Muscarinic stimulation exerts both stimulatory and inhibitory effects on the concentration of cytoplasmic Ca^{2+} in the electrically excitable pancreatic B-cell

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Mouse pancreatic islets were used to investigate how muscarinic stimulation influences the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in insulin-secreting B-cells. In the absence of extracellular Ca^{2+}, acetylcholine (ACh) triggered a transient, concentration-dependent and thapsigargin-inhibited increase in $[Ca^{2+}]_i$. In the presence of extracellular Ca^{2+} and 15 mM glucose, ACh induced a biphasic rise in $[Ca^{2+}]_i$. The initial, transient, phase increased with the concentration of ACh, whereas the second, sustained, phase was higher at low (0.1–1 μM) than at high ($\geq$ 10 μM) concentrations of ACh. Thapsigargin attenuated (did not suppress) the first phase of the $[Ca^{2+}]_i$ rise and did not affect the sustained response. This sustained rise was inhibited by omission of extracellular Na^+ (which prevents the depolarizing action of ACh) and by D600 or diazoxide (which prevent activation of voltage-dependent Ca^{2+} channels). During steady-state stimulation, the Ca^{2+} action potentials in B-cells were stimulated by 1 μM ACh but inhibited by 100 μM ACh. When B-cells were depolarized by 45 mM K^+, ACh induced a concentration-dependent, biphasic change in $[Ca^{2+}]_i$, consisting of a first peak rapidly followed by a decrease. Thapsigargin suppressed the peak without affecting the drop in $[Ca^{2+}]_i$. Measurements of $^{45}Ca^{2+}$ efflux under similar conditions indicated that ACh decreases Ca^{2+} influx and slightly increases the efflux. All effects of ACh were blocked by atropine. In conclusion, three mechanisms at least are involved in the biphasic change in $[Ca^{2+}]_i$ that muscarinic stimulation exerts in excitable pancreatic B-cells. A mobilization of Ca^{2+} from the endoplasmic reticulum contributes significantly to the first peak, but little to the steady-state rise in $[Ca^{2+}]_i$. This second phase results from an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels activated by a Na^+-dependent depolarization. However, when high concentrations of ACh are used, Ca^{2+} influx is attenuated.

INTRODUCTION

Muscarinic acetylcholine (ACh) receptors subserve an array of biological functions [1] which are mediated by distinct subtypes of receptors coupled to various transduction pathways, including changes in the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$). The characteristics of these changes have been investigated widely in non-excitable cells [2,3], but less so in excitable tissues. Here we have investigated how ACh influences $[Ca^{2+}]_i$ in the electrically excitable pancreatic B-cell. ACh, released by parasympathetic nerve endings during the cephalic and intestinal phases of feeding, increases insulin secretion [4] by activating muscarinic receptors in B-cells. These receptors display the pharmacological characteristics of M_3 receptors [5,6].

Several transduction pathways are involved in mediating the effects of ACh on insulin secretion. The most classical one is the stimulation of a phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate. This generates $Ins(1,4,5)P_3$, which mobilizes Ca^{2+} from intracellular stores, and diacylglycerol (DAG), which activates protein kinase C (PKC) [7–10]. Muscarinic agonists also generate DAG by hydrolysis of phosphatidylinositol [11]. In addition, by stimulating phospholipase A_2, they lead to an accumulation of lysophospholipids and arachidonic acid [12]. How lysophospholipids may affect insulin secretion remains unclear [13], but arachidonic acid may contribute to the stimulation of insulin secretion by activating PKC, mobilizing intracellular Ca^{2+} or facilitating Ca^{2+} influx [10,13]. Besides these complex effects on phospholipid metabolism, ACh also causes a Na^+-dependent depolarization of B-cells [14], which potentiates the influx of Ca^{2+} triggered by glucose or other secretagogues [15].

Measurements of $[Ca^{2+}]_i$ in insulin-secreting cells stimulated by muscarinic agonists have yielded controversial results. The transient rise in $[Ca^{2+}]_i$ brought about by ACh in HIT cells [16] and MIN6 cells [17] was mainly ascribed to an influx of Ca^{2+}. In contrast, mobilization of Ca^{2+} from intracellular stores seems to explain the transient increase in $[Ca^{2+}]_i$ brought about by carbachol in RINm5F cells [18], HIT cells [19,20], rat B-cells [21,22] and *ob/ob* mouse B-cells [23–25]. This increase was sometimes followed by a small sustained elevation, attributed to Ca^{2+} influx [19,21,24] or to mobilization of Ca^{2+} [25].

It has become clear that the changes in $[Ca^{2+}]_i$ brought about by glucose and certain other secretagogues in isolated insulin-secreting cells substantially differ from those occurring in the more physiological preparation of whole pancreatic islets [26–28]. In the present study, therefore, intact mouse islets loaded with the Ca^{2+} indicator fura-2 were used to characterize the effects of ACh on $[Ca^{2+}]_i$ in B-cells. Thus the respective contributions of Ca^{2+} mobilization and Ca^{2+} influx could be established. In addition, careful examination of the concentration- and time-dependence of the effects of ACh unexpectedly revealed that activation of muscarinic receptors can both increase and decrease $[Ca^{2+}]_i$ in pancreatic B-cells.

MATERIALS AND METHODS

Solutions

Different types of solutions were used, all of which had a pH of 7.4 at 37 °C. They were supplemented with 1 mg/ml BSA (fraction V; Boehringer-Mannheim, Mannheim, Germany), except in electrophysiological experiments. The medium used

Abbreviations used: ACh, acetycholine; DAG, diacylglycerol; PKC, protein kinase C; $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration.

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during isolation of the islets and for most experiments 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 $NaHCO_3$, and was gassed with O_2/CO_2 (94:6). When the concentration of KCl was increased to 45 mM, that of NaCl was decreased accordingly. Ca^{2+}-free solutions were prepared by substituting $MgCl_2$ for $CaCl_2$. In one series of experiments (see Table 1), the concentration of free Ca^{2+} was decreased to 1 μM by adding 2.67 mM EGTA to the medium containing 2.5 mM $CaCl_2$. The experiments aiming at testing the Na^+-dependence of the effects of ACh were performed in a Hepes-buffered solution gassed with O_2. The control solutions contained 135 mM NaCl, 4.8 mM KOH, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 10 mM Hepes. Na^+-free solutions were prepared by substituting *N*-methyl-D-glucamine chloride for NaCl [14]. The concentration of glucose was always 15 mM.

Preparation

All experiments were performed with pancreatic islets from fed female NMRI mice (25–30 g). Except for electrophysiological recordings, islets were isolated after collagenase digestion of the pancreas. They were then cultured for 1 or 2 days in RPMI 1640 medium (Flow Laboratories, ICN Biomedicals Ltd., Irvine, Scotland, U.K.) containing 10 mM glucose [27].

Measurements of $[Ca^{2+}]_i$

Cultured islets were loaded with fura-2 during 40 min of incubation at 37 °C in a bicarbonate-buffered solution containing 10 mM glucose and 1 μM fura-2-acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.). They were then transferred into a temperature-controlled perifusion chamber (Applied Imaging, Sunderland, U.K.). The tissue was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science Ltd., Tunbridge Wells, U.K.). The time interval between series of 340/380 images was 3 s. The images were analysed by the MagiCal system (Applied Imaging). The technique has been described in detail previously [27].

Measurements of ^{45}Ca efflux

Cultured islets were first loaded with ^{45}Ca during 90 min of incubation in 0.25 ml of bicarbonate-buffered medium supplemented with $^{45}CaCl_2$. After washing, batches of 40–50 islets were placed in perifusion chambers and the radioactivity lost by the islets was measured in the effluent fractions collected every 1 min [14].

Measurements of inositol phosphates

Cultured islets were loaded with *myo*-[2-^{3}H]inositol during a preincubation of 2 h. After washing, batches of 40 islets were then incubated as described in the legend of Table 1. The incubation was stopped by addition of 3 ml of chloroform/methanol/conc. HCl (200:100:1, by vol.) and 100 μl of EGTA (100 μM). Inositol monophosphate, bisphosphate and trisphosphate were then separated by anion exchange chromatography [29] as described previously [30].

Electrophysiological recordings

A piece of pancreas was fixed in a small perifusion chamber and the membrane potential of a single cell within an islet was continuously recorded with a high-resistance microelectrode [31]. B-cells were identified by the typical electrical activity that they display in the presence of 15 mM glucose.

Drugs

ACh chloride, atropine sulphate, phorbol 12-myristate 13-acetate, 4α-phorbol 12,13-didecanoate and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diazoxide was from Schering-Plough Avondale (Rathdrum, Ireland), and *N*-methyl-D-glucamine was from Janssen Chimica (Beerse, Belgium). D-600 was a gift from Knoll (Ludwigshafen, Germany) and nimodipine was from Bayer AG (Wuppertal, Germany). Radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.).

Presentation of results

Most measurements of $[Ca^{2+}]_i$ and all electrophysiological experiments are illustrated by recordings which are representative of results obtained with the indicated number of islets. For membrane potential measurements, this number of islets corresponds to the number of different mice. For $[Ca^{2+}]_i$ measurements, several islets from the same culture were tested with the same protocol, but each protocol was repeated with islets from at least three different cultures. Results from some $[Ca^{2+}]_i$ measurements, and all $^{45}Ca^{2+}$ efflux and [^{3}H]inositol phosphate experiments, are presented as means $\pm$ S.E.M. for a certain number of experiments or batches of islets. To assess the statistical significance of observed differences, a paired *t* test was used for the comparison of $[Ca^{2+}]_i$ in the same islet before and after ACh, and an analysis of variance followed by a Neuman–Keuls test was used for multiple comparisons.

RESULTS

Effects of ACh in the absence of extracellular Ca^{2+}

In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ in islet cells was low (39 ± 1.2 nM; $n = 54$) and stable, despite the presence of 15 mM glucose in the medium. Addition of ACh induced a transient increase in $[Ca^{2+}]_i$ that was blocked by atropine (Figures 1a–1c). This effect increased with the concentration of ACh (K_m 10 μM) (Figure 1d). A second, more sustained, increase in $[Ca^{2+}]_i$ was also produced by the highest concentrations of ACh, as illustrated by the small decrease following addition of atropine (Figure 1b). This second phase could not be reliably quantified because of its inconsistency and very small amplitude in the presence of low ACh concentrations.

To identify the origin of intracellular Ca^{2+} mobilized by ACh, the islets were perifused with thapsigargin, an inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum [32]. Addition of 1 μM thapsigargin to a medium without Ca^{2+}, 5 or 10 min before the application of 100 μM ACh, did not significantly affect the mobilization of Ca^{2+} induced by ACh (results not shown). However, when the islets were incubated with thapsigargin for 45 min before ACh stimulation, the transient peak of $[Ca^{2+}]_i$ was markedly reduced. This inhibitory effect was directly dependent on the concentration of thapsigargin and it was nearly complete with 1 μM of the drug (Figure 2). The inhibitory action of thapsigargin did not result from an alteration of the stimulation of phosphoinositide metabolism by ACh (Table 1). Indeed, 100 μM ACh increased inositol phosphate levels to a similar extent in islets preincubated or not with thapsigargin.

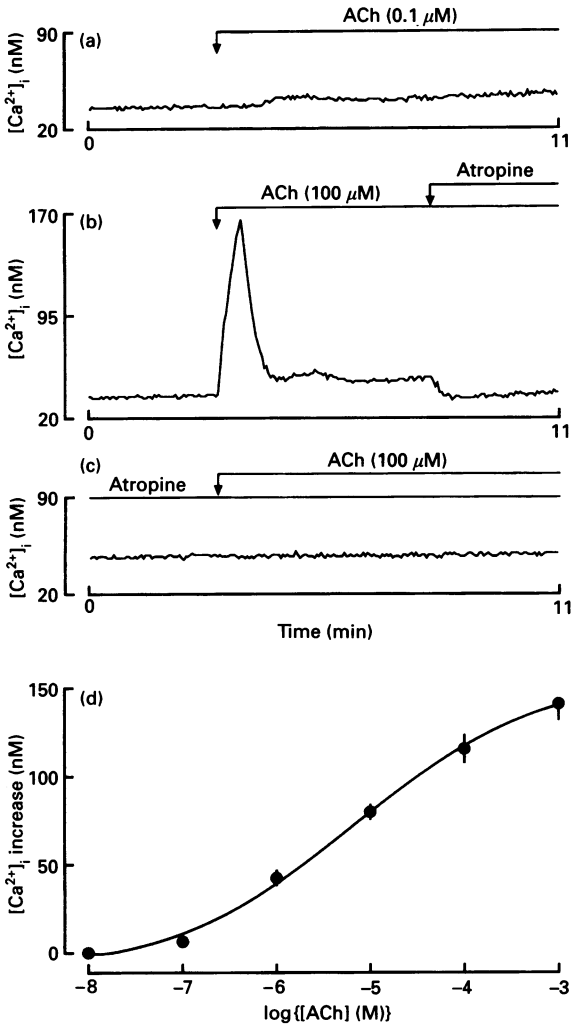

Figure 1 Effects of ACh on $[Ca^{2+}]_i$ in mouse islets perifused with a Ca^{2+}-free medium

Atropine (10 μM) was added as indicated by the arrows (**b**) or was present throughout the experiment (**c**). The traces are representative of results obtained with between six and nine islets. Panel (**d**) shows the concentration-dependence of the peak increase in $[Ca^{2+}]_i$ produced by ACh. The results are presented as absolute $[Ca^{2+}]_i$ changes. Values are means ± S.E.M. for nine islets.

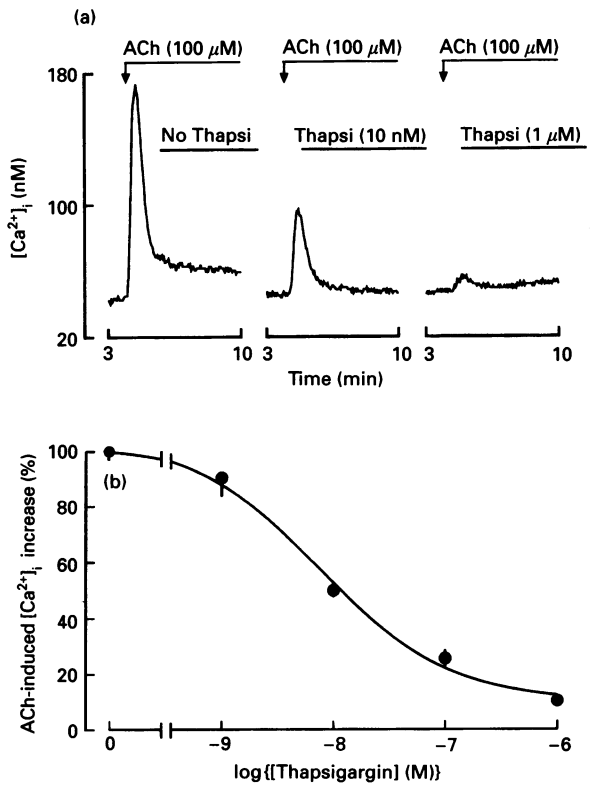

Figure 2 Effects of thapsigargin on the rise in $[Ca^{2+}]_i$ induced by 100 μM ACh in mouse islets perifused with a Ca^{2+}-free medium

Except in control experiments, both the medium used during the loading with fura-2 and the perifusion medium were supplemented with the indicated concentrations of thapsigargin (Thapsi). The traces shown in (**a**) are representative of results obtained in nine islets for each concentration of thapsigargin. Panel (**b**) shows the concentration-dependence of the inhibitory effect of thapsigargin. The results are presented as percentages of the increase in $[Ca^{2+}]_i$ brought about by 100 μM ACh in the absence of thapsigargin. This increase amounted to 108 ± 3 nM. Values are means ± S.E.M. for nine islets.

Effects of ACh in the presence of extracellular Ca^{2+}

When the perifusion medium contained 4.8 mM K^+, 2.5 mM Ca^{2+} and 15 mM glucose, $[Ca^{2+}]_i$ in islet cells was found to oscillate as reported previously [26–28]. These oscillations were very regular in certain islets (Figure 3a), but exhibited a more complex, periodic, pattern in others (Figures 3b and 3c) [26,33]. Addition of 0.01 μM ACh faintly increased $[Ca^{2+}]_i$ and accelerated the oscillations (results not shown). Higher concentrations of ACh (0.1 μM–1 mM) induced a biphasic rise in $[Ca^{2+}]_i$. At 0.1 μM ACh, this effect was characterized by a rapid upward shift of the oscillations of $[Ca^{2+}]_i$, followed by rapid fluctuations of smaller amplitude on a less elevated level (Figure 3a). At higher concentrations of ACh, the first peak of $[Ca^{2+}]_i$ was large and transient, and it was followed by a more sustained elevation that decreased with time to eventually reach a new steady-state level (Figure 3b). When the islets were sequentially challenged with 1 and 100 μM ACh, the initial peak of $[Ca^{2+}]_i$ otherwise produced by the high concentration was depressed, whereas its lowering effect on steady-state $[Ca^{2+}]_i$ was more rapidly apparent (Figure 3c). Atropine (10 μM) did not affect the control oscillations of $[Ca^{2+}]_i$, but it completely blocked the effects of ACh (results not shown).

Figure 4 illustrates the concentration-dependence of the effects of ACh on the initial peak and the steady-state elevation of $[Ca^{2+}]_i$. With the lowest concentration of ACh that was tested (0.01 μM), no significant peak was recorded, but a steady-state elevation was consistently present ($P < 0.05$). The initial peak of $[Ca^{2+}]_i$ increased with the concentration of ACh. In contrast, the steady-state elevation of $[Ca^{2+}]_i$ was maximal at intermediate concentrations of ACh (0.1–1 μM) and decreased when higher concentrations of the neurotransmitter were tested, to become statistically not different from the prestimulatory control levels at 1 mM ACh (Figure 4).

In a medium containing 15 mM glucose and 2.5 mM Ca^{2+}, B-cells exhibited a characteristic electrical activity consisting of bursts of spikes superimposed on the plateau of slow waves of the membrane potential (Figure 5). Upon addition of 1 μM ACh, the membrane remained persistently depolarized and the spike activity became continuous in five out of seven islets (Figure 5a). In the other two islets, the frequency of the slow waves increased, as observed previously in the presence of 10 mM glucose [14,15].

Table 1 Effects of ACh and thapsigargin on inositol phosphate levels in mouse islets

After preincubation with *myo*-[^{3}H]inositol, the islets were washed and subdivided into batches of 40. Each batch was placed in 0.5 ml of medium containing 15 mM glucose, 1 mM inositol and 10 mM LiCl. The other components of the medium were changed in a sequence that tried to mimic the protocol of the $[Ca^{2+}]_i$ measurement experiments as closely as possible. In the first series of experiments, the medium (500 μl) initially contained 2.5 mM Ca^{2+}, and was supplemented or not with 1 μM thapsigargin. After 40 min, 250 μl of medium containing 8 mM EGTA was added to lower the concentration of free Ca^{2+} to approx. 1 μM; 15 min later (i.e. after 55 min of incubation), another 250 μl of medium containing 400 μM ACh was added, and the incubation was eventually stopped 5 min later. All these solutions were prewarmed and were supplemented or not with thapsigargin as appropriate. In the second series of experiments, the medium (500 μl) initially contained 4.8 mM K^+ and 250 μM diazoxide, and was supplemented or not with 1 μM thapsigargin. After 40 min, 250 μl of medium containing 125.4 mM K^+ was added to increase the concentration of K^+ to 45 mM; 15 min later (i.e. after 55 min of incubation) another 250 μl of medium containing 400 μM ACh was added, and the incubation was eventually stopped 5 min later. All these solutions were prewarmed, contained 250 μM diazoxide, and were supplemented or not with thapsigargin as appropriate. Values are means ± S.E.M. for six batches of islets. Significance of differences is indicated by: $^*P < 0.01$ for the effect of ACh in absence or presence of thapsigargin; $\dagger P < 0.05$ for the effect of thapsigargin.

Experimental conditions	Inositol phosphates (d.p.m./islet)		
	InsP_1	InsP_2	InsP_3
(1) Experiments in 0 mM Ca^{2+} and 4.8 mM K^+			
Glucose (15 mM)	114±9	30±2	24±2
+ ACh (100 μM)	527±25*	145±10*	40±2*
+ Thapsigargin (1 μM)	190±11†	31±2	21±1
+ ACh and thapsigargin	576±27*	145±11*	37±2*
(2) Experiments in 2.5 mM Ca^{2+} and 45 mM K^+			
Glucose 15 mM	195±20	39±4	27±2
+ ACh (100 μM)	790±106*	185±22*	59±7*
+ Thapsigargin (1 μM)	268±34	47±5	28±2
+ ACh and thapsigargin	767±123*	168±24*	51±7*

When the concentration of ACh was raised to 100 μM, the spike frequency increased transiently and then decreased, while the spike amplitude became very small (Figure 5b).

Role of intracellular Ca^{2+} stores in the effects of ACh in the presence of extracellular Ca^{2+}

The role of Ca^{2+} mobilization from the endoplasmic reticulum in the $[Ca^{2+}]_i$ changes brought about by ACh in the presence of extracellular Ca^{2+} was then evaluated. The experiments shown above (Figure 2) have established that the ACh-sensitive Ca^{2+} pool is almost completely emptied by 1 μM thapsigargin. After pretreatment of the islets with 1 μM thapsigargin, addition of 1 μM ACh to a medium containing Ca^{2+} increased $[Ca^{2+}]_i$ to a plateau, whereas 100 μM ACh lowered $[Ca^{2+}]_i$ to a new steady-state level (Figure 3d). This indicates that Ca^{2+} influx plays a major role in the rise in $[Ca^{2+}]_i$ induced by 1 μM ACh, since the intracellular pool has been depleted. This also shows that sequestration in the endoplasmic reticulum plays no role in the lowering of $[Ca^{2+}]_i$ by 100 μM ACh, since thapsigargin prevents any refilling of this store.

The response to ACh was then tested under conditions where extracellular Ca^{2+} was present, but Ca^{2+} influx through voltage-dependent Ca^{2+} channels was prevented by D600 or diazoxide. Blocking of voltage-dependent Ca^{2+} channels with D600 (Figure 6a) or repolarizing the B-cell membrane by opening ATP-sensitive K^+ channels with diazoxide (Figure 6b) abolished the oscillations of $[Ca^{2+}]_i$ induced by 15 mM glucose and lowered $[Ca^{2+}]_i$ to basal levels. Addition of 100 μM ACh to a medium containing D600 or diazoxide induced a large transient peak of $[Ca^{2+}]_i$ followed by a small sustained phase which was suppressed by atropine (Figures 6a and 6b). Pretreatment of the islets with 1 μM thapsigargin suppressed the ACh-induced $[Ca^{2+}]_i$ increase in the presence of D600 (results not shown).

We have previously shown that ACh no longer depolarizes the B-cell membrane when Na^+ is omitted from the extracellular medium [14]. As shown in Figures 7(a) and 7(b), replacement of Na^+ by the impermeant *N*-methyl-D-glucamine in a medium containing Ca^{2+} and 15 mM glucose induced three phases of $[Ca^{2+}]_i$ changes: an initial transient decrease was followed by a large and long elevation of $[Ca^{2+}]_i$, and then by the reappearance of $[Ca^{2+}]_i$ oscillations. These oscillations were large and their frequency decreased with time. These characteristics correspond

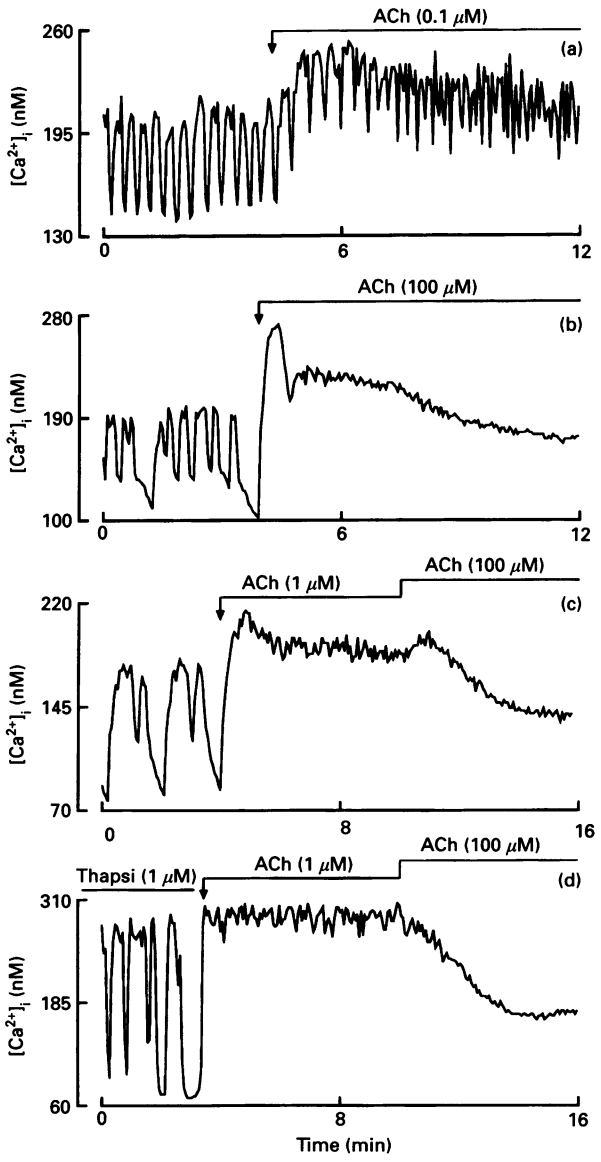

Figure 3 Effects of ACh on $[Ca^{2+}]_i$ in mouse islets perifused with a medium containing 2.5 mM Ca^{2+}

In the experiment shown in (**d**), both the medium used during the loading with fura-2 and the perifusion medium were supplemented with 1 μM thapsigargin (Thapsi). These traces are representative of results obtained in 12–13 islets.

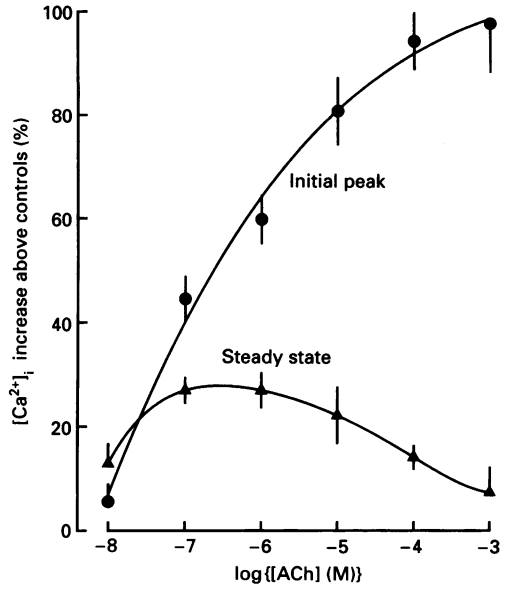

Figure 4 Concentration-dependence of the biphasic changes in $[Ca^{2+}]_i$ produced by ACh in islets perifused with a medium containing 2.5 mM Ca^{2+}

The results are presented as percentage increases above control levels, which were computed by integrating $[Ca^{2+}]_i$ during the last 3 min before addition of ACh. This prestimulatory $[Ca^{2+}]_i$ averaged 157 ± 3 nM ($n = 60$). When no well-defined peak of $[Ca^{2+}]_i$ was observed, as after addition of 0.01 and 0.1 μM ACh, the initial 'peak' value was obtained by integrating $[Ca^{2+}]_i$ over the first 1 min after ACh stimulation. The steady-state effect of ACh was measured between 5 and 8 min after ACh application. Values are means $\pm$ S.E.M. for 9–12 islets.

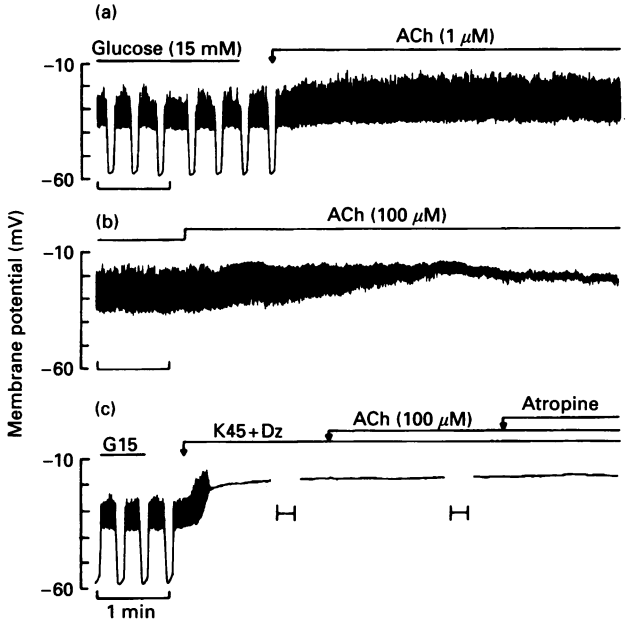

Figure 5 Effects of ACh on the membrane potential of mouse islets perifused with a medium containing 2.5 mM Ca^{2+}

Panels (**a**) and (**b**) show, without interruption, an experiment in which 1 μM and 100 μM ACh was successively added to the medium. In (**c**), the control medium containing 15 mM glucose (G) and 4.8 mM K^+ was replaced by a medium containing 15 mM glucose, 45 mM K^+ and 250 μM diazoxide (K45 + Dz) before the successive addition of ACh and atropine (10 μM). The interruptions correspond to periods of 4.4 min. These recordings are representative of results obtained in seven (**a**, **b**) and four (**c**) islets from different mice.

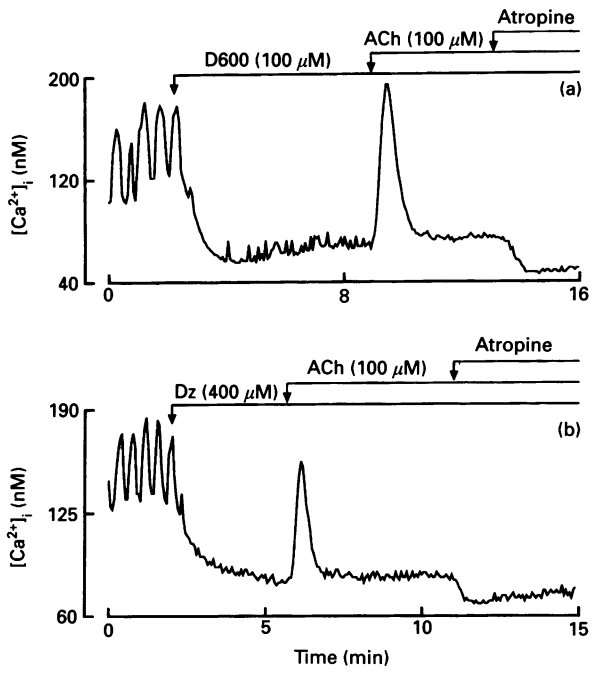

Figure 6 Effects of ACh on $[Ca^{2+}]_i$ in mouse islets perifused under conditions inhibiting Ca^{2+} influx

The medium contained 2.5 mM Ca^{2+} and 15 mM glucose. D600 or diazoxide (Dz) was first added to block Ca^{2+} influx before stimulation with 100 μM ACh and the eventual addition of 10 μM atropine, as indicated by the arrows. The traces are representative of results obtained in nine (**a**) and six (**b**) islets.

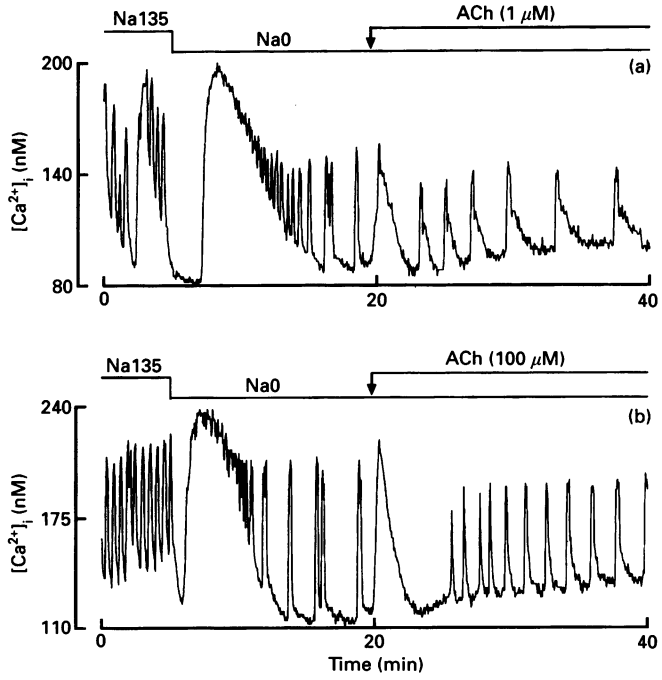

Figure 7 Role of extracellular Na^+ in the $[Ca^{2+}]_i$ changes produced by ACh in mouse islets perifused with a medium containing 2.5 mM Ca^{2+}

The control perifusion medium containing 135 mM Na^+ (Na135) was replaced by a Na^+-free medium (Na0) with *N*-methyl-D-glucamine as substitute. ACh was then added as indicated. The traces are representative of results obtained in 6–9 islets.

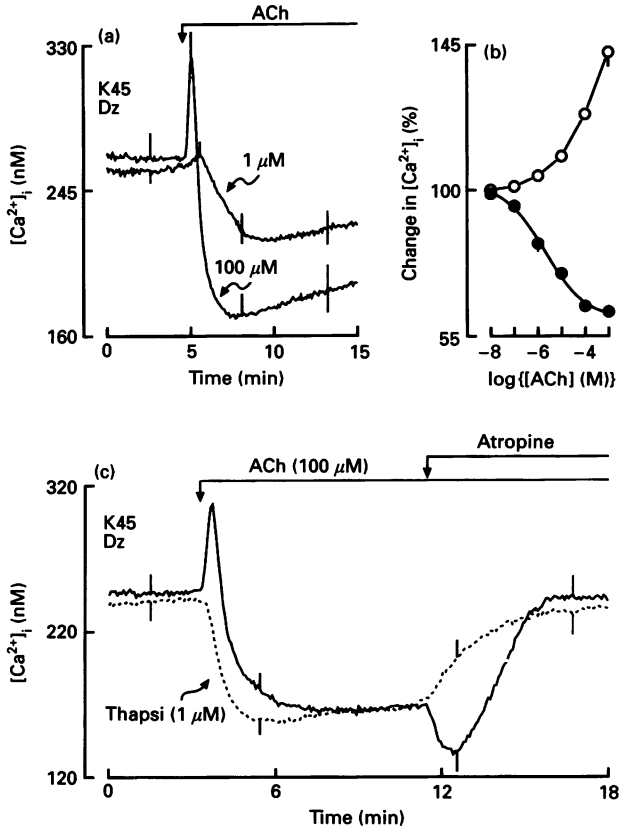

Figure 8 Effects of ACh on $[Ca^{2+}]_i$ in mouse islets depolarized by high K^+

The perifusion medium contained 2.5 mM Ca^{2+}, 15 mM glucose, 45 mM K^+ (K45) and 250 μM diazoxide (Dz). In (**a**), the traces correspond to the mean responses (±S.E.M.) for 6–7 islets. Panel (**b**) shows the concentration-dependence of the changes in $[Ca^{2+}]_i$ produced by ACh. The results are presented as percentage changes from the control levels measured during the last 3 min before addition of ACh. This prestimulatory $[Ca^{2+}]_i$ averaged 257 ± 5 nM ($n = 47$). The changes induced by ACh were calculated at the peak (○) or at the minimum (●). Values are means ± S.E.M. for 6–9 islets. In panel (**c**), ACh and atropine (10 μM) were successively added as indicated. The broken line illustrates the $[Ca^{2+}]_i$ changes occurring in islets treated with 1 μM thapsigargin (Thapsi) during the loading period with fura-2 and the whole perifusion. Values are means ± S.E.M. for 5–9 islets.

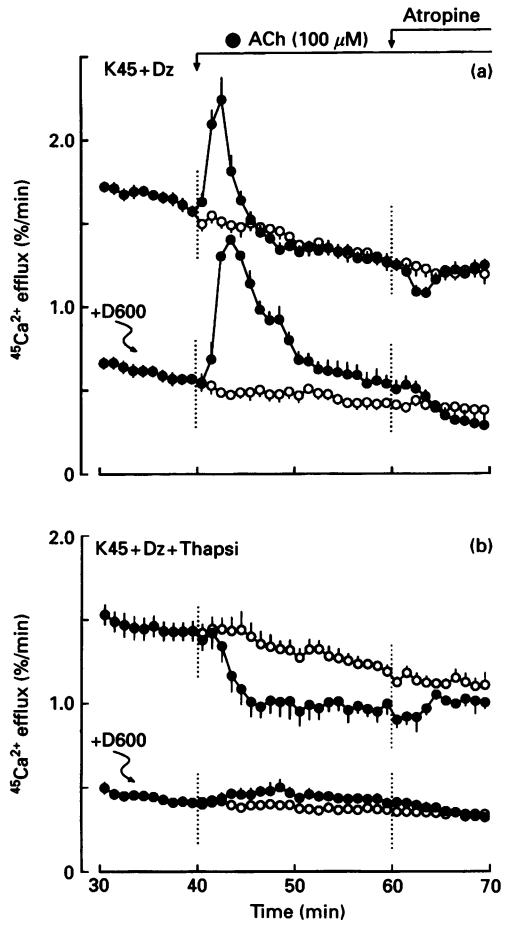

Figure 9 Effects of ACh on $^{45}Ca^{2+}$ efflux from perifused mouse islets

The perifusion medium contained 2.5 mM Ca^{2+}, 15 mM glucose, 45 mM K^+ (K45) and 250 μM diazoxide (Dz). In the experiments shown in (**b**), both the medium used during the loading with $^{45}Ca^{2+}$ and the perifusion medium were supplemented with 1 μM thapsigargin (Thapsi). In two series of experiments, 100 μM D600 was present during the whole perifusion. In each series of experiments, ACh was added from 40 to 60 min and atropine from 60 to 70 min to one group of islets (●), whereas the other group served as control (○). Values are means ± S.E.M. for 4–5 experiments.

well with the changes in B-cell membrane potential that occur under these conditions [34]. Addition of 1 or 100 μM ACh to the Na^+-free medium induced a large but transient peak of $[Ca^{2+}]_i$ (Figures 7a and 7b) that was abolished by pretreatment of the islets with thapsigargin (results not shown). However, neither concentration of ACh was able to induce a sustained increase in $[Ca^{2+}]_i$ in the absence of Na^+ (Figures 7a and 7b). It is thus clear that this sustained rise of $[Ca^{2+}]_i$ depends on a depolarization-mediated acceleration of Ca^{2+} influx.

Effects of ACh in islet cells depolarized by 45 mM K^+

To test the effects of ACh independently of changes in B-cell membrane potential and in the presence of a steadily elevated $[Ca^{2+}]_i$, experiments were performed in a medium containing 15 mM glucose, 45 mM K^+ and 250 μM diazoxide. When this medium was substituted for the control medium, the B-cell membrane rapidly depolarized and the spike frequency transiently increased before ceasing abruptly (Figure 5c). The membrane then remained depolarized at −20 ± 1 mV. Neither 100 μM ACh nor the subsequent addition of atropine affected the membrane potential under these conditions.

In the presence of high K^+ and diazoxide, $[Ca^{2+}]_i$ was high and stable (Figure 8a). Addition of ACh induced a concentration-dependent, biphasic, change in $[Ca^{2+}]_i$, with an initial peak followed by a rapid decrease. The decrease was relatively more marked than the increase at low ACh concentrations (e.g. 1 μM), but reached a maximum at lower ACh concentrations than did the initial peak (Figure 8b). When atropine was added after ACh had decreased the $[Ca^{2+}]_i$, a further, transient, decrease occurred before $[Ca^{2+}]_i$ eventually returned to control levels (Figure 8c). To test the role of the endoplasmic reticulum in these changes, similar experiments were performed in the presence of thapsigargin. Under these conditions, 100 μM ACh no longer increased $[Ca^{2+}]_i$, but decreased it to a similar extent as in the islets which had not been pretreated with the Ca^{2+}-ATPase inhibitor (Figure 8c). However, the onset of the decrease was immediate, suggesting that the peak of $[Ca^{2+}]_i$ induced by ACh in control islets is the result of two antagonistic effects. In the islets treated with thapsigargin, atropine immediately reversed the

effect of ACh on $[Ca^{2+}]_i$ without producing any transient drop (Figure 8c). The suppression of this transient fall by thapsigargin demonstrates that it corresponds to atropine-induced refilling of intracellular pools after ACh stimulation. Control experiments showed that thapsigargin did not alter the effects of ACh on phosphoinositide metabolism in the presence of high K^+ and diazoxide (Table 1).

The possible ionic mechanisms of the decrease in $[Ca^{2+}]_i$ brought about by ACh were evaluated in experiments measuring $^{45}Ca^{2+}$ efflux. The rate of $^{45}Ca^{2+}$ efflux was high because Ca^{2+} influx was constantly stimulated by the high concentration of K^+ (Figure 9a). Upon addition of 100 μM ACh, the efflux rate markedly increased before returning to control levels within a few minutes. Subsequent addition of atropine transiently decreased $^{45}Ca^{2+}$ efflux. Thus the steady-state lowering of $[Ca^{2+}]_i$ by ACh was not associated with an increase in $^{45}Ca^{2+}$ efflux. This does not necessarily imply that no acceleration of Ca^{2+} efflux occurred because ACh could, at the same time, have decreased Ca^{2+} influx. This was tested in the presence of D600. When Ca^{2+} influx was inhibited by D600, the rate of $^{45}Ca^{2+}$ efflux was much lower. ACh produced a biphasic increase, with a large initial peak that was now followed by a small sustained phase, reversed by atropine (Figure 9a). To circumvent the difficulties linked to the mobilization of intracellular Ca^{2+}, similar experiments were repeated with islets treated with 1 μM thapsigargin. Under these conditions, the efflux rate of $^{45}Ca^{2+}$ was similar to that seen in islets that had not been treated with the Ca^{2+}-ATPase inhibitor (Figure 9b). ACh induced a sustained decrease in $^{45}Ca^{2+}$ efflux that was reversed by atropine. In the presence of D600, the low efflux rate of $^{45}Ca^{2+}$ was slightly increased by ACh. Taken together, these results indicate that the fall in $[Ca^{2+}]_i$ brought about by ACh may be ascribed to a reduced influx and a slightly accelerated efflux of Ca^{2+}.

DISCUSSION

The present study demonstrates that the changes in $[Ca^{2+}]_i$ brought about by ACh in islet cells result from both stimulatory and, heretofore unsuspected, inhibitory effects of the neurotransmitter. These antagonistic effects do not display the same concentration-dependence and involve distinct transduction pathways. Although intact islets contain non-B-cells (about 20% in the mouse), they have the advantage of preserving the architecture of the micro-organ constituting the endocrine pancreas. Recent studies from our [27,33] and other [26,28,35] laboratories have shown that whole islets can reliably be used to study $[Ca^{2+}]_i$ changes in B-cells.

It is well established that muscarinic agonists stimulate phosphoinositide hydrolysis in pancreatic B-cells, as in other cells [18,36,37]. This hydrolysis produces Ins(1,4,5)P_3, which may mobilize Ca^{2+} from intracellular stores, as shown by the acceleration of $^{45}Ca^{2+}$ efflux [37,38] and the rise in $[Ca^{2+}]_i$ [18,19,21,25] in insulin-secreting cells incubated or perifused in a Ca^{2+}-free medium. In the present study, we characterized this mobilization of Ca^{2+} under different conditions chosen to inhibit Ca^{2+} influx: a blockade of voltage-dependent Ca^{2+} channels by D600, a hyperpolarization of the membrane with diazoxide, and the omission of extracellular Ca^{2+}. ACh caused a large peak increase of $[Ca^{2+}]_i$ followed by a much smaller, sustained, rise. Our observation that both phases were abolished by thapsigargin pretreatment suggest that the ACh-releasable intracellular store is entirely located in the endoplasmic reticulum. No oscillations of $[Ca^{2+}]_i$ were detected during Ca^{2+} mobilization by ACh, in contrast to observations made in HIT or single B-cells [19,25]. This does not imply that such oscillations do not occur. However, to be detected in a whole islet, they should be synchronized in all cells, which is unlikely to be the case, unlike the oscillations of $[Ca^{2+}]_i$ driven by the changes in membrane potential [27,33,35]. Moreover, the pattern of the oscillations induced by ACh in HIT cells was found to be quite variable from cell to cell [19].

ACh also produced a biphasic increase in $[Ca^{2+}]_i$ in islets perifused with a medium containing 15 mM glucose and 2.5 mM Ca^{2+}. The initial rise results from both an acceleration of Ca^{2+} influx and a mobilization of cellular Ca^{2+}. That the influx of Ca^{2+} plays a major role is supported by several observations. The $[Ca^{2+}]_i$ rise was accompanied by a stimulation of the Ca^{2+}-dependent electrical activity in B-cells, was also produced by low ACh concentrations which hardly mobilized intracellular Ca^{2+}, and persisted after treatment of the islets with thapsigargin. That the mobilization of Ca^{2+} contributes to the effect of high concentrations of ACh is shown by the attenuation of the initial rise of $[Ca^{2+}]_i$ after pretreatment with thapsigargin. In contrast, both the lack of a clear effect of thapsigargin on the second, sustained, phase of the $[Ca^{2+}]_i$ rise, and the almost complete suppression of this phase by D600 and diazoxide, strongly indicate that the acceleration of Ca^{2+} influx through voltage-dependent Ca^{2+} channels plays a predominant role. Conversely, the fact that thapsigargin pretreatment did not prevent the sustained rise in $[Ca^{2+}]_i$ induced by ACh strongly suggests that the neurotransmitter does not act as in non-excitable cells, where it triggers a 'capacitative Ca^{2+} entry', i.e. an influx of Ca^{2+} activated by depletion of intracellular Ca^{2+} pools [3].

We have shown previously that the depolarization of pancreatic B-cells by ACh is Na^+-dependent [14]. In the absence of extracellular Na^+, ACh caused a rapid, large, increase in $[Ca^{2+}]_i$ that can be ascribed entirely to mobilization of intracellular Ca^{2+} because of its suppression by thapsigargin. This peak was somewhat more prolonged than that occurring in the absence of Ca^{2+} or in the presence of diazoxide or D600, probably because of the inhibition of the Na^+/Ca^{2+} exchange and the slower correction of the $[Ca^{2+}]_i$ rise in the Na^+-free medium. More importantly, no sustained rise in $[Ca^{2+}]_i$ followed the initial peak under these conditions. This is entirely consistent with the conclusion that the sustained rise of $[Ca^{2+}]_i$ is the consequence of an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels activated by the Na^+-dependent depolarization of the B-cell membrane. The mechanism by which activation of muscarinic receptors increases Na^+ influx and depolarizes B-cells [14,39] is unknown. It does not involve activation of tetrodotoxin-sensitive Na^+ channels or blockade of the Na^+ pump [14,40,41]. It has been suggested recently that Ca^{2+} store depletion might induce a depolarizing Na^+ current in B-cells [42], as in non-excitable cells [43]. However, this proposal is not supported by our observation that thapsigargin did not prevent the Na^+-dependent rise in $[Ca^{2+}]_i$ induced by ACh in the presence of extracellular Ca^{2+}.

Another important novel finding of the present study is that ACh not only increases, but also decreases, $[Ca^{2+}]_i$ in islet cells. One should first consider that this decrease may be artefactual, like that observed secondarily in hepatocytes stimulated with phospholipase C-linked agonists [44]. In these cells, compartmentalization of fura-2 is extensive after loading with the membrane-permeant ester, and the emptying of the endoplasmic reticulum causes an apparent fall in $[Ca^{2+}]_i$. However, this is not the case in islet cells, for two reasons. First, the decrease in $[Ca^{2+}]_i$ brought about by ACh occurred only when Ca^{2+} influx was activated. Secondly, this decrease persisted when ACh was applied after emptying of the endoplasmic reticulum with thapsigargin.

Under control conditions (normal K^+ and presence of extracellular Ca^{2+}), the initial peak of $[Ca^{2+}]_i$ produced in B-cells by

ACh increased with the concentration of the neurotransmitter, whereas the magnitude of the steady-state rise declined when ACh exceeded 1 μM. This peculiarity agrees well with the evidence that 1 μM ACh increased the electrical activity in B-cells, whereas 100 μM ACh progressively inhibited the Ca^{2+} spikes, as also observed by others [45]. The interpretation of these experiments is difficult because of the changes in membrane potential that ACh produces. In particular, it is unclear whether low concentrations of ACh did not produce an inhibitory effect on $[Ca^{2+}]_i$ or whether this effect was masked by the stimulation of Ca^{2+} influx.

The experiments carried out in the presence of 45 mM K^+ and diazoxide markedly helped to clarify this question. It was first evident that concentrations of ACh as low as 0.1 μM already produced an inhibitory effect on $[Ca^{2+}]_i$ (in addition to the stimulatory one). Since ACh did not affect the membrane potential under these conditions, it is clear that a mechanism distinct from a voltage-mediated closure or inactivation of Ca^{2+} channels is involved. The persistence of this inhibitory effect after thapsigargin treatment also makes it clear that the endoplasmic reticulum plays no direct role in the lowering of $[Ca^{2+}]_i$ by ACh. To identify the ionic mechanisms implicated in the phenomenon, $^{45}Ca^{2+}$ efflux from islet cells was measured. The results show that neither an acceleration of Ca^{2+} efflux nor an inhibition of Ca^{2+} influx alone is sufficient to explain the decrease in $[Ca^{2+}]_i$. The two effects seem to co-exist. The small decrease in $^{45}Ca^{2+}$ efflux, caused by ACh when Ca^{2+} mobilization from and re-uptake within the endoplasmic reticulum were blocked by thapsigargin, is best explained by an inhibition of Ca^{2+} influx. On the other hand, the small rise in $^{45}Ca^{2+}$ efflux, produced by ACh when Ca^{2+} mobilization and influx were prevented by thapsigargin and D600 respectively, suggests that Ca^{2+} efflux was also accelerated. This acceleration could be ascribed to the activation of PKC, because phorbol esters are known to produce such an effect [46], possibly by activating the plasma membrane Ca^{2+}-ATPase [47]. On the other hand, the effects of PKC on Ca^{2+} influx in insulin-secreting cells are controversial [48], and we have no personal evidence that PKC could be involved in the ACh-mediated decrease in Ca^{2+} influx. In various cells types, muscarinic agonists directly inhibit Ca^{2+} currents by acting on M_1, M_2 or M_4 receptors [49,50]. The muscarinic receptors in pancreatic B-cells display the pharmacological characteristics of the M_3 type [5,6] but are somewhat atypical in influencing a Na^+ permeability [14,40]. Whether more than one type of muscarinic receptor is present in B-cells, and how these receptors might affect several transduction pathways, remains to be established.

Insulin secretion was not measured in the present study, but we have previously shown that the monophasic rise of $[Ca^{2+}]_i$ brought about by ACh in the absence of Ca^{2+} influx triggers a monophasic peak of secretion, at least when it is of sufficient amplitude [51–53]. Both events are directly dependent on the concentration of ACh above 1 μM. When Ca^{2+} influx is possible and glucose is present, the increase in insulin secretion induced by ACh is not always clearly biphasic [15,52,53], and even in the steady state is directly dependent on the concentration of ACh [52]. These two differences that occur with the changes in $[Ca^{2+}]_i$ can be attributed to the activation of PKC that ACh also produces. The amplification of insulin secretion by ACh results from both an increase in $[Ca^{2+}]_i$ and a PKC-mediated sensitization of the secretory system to the action of elevated $[Ca^{2+}]_i$ [8,16,52,53]. This may explain how the sustained effect of 100 μM ACh on insulin secretion is larger than that of 1 μM, in spite of a smaller rise in $[Ca^{2+}]_i$.

In conclusion, the present study demonstrates that muscarinic receptor stimulation causes more complex changes in $[Ca^{2+}]_i$ in an excitable cell type, like the pancreatic B-cell, than in non-excitable cells [2,3]. ACh exerts both inhibitory and stimulatory effects on $[Ca^{2+}]_i$. ACh increases $[Ca^{2+}]_i$ by releasing Ca^{2+} from thapsigargin-sensitive intracellular stores and by augmenting Ca^{2+} influx through voltage-dependent Ca^{2+} channels. The latter effect, which results from a Na^+-dependent depolarization of the B-cell membrane, predominates during sustained stimulation. Once $[Ca^{2+}]_i$ is high, ACh also exerts effects which tend to lower $[Ca^{2+}]_i$ by decreasing the influx and by stimulating the efflux of the ion. The distinct time- and concentration-dependencies of the activation and inactivation of these processes explain the biphasic effect of ACh on $[Ca^{2+}]_i$.

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