -labelled products released from islets preloaded with *myo*-[2- ^3H]inositol consist of free inositol (> 70 %) and inositol phosphates. Those four studies concluded that the acceleration of ^3H efflux brought about by several secretagogues, including muscarinic agonists [28], reflects phosphoinositide breakdown. During the experiments of $^{86}\text{Rb}^+$ efflux, a portion of each effluent fraction was taken for measurement of immunoreactive insulin [30], with rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark). $^{45}\text{Ca}^{2+}$ uptake by islet cells was measured as described previously [30], by using [6,6'- ^3H]sucrose as marker of the extracellular space.

Acetylcholine chloride, carbamoylcholine chloride and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were from Merck A.G. (Darmstadt, Germany). Radiochemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

All data are presented as means ($\pm$ S.E.M.) for a certain number of experiments, performed with different islet preparations. The statistical significance of the effects of the different concentrations of ACh was assessed by analysis of variance, followed by a Dunnett test.

RESULTS

Effects of ACh in the presence of 10 mM-glucose and 2.5 mM- Ca^{2+}

The effects of a wide range of ACh concentrations (0.1–100 μM) were studied in islets perfused with a medium containing 10 mM-glucose and 2.5 mM- Ca^{2+} . Fig. 1 illustrates the time courses of the responses to the lowest and highest concentrations of ACh. At 100 μM , ACh caused rapid, marked, sustained and reversible increases in $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux and insulin release. It also strongly accelerated ^3H efflux from islets prelabelled with *myo*-[2- ^3H]inositol. A 1000-fold lower concentration of ACh (0.1 μM) was already sufficient to increase $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^+$ efflux and to double insulin release (Fig. 1). Whereas the rate of ^3H efflux steadily declined in the presence of 10 mM-glucose alone, it consistently increased on addition and decreased on withdrawal of 0.1 μM -ACh. Ca^{2+} influx in islet cells, estimated by the 5 min uptake of $^{45}\text{Ca}^{2+}$, was increased by both 0.1 μM - and 100 μM -ACh (Table 1).

Fig. 2 shows the concentration-dependence of ACh effects during the initial and last minutes of a 30 min period of stimulation. Except for ^3H efflux, all measured parameters were already significantly increased by 0.1 μM -ACh, and their stimulation usually reached a maximum or was close to it at 10 μM -ACh. Fig. 2 also shows that the effects of 100 μM -CCh were similar to those of an equimolar concentration of ACh. All effects of ACh were suppressed by atropine (results not shown).

Effects of ACh in the presence of 10 mM-glucose and in the absence of Ca^{2+}

When Ca^{2+} was omitted from the perfusion medium, 100 μM -ACh still accelerated ^3H , $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^+$ efflux, and triggered insulin release. However, the amplitude and the time course of these effects were strikingly different from those observed in the presence of extracellular Ca^{2+} (compare Fig. 3 with Fig. 1). The increase in ^3H efflux was of smaller amplitude. The acceleration of $^{45}\text{Ca}^{2+}$ efflux was initially larger and then smaller, whereas that of $^{86}\text{Rb}^+$ efflux was markedly

Table 1. Effects of acetylcholine on $^{45}\text{Ca}^{2+}$ uptake by mouse islets

Batches of 10 islets were incubated for 25 min in 50 μl of medium layered on silicone oil. The medium contained the indicated concentration of glucose. Then 50 μl of the same prewarmed medium containing the tracers and supplemented or not with a double concentration of ACh was added. The incubation was terminated 5 min later by centrifuging the islets through the oil. Values are means $\pm$ S.E.M. for 10, 14 and 15 batches of islets at 3 mM-, 10 mM- and 30 mM-glucose respectively. * P < 0.05, ** P < 0.01 versus controls.

Glucose (mM)	$^{45}\text{Ca}^{2+}$ uptake (pmol/5 min per islet)			
	Controls	0.1 μM -ACh	1 μM -ACh	100 μM -ACh
3	2.15 $\pm$ 0.11	–	2.31 $\pm$ 0.13	2.56 $\pm$ 0.16*
10	4.37 $\pm$ 0.18	5.41 $\pm$ 0.21**	6.20 $\pm$ 0.22**	6.57 $\pm$ 0.19**
30	8.30 $\pm$ 0.26	–	9.53 $\pm$ 0.25**	9.25 $\pm$ 0.26**

Fig. 1. Effects of acetylcholine on ^3H efflux, $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux and insulin release from mouse islets perfused with a medium containing 2.5 mM-Ca^{2+}

Islets were preloaded with *myo*-[2- ^3H]inositol, $^{45}\text{Ca}^{2+}$ or $^{86}\text{Rb}^+$ as described in the Experimental section. The concentration of glucose (G) was 10 mM throughout, and ACh [$0.1\text{ }\mu\text{M}$ ($\circ$) or $100\text{ }\mu\text{M}$ ($\bullet$)] was added between 40 and 70 min. Control experiments without ACh are shown by the broken lines. Values are means $\pm$ S.E.M. for four to six experiments.

augmented. The stimulation of insulin release was rapid, but only transient. Total insulin response (above control release) induced by 30 min of stimulation with $100\text{ }\mu\text{M}$ -ACh in a Ca^{2+} -free medium amounted to less than 5% of the corresponding response in the presence of Ca^{2+} . It was also 50% less than the response to as little as $0.1\text{ }\mu\text{M}$ -ACh in the presence of Ca^{2+} . In the absence of Ca^{2+} , this low concentration of ACh ($0.1\text{ }\mu\text{M}$) caused a slight and

Fig. 2. Effects of various concentrations of acetylcholine or carbamoylcholine on $^{45}\text{Ca}^{2+}$ uptake by incubated mouse islets, and on ^3H efflux, $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux and insulin release from perfused islets

The concentration of glucose was always 10 mM and that of Ca^{2+} 2.5 mM . The effects on $^{45}\text{Ca}^{2+}$ uptake were measured over 5 min periods starting either with the addition of ACh (early effect; $\triangle$, $\blacktriangle$) or 25 min later (late effect; $\circ$, $\bullet$). The other experiments were performed as described in Fig. 1. For $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux and insulin release, the early effect corresponds to the mean rates of efflux or release between 42 and 46 min (2–6 min after addition of ACh), and the late effect corresponds to the mean values between 66 and 70 min. For ^3H efflux, early and late effects were measured between 43 and 46 min and between 64 and 70 min respectively. Values are means $\pm$ S.E.M. for four to six experiments, or for 14 batches of islets ($^{45}\text{Ca}^{2+}$ uptake). Black symbols denote a statistically significant difference from controls ($P < 0.05$ or less).

Fig. 3. Effects of acetylcholine on ^3H efflux, $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux, and insulin release from mouse islets perfused with a Ca^{2+} -free medium

Experimental conditions were the same as in Fig. 1, except for the absence of extracellular Ca^{2+} . Values are means $\pm$ S.E.M. for four or five experiments. Key: G, glucose; $\bullet$, 100 μM -ACh; $\circ$, 0.1 μM -ACh.

delayed increase in ^3H and $^{45}\text{Ca}^{2+}$ efflux, a marked and rapid acceleration of $^{86}\text{Rb}^+$ efflux and no change in insulin release (Fig. 3).

The concentration-dependence of ACh effects in the absence of Ca^{2+} is shown in Fig. 4. Early and late stimulations of ^3H efflux similarly increased with the concentration of ACh. A rapid acceleration of $^{45}\text{Ca}^{2+}$ efflux was produced by 1–100 μM -ACh, but a rapid phase of insulin release was triggered only by 10 μM - and 100 μM -ACh. In contrast, the small late phase of $^{45}\text{Ca}^{2+}$ efflux produced by ACh was not accompanied by signifi-

Fig. 4. Effects of various concentrations of acetylcholine or carbamoylcholine on ^3H efflux, $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^+$ efflux and insulin release from mouse islets perfused with a Ca^{2+} -free medium

The concentration of glucose was 10 mM. The experiments were performed as shown in Fig. 3. The early effects ($\triangle$, $\blacktriangle$) correspond to the mean values obtained between 43 and 46 min for ^3H efflux, 42 and 46 min for $^{86}\text{Rb}^+$ efflux, and 42 and 44 min for $^{45}\text{Ca}^{2+}$ efflux and insulin release. The late effects ($\circ$, $\bullet$) correspond to the mean values obtained between 66 and 70 min, except for ^3H efflux, where they were obtained between 64 and 70 min. Values are means $\pm$ S.E.M. for four or five experiments. Black symbols denote a statistically significant difference from controls ($P < 0.05$ or less).

cant changes in insulin release. The acceleration of $^{86}\text{Rb}^+$ efflux was very strong and already maximal at 1–10 μM -ACh. Fig. 4 also shows that the effects of 100 μM -CCh were similar to those of an equimolar concentration of

Fig. 5. Effects of acetylcholine on $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^{+}$ efflux and insulin release from mouse islets perfused with a medium containing 2.5 mM- Ca^{2+} and 3 mM-glucose

The concentration of glucose (G) was 3 mM throughout, and ACh [$1\ \mu\text{M}$ (○) or $100\ \mu\text{M}$ (●)] was added between 40 and 70 min. Control experiments without ACh are shown by the broken lines, except when indistinguishable from test experiments. Values are means $\pm$ S.E.M. for four or five experiments.

ACh. All effects of ACh were suppressed by atropine (results not shown).

Experiments were also performed in a Ca^{2+} -free medium supplemented with 0.5 mM-EGTA. All effects of $100\ \mu\text{M}$ -ACh persisted and displayed the same time course as that shown in Fig. 3. Compared with the effects measured in the absence of Ca^{2+} chelator, the amplitude of the early effects of ACh measured in the presence of EGTA amounted to 81 % for ^3H efflux, 91 % for $^{45}\text{Ca}^{2+}$ efflux, 111 % for $^{86}\text{Rb}^{+}$ efflux and 67 % for insulin release.

Effects of ACh in the presence of 3 mM-glucose

In the presence of a non-stimulatory concentration of glucose (3 mM) and 2.5 mM- Ca^{2+} , $1\ \mu\text{M}$ -ACh caused only a slight but sustained increase in $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^{+}$ efflux, and did not affect basal insulin release (Fig. 5). When ACh was used at a higher concentration ($100\ \mu\text{M}$), the acceleration of $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^{+}$ efflux was of larger amplitude, but its time course was not modified. A small, progressive and reversible increase in insulin release was

Fig. 6. Effects of acetylcholine on $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^{+}$ efflux and insulin release from mouse islets perfused with a Ca^{2+} -free medium containing 3 mM-glucose (G)

ACh [$1\ \mu\text{M}$ (○) or $100\ \mu\text{M}$ (●)] was added between 40 and 70 min. Values are means $\pm$ S.E.M. for four or five experiments.

also observed, which amounted to no more than 1.6 % of the corresponding response induced in the presence of 10 mM-glucose (Fig. 5). The high, but not the low, concentration of ACh marginally stimulated $^{45}\text{Ca}^{2+}$ uptake by islets incubated with 3 mM-glucose (Table 1).

In the absence of extracellular Ca^{2+} , the effects of both concentrations of ACh on $^{45}\text{Ca}^{2+}$ and $^{86}\text{Rb}^{+}$ efflux were augmented, but $100\ \mu\text{M}$ -ACh no longer induced insulin release (Fig. 6).

Effects of ACh in the presence of 30 mM-glucose

In the presence of a maximally effective concentration of glucose (30 mM), $1\ \mu\text{M}$ - and $100\ \mu\text{M}$ -ACh induced a similar increase in $^{45}\text{Ca}^{2+}$ efflux, but the acceleration of $^{86}\text{Rb}^{+}$ efflux was still dose-dependent (Fig. 7). The insulin response to $1\ \mu\text{M}$ -ACh was 85 % of the response to $100\ \mu\text{M}$ -ACh, the latter being similar to (95 %) and the former being larger than (195 %) the corresponding responses measured at 10 mM-glucose. Both concentrations of ACh augmented the already high rate of $^{45}\text{Ca}^{2+}$ influx by 10–15 % (Table 1).

In the absence of extracellular Ca^{2+} , ACh triggered a biphasic efflux of $^{45}\text{Ca}^{2+}$, with a prominent rapid phase followed by a considerably smaller sustained phase, the

Fig. 7. Effects of acetylcholine on $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^{+}$ efflux and insulin release from mouse islets perfused with a medium containing 2.5 mM- Ca^{2+} and 30 mM-glucose

The concentration of glucose (G) was 30 mM throughout, and ACh [$1\ \mu\text{M}$ (○) or $100\ \mu\text{M}$ (●)] was added between 40 and 70 min. Control experiments without ACh are shown by the broken lines, except when indistinguishable from test experiments. Values are means $\pm$ S.E.M. for four or five experiments.

Fig. 8. Effects of acetylcholine on $^{45}\text{Ca}^{2+}$ efflux, $^{86}\text{Rb}^{+}$ efflux and insulin release from mouse islets perfused with a Ca^{2+} -free medium containing 30 mM-glucose (G)

ACh [$1\ \mu\text{M}$ (○) or $100\ \mu\text{M}$ (●)] was added between 40 and 70 min. Values are means $\pm$ S.E.M. for four or five experiments.

existence of which was, however, clearly attested by the decrease in efflux rate consistently seen on withdrawal of ACh (Fig. 8). The initial phase was much larger with $100\ \mu\text{M}$ - than with $1\ \mu\text{M}$ -ACh, whereas the late phase was similar with both concentrations. The only significant change in insulin release was a rapid short-lived stimulation by $100\ \mu\text{M}$ -ACh. $^{86}\text{Rb}^{+}$ efflux was similarly accelerated by $1\ \mu\text{M}$ - and $100\ \mu\text{M}$ -ACh (Fig. 8).

DISCUSSION

Concentration-dependence of ACh effects

Islets freshly isolated from normal mice proved very sensitive to ACh. Significant responses were produced by $0.1\ \mu\text{M}$ -, and maximum or near-maximum responses were usually obtained with $10\ \mu\text{M}$ -ACh. A similar dose-dependence has been reported for ACh-induced insulin release by incubated *ob/ob*-mouse islets [12] or by the perfused rat pancreas [19,31]. On the other hand, a lower

sensitivity to muscarinic agonists is usually found in freshly isolated rat islets [9,32; J.-C. Henquin, unpublished work], which sometimes must be cultured for the insulinotropic effect of ACh to be disclosed [24]. Two puzzling exceptions are the reports that as little as 0.1 – $10\ \text{nM}$ -ACh triggers insulin release in the presence of 2.4 – $2.8\ \text{mM}$ -glucose in freshly isolated [20] or cultured [10] rat islets.

Glucose has no short-term effects on the number and affinity of muscarinic receptors, but prolonged incubation of islets in a Ca^{2+} -free medium has been shown to decrease methylscopolamine binding to these receptors [21]. However, Ca^{2+} omission did not cause a generalized decrease in ACh efficiency. Thus, the absolute and relative (% of maximal) accelerations of $^{86}\text{Rb}^{+}$ efflux by $0.1\ \mu\text{M}$ -ACh were more than twice as large in the absence as in the presence of Ca^{2+} .

Ca^{2+} -dependence of ACh effects

Several studies have established that the acceleration of ^3H efflux from islets prelabelled with *myo*-[2- ^3H]-

inositol and then perfused under conditions similar to ours is an index of phosphatidylinositol breakdown [26–29]. Our results show that the acceleration of ^3H efflux by ACh was dose-dependent, and confirm that it is only partially inhibited by omission of extracellular Ca^{2+} [28]. These observations of a partial Ca^{2+} -dependency of ACh-induced ^3H efflux contrast with one report [16], but agree with two other studies [33,34], which directly showed that inositol phosphate formation in response to CCh was lower in the absence than in the presence of Ca^{2+} .

As previously reported [8,11], the large acceleration of $^{86}\text{Rb}^{+}$ efflux produced by ACh did not require extracellular Ca^{2+} , and was even increased in a Ca^{2+} -free medium. It appears to be the most sensitive response to ACh, being consistently observed with $0.1\ \mu\text{M}$ -ACh, and being often maximally stimulated by $1\ \mu\text{M}$ -ACh. The mechanism of this stimulation of $^{86}\text{Rb}^{+}$ efflux is still unclear. The present results show that it is not directly related to the magnitude of $^{45}\text{Ca}^{2+}$ mobilization or phosphatidylinositol turnover. Our recent evidence that it is suppressed in Na^{+} -free solutions, when ACh no longer depolarizes the β -cell membrane [35], suggests that the increase in $^{86}\text{Rb}^{+}$ efflux could be the consequence of Na^{+} influx and membrane depolarization.

Regardless of the concentration of glucose, ACh stimulated a biphasic efflux of $^{45}\text{Ca}^{2+}$ from the islets. The persistence of this stimulation in the absence of extracellular Ca^{2+} is interpreted as evidence for ACh-induced mobilization of intracellular Ca^{2+} [8,9,11,14,16]. Since, as shown by the present data, ACh also stimulates Ca^{2+} influx, the changes in $^{45}\text{Ca}^{2+}$ efflux occurring in a Ca^{2+} -containing medium result from mixed phenomena and their interpretation is not always straightforward. Two observations deserve comment, however. Although one could have expected the stimulation of Ca^{2+} influx to enhance $^{45}\text{Ca}^{2+}$ efflux further (through an exchange of cellular radioactive with incoming non-radioactive Ca^{2+}), the initial phase of efflux was larger in the absence than in the presence of extracellular Ca^{2+} (except with $0.1\ \mu\text{M}$ -ACh). This does not necessarily mean that ACh mobilizes less Ca^{2+} under the latter conditions. The smaller $^{45}\text{Ca}^{2+}$ efflux is more likely to be due to a progressive exchange of radioactive $^{45}\text{Ca}^{2+}$ present in ACh-sensitive pools with non-radioactive Ca^{2+} entering the cells before stimulation with the neurotransmitter. On the other hand, the second phase of $^{45}\text{Ca}^{2+}$ efflux was larger in the presence than in the absence of Ca^{2+} , when Ca^{2+} influx was markedly stimulated (1 – $100\ \mu\text{M}$ -ACh at $10\ \text{mM}$ -glucose), but not when it was not or only marginally stimulated (at $3\ \text{mM}$ - or $30\ \text{mM}$ -glucose).

In the absence of extracellular Ca^{2+} , a low concentration of ACh ($1\ \mu\text{M}$) did not induce insulin release even when the medium contained $10\ \text{mM}$ - or $30\ \text{mM}$ -glucose, and in spite of a rapid mobilization of cellular Ca^{2+} . However, a short-lived peak of insulin release accompanied the rapid mobilization of Ca^{2+} triggered by high concentrations of ACh (10 – $100\ \mu\text{M}$). It is evident that there is no tight relationship between the amount of Ca^{2+} that is mobilized and the stimulation of release. This may mean either that the concentration of cytoplasmic Ca^{2+} must exceed a certain threshold (not reached on stimulation by $1\ \mu\text{M}$ -ACh) for insulin release to be triggered, or that high concentrations of ACh have additional effects, which increase the efficiency of mobilized Ca^{2+} on the releasing machinery.

Glucose-dependence of ACh effects

At a non-stimulatory concentration of glucose ($3\ \text{mM}$), a small and progressive increase in insulin release was caused by $100\ \mu\text{M}$ -, but not by $1\ \mu\text{M}$ -ACh. This observation agrees both with our previous reports that $1\ \mu\text{M}$ -ACh is ineffective [8,11] and with reports by others that high concentrations of muscarinic agonists slightly stimulate release by islets incubated at sub-stimulatory concentrations of glucose [21,22]. The kinetics, magnitude and Ca^{2+} -dependency of the release of insulin evoked by $100\ \mu\text{M}$ -ACh in the presence of $3\ \text{mM}$ -glucose are strikingly similar to those of insulin release induced by $20\ \text{mM}$ -arginine or by a moderately elevated ($14\ \text{mM}$) concentration of K^{+} [11,36]. These three conditions have in common that they rapidly depolarize the β -cell membrane and increase Ca^{2+} influx without triggering electrical activity [11,36].

The mobilization of intracellular Ca^{2+} by ACh consistently displayed a biphasic time course with an initial phase that was smaller and a late phase that was larger at low than at high glucose. These results support the suggestion that the initial phase of efflux involves a pool of Ca^{2+} that is filled under the influence of glucose, whereas mobilization from another pool during the late phase is rather inhibited by the sugar [14]. It is possible that mobilization of Ca^{2+} by ACh failed to trigger insulin release in the presence of $3\ \text{mM}$ -glucose because the concentration of the sugar was too low to produce a necessary amplifying signal. However, the simplest explanation is that not enough Ca^{2+} was rapidly mobilized to increase cytoplasmic free Ca^{2+} to the value where release is activated. Thus the peak of $^{45}\text{Ca}^{2+}$ efflux induced by $100\ \mu\text{M}$ -ACh at $3\ \text{mM}$ -glucose was one-third smaller than that induced by $1\ \mu\text{M}$ -ACh at $10\ \text{mM}$ -glucose, and the latter was also not accompanied by any release of insulin. Nevertheless, only measurements of cytoplasmic Ca^{2+} under these various conditions could definitively solve the question.

In perfused islets of normal mice, maximal glucose stimulation is achieved with $30\ \text{mM}$; a rise to $40\ \text{mM}$ -glucose has no consistently detectable effect (J.-C. Henquin, unpublished work). The present study shows that, in the presence of a maximally effective concentration of glucose, ACh still produced ionic and secretory effects qualitatively similar to those produced at $10\ \text{mM}$ -glucose. In particular, insulin release was increased more than 2-fold. Although the β -cell membrane is persistently depolarized at the plateau potential during perfusion with $30\ \text{mM}$ -glucose [37], ACh still slightly stimulated $^{45}\text{Ca}^{2+}$ influx. An increase in spike frequency was observed under these conditions (J.-C. Henquin, unpublished work). However, the stimulation of release was surprisingly large, in consideration of the minor stimulation of Ca^{2+} influx. This discrepancy strongly suggests additional effects of ACh. The existence of the latter is further supported by the observation that $^{45}\text{Ca}^{2+}$ influx was much larger in the presence of $30\ \text{mM}$ -glucose alone than when $10\ \text{mM}$ -glucose and $1\ \mu\text{M}$ -ACh were combined, whereas the rate of insulin release was similar under both conditions. These additional effects could involve changes in intracellular Ca^{2+} handling and improvement of the efficiency of cytoplasmic Ca^{2+} . The latter could be linked to the ability of ACh to enhance production of diacylglycerol and activate protein kinase C. Experiments using a phorbol ester as a substitute for

diacylglycerol in permeabilized [38,39] or in intact islets [24,40,41] have indeed concluded that activation of protein kinase C sensitizes the releasing machinery to Ca^{2+} .

Conclusions

Though the concentration of ACh at parasympathetic nerve endings is not known, a conservative conclusion is that the effects observed here with the lowest concentrations of the neurotransmitter have the greatest physiological relevance. β -Cells exhibit their highest sensitivity to ACh in the presence of a stimulatory concentration of glucose and of extracellular Ca^{2+} . Under these conditions, the large increase in insulin release brought about by the neurotransmitter is the result of combined effects on Ca^{2+} influx, on intracellular Ca^{2+} stores and on the efficiency with which Ca^{2+} activates the releasing machinery. When Ca^{2+} influx is not possible (Ca^{2+} -free solutions), high concentrations of ACh must be used to mobilize enough cellular Ca^{2+} and to trigger a small, short-lived, release of insulin. At a non-stimulatory concentration of glucose, or when glucose-induced depolarization is prevented by diazoxide [11], basal Ca^{2+} influx is low and hardly increased by ACh. The intracellular effects of the neurotransmitter are insufficient to trigger substantial insulin release, which only marginally and belatedly increases when a high concentration of ACh is added to a medium containing Ca^{2+} . At a maximally effective concentration of glucose, Ca^{2+} influx is strongly stimulated and only slightly further augmented by ACh. However, insulin release is markedly amplified, probably through operation of intracellular effects already produced by low concentrations of the neurotransmitter.

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