G protein-dependent inhibition of L-type Ca^{2+} currents by acetylcholine in mouse pancreatic B-cells

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1. The effect of acetylcholine (ACh) on voltage-dependent Ca^{2+} currents in mouse pancreatic B-cells was studied using the whole-cell configuration of the patch-clamp technique.

2. ACh (0·25–250 μM) reversibly and dose-dependently inhibited the Ca^{2+} current elicited by depolarizations from −80 mV to +10 mV. Maximal inhibition was observed at concentrations > 25 μM where it amounted to ~35%. The effect was voltage independent and prevented by atropine (10 μM) suggesting that it was mediated by muscarinic receptors.

3. The inhibitory action of ACh on the Ca^{2+} current was abolished when the cytoplasmic solution contained GDPβS (2 mM) and became irreversible when the non-hydrolysable GTP analogue GTPγS (10 μM) was included in the pipette. This indicates the participation of G proteins in the inhibitory effect of ACh but pretreatment of the cells with either pertussis or cholera toxin failed to prevent the effect of ACh on the Ca^{2+} current.

4. ACh remained equally effective as an inhibitor of the whole-cell Ca^{2+} current in the presence of the L-type Ca^{2+} channel agonist (−)-Bay K 8644 and after partial inhibition of the current by nifedipine. Addition of ω-agatoxin IVA, ω-conotoxin GVIA or ω-conotoxin MVIIC neither affected the peak Ca^{2+} current amplitude nor the extent of inhibition produced by ACh. These pharmacological properties indicate that ACh acts by inhibiting L-type Ca^{2+} channels.

5. The inhibitory action of ACh on the B-cell Ca^{2+} current was not secondary to elevation of $[Ca^{2+}]_i$ and ACh remained equally effective as an inhibitor when Ba^{2+} was used as the charge carrier, when $[Ca^{2+}]_i$ was buffered to low concentrations using EGTA and under experimental conditions preventing the mobilization of Ca^{2+} from intracellular stores.

6. These results suggest that ACh reduces the whole-cell Ca^{2+} current in the B-cell through a G protein-regulated, voltage- and Ca^{2+}-independent inhibition of L-type Ca^{2+} channels.

Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels plays a central role in the control of the exocytotic process in a variety of excitable cells including the pancreatic B-cell (for a review see Ashcroft & Rorsman, 1989). Modulation of the Ca^{2+} channels by hormones and neurotransmitters controls the extent of Ca^{2+} entry and thus the exocytotic response (for a review see Hille, 1994). Glucose-induced insulin secretion is modulated by hormones and neurotransmitters (for a review see Rasmussen, Zawalich, Ganesan, Calle & Zawalich, 1990). Acetylcholine is released by parasympathetic nerve endings within the pancreatic islets (Ahrén, Taborsky & Porte, 1986) and stimulates insulin secretion by activating muscarinic receptors with the pharmacological characteristics of M_3 receptors (Henquin & Nenquin, 1988; Verspohl, Tacke, Mutschler & Lambrecht, 1990). Several intracellular signal transduction pathways are activated by ACh in the B-cell. These include stimulation of phospholipase C, which generates diacylglycerol (an activator of protein kinase C) and Ins(1,4,5)P_3 (which mobilizes Ca^{2+} from intracellular stores). ACh also induces a Na^+-dependent depolarization in B-cells (Henquin, Garcia, Bozem, Hermans & Nenquin, 1988), which leads to membrane depolarization

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and opening of voltage-gated Ca^{2+} channels. Activation of the two latter transduction pathways increases $[Ca^{2+}]_i$ (Gao, Gilon & Henquin, 1994; Yada, Hamakawa & Yaekura, 1995) and thus enhances Ca^{2+}-dependent insulin secretion. Recent data indicate that ACh also exerts a paradoxical $[Ca^{2+}]_i$-lowering action attributable to inhibition of Ca^{2+} influx and stimulation of Ca^{2+} extrusion (Gilon, Nenquin & Henquin, 1995). Here we have used patch electrode voltage-clamp recordings to explore the suppressor action of ACh on the voltage-gated Ca^{2+} current in the pancreatic B-cell. We demonstrate that ACh, via activation of muscarinic receptors, causes a concentration-dependent inhibition of L-type Ca^{2+} channels. The effect involves G proteins and may serve to protect B-cells from overloading with Ca^{2+} during the intense electrical activity evoked by the neurotransmitter.

METHODS

Preparation of cells

Pancreatic glands were taken from NMRI (Bomholtgård, Ry, Denmark) or CD1 (Charles Rivers, Raleigh, NC, USA) mice. The mice were stunned by a blow to the head and killed by cervical dislocation. Pancreatic islets were isolated by collagenase digestion and were dispersed into single cells by shaking in a Ca^{2+}-free medium. The cells were plated on 35 mm-diameter Petri dishes or, for $[Ca^{2+}]_i$ measurements, on 22 mm-diameter coverslips, and maintained for up to 6 days in RPMI 1640 tissue-culture medium (Gibco BRL, Life Technologies Ltd, Paisley, UK), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Solutions

The standard extracellular solution contained (mM): 125 NaCl, 4·8 KCl, 10 $CaCl_2$ (or $BaCl_2$), 1·2 $MgCl_2$, 10 tetraethylammonium chloride (TEA-Cl), 5 Hepes (pH adjusted to 7·4 using NaOH), 15 glucose and 0·3 μM tetrodotoxin. The standard pipette solution for conventional whole-cell recording was composed of (mM): 125 CsCl, 30 KOH, 1 $MgCl_2$, 10 EGTA, 3 MgATP, 0·1 Na_2GTP and 5 Hepes (pH adjusted to 7·15 with KOH). Guanine nucleotides other than GTP were included in the pipette solution as specified in the text and the legends. For the measurements of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), an EGTA-free solution was used which, unless otherwise indicated, contained (mM): 135 CsCl, 10 NaCl, 1 $MgCl_2$, 5 Hepes (pH 7·15 with KOH), 3 MgATP and 0·1 Na_2GTP.

Some Ca^{2+} current measurements were carried out in metabolically intact B-cells using perforated patch whole-cell recordings. In these experiments, the pipette solution contained (mM): 76 Cs_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$ and 5 Hepes (pH 7·35 with CsOH). Electrical contact was established by addition of the pore-forming antibiotic amphotericin B (Rae, Cooper, Gates & Watsky, 1991). Briefly, a stock solution was prepared by dissolving 6 mg of amphotericin B in 100 μl dimethyl sulphoxide (DMSO). Twenty microlitres of this stock solution were then added to 5 ml of the pipette solution yielding a final concentration of 0·24 mg ml^{-1}. The tip of the pipette was filled with amphotericin-free solution and the pipette was then back-filled with the amphotericin-containing solution. Perforation required a few minutes and the voltage clamp was considered satisfactory when series resistance had fallen below 20 MΩ.

In a few experiments, the cells were pretreated at 37 °C with 100 or 500 ng ml^{-1} pertussis toxin (PTX) or with 500 ng ml^{-1} cholera toxin for 16–24 h. To test for the involvement of intracellular Ca^{2+} stores, some cells were pretreated with 0·5 μM thapsigargin for 20 min prior to the electrophysiological recordings. ACh, atropine, nifepidine, amphotericin B, tetrodotoxin, PTX, GTP, GDPβS and GTPγS were all obtained from Sigma. Thapsigargin and the Ca^{2+} channel blockers ω-agatoxin IVA, ω-conotoxin GVIA and ω-conotoxin MVIIC were from Alomone Labs (Jerusalem). Cholera toxin and the Ca^{2+} channel agonist (−)-Bay K 8644 were purchased from Research Biochemicals International. All experiments were performed at room temperature (22–24 °C).

Electrophysiological recordings

Voltage-gated Ca^{2+} currents were recorded using the whole-cell configuration of the patch-clamp method. Pipettes were pulled from borosilicate glass (Hilgenberg GmbH, Malsfeld, Germany or Corning 7052, Garner Glass Co., Claremont, CA, USA), coated with Sylgard 184 (Dow Corning) near their tips and fire polished. They had a resistance of 2–5 MΩ when filled with the standard pipette solution. Membrane currents were measured using either an EPC-9 patch-clamp amplifier (Heka Electronics, Lambrecht/Pfalz, Germany) and the software Pulse (version 7.62), or an Axopatch-1D amplifier and the software pCLAMP 6 (Axon Instruments). The holding potential was −80 mV and depolarizing voltage pulses were applied at low frequency (0·1–0·2 Hz) to minimize the rundown of the Ca^{2+} current. The currents were filtered at 1 kHz, digitized at 4–5 kHz and stored in a computer pending later analysis. For tail current measurements, currents were filtered at 8 kHz and digitized at 20 kHz. Leak currents and capacitative transients were removed on-line using a $P/4$ or a $P/16$ routine. The reference potential for all measurements was the zero-current potential of the pipette obtained immediately after junction potential correction and before seal establishment.

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

$[Ca^{2+}]_i$ was measured using an Axiovert 100 inverted microscope equipped with a Plan-Neofluar ×100/1·30 objective (Carl Zeiss, Oberkochen, Germany) and an Ionoptix (Milton, MA, USA) fluorescence imaging system as described elsewhere (Bokvist, Eliasson, Ämmälä, Renström & Rorsman, 1995). Excitation was effected at 340 and 380 nm and emitted light recorded at 510 nm with an interval between each data point of 200 ms. The experiments were carried out using the standard whole-cell configuration and fura-2 (0·1 mM of the pentapotassium salt; Molecular Probes) was loaded into the cell by inclusion in the pipette solution.

Data analysis

The experiments are illustrated by representative recordings. Data are presented as mean values ± S.E.M. for n experiments. Statistical significances were evaluated using Student's t test for paired or unpaired data. Multiple comparisons were performed by analysis of variance followed by a Newman–Keuls test.

RESULTS

ACh reversibly inhibits Ca^{2+} current by activation of muscarinic receptors

Voltage-dependent Ca^{2+} currents were elicited by 100 ms voltage steps from −80 to +10 mV (Fig. 1*A*). The resulting currents were typically biphasic, consisting of a sustained and an inactivating component. Addition of a high concentration of ACh (250 μM) inhibited both components by ~35%. Figure 1*B* summarizes the changes of the peak Ca^{2+} current amplitude produced by application of ACh as a

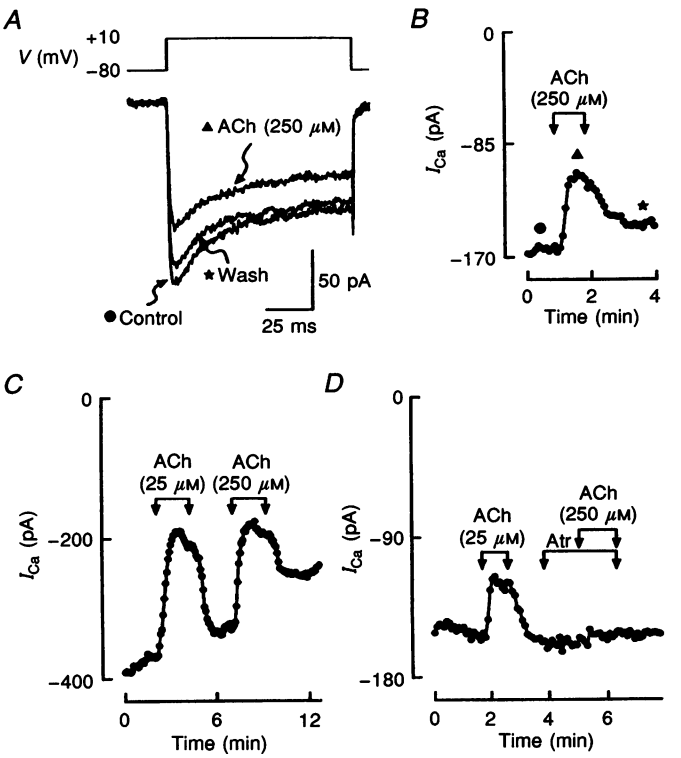

Figure 1. ACh reversibly inhibits the voltage-dependent Ca^{2+} current by activating muscarinic receptors in mouse pancreatic B-cells

A, effect of ACh (250 μM) on the whole-cell Ca^{2+} currents evoked by 100 ms depolarizing pulses from −80 to +10 mV. The sweeps were taken before addition (●), in the presence of (▲) and following the washout of ACh (★). *B*, peak Ca^{2+} current (I_{Ca}) plotted *versus* time. ACh was applied as indicated. The symbols ●, ★ and ▲ indicate the records selected for display in *A*. Note reversibility of ACh action. *C*, effects of sequential application of 25 and 250 μM ACh on peak Ca^{2+} current. *D*, same as *C*, but effect of 250 μM ACh fully antagonized by atropine (10 μM). Depolarizations were applied at 5 s intervals. The traces are representative of results obtained in 33 (*A* and *B*), 6 (*C*) and 10 cells (*D*).

function of time. It can be observed that the inhibitory effect of ACh developed rapidly upon application of ACh and was readily reversed upon removal of the neurotransmitter from the perfusion medium. Lower concentrations of ACh were also effective, with 25 μM producing a rapid and reversible inhibition of the current (Fig. 1*C*). The action of ACh on the Ca^{2+} current is mediated by activation of muscarinic receptors as suggested by the experiment displayed in Fig. 1*D*. It was first ascertained that 25 μM ACh was capable of reducing the peak Ca^{2+} current amplitude. Following the washout of ACh and the return of the current amplitude to the control level, atropine (10 μM) was added to the bath medium and ACh applied at a concentration of 250 μM in the continued presence of the antagonist. It is clear that ACh under these conditions failed to inhibit the Ca^{2+} current (compare with Fig. 1*C*).

Concentration-dependent inhibition of Ca^{2+} current by ACh

The concentration dependence of the ACh inhibitory action is summarized in Fig. 2. ACh was without inhibitory action at concentrations below 1 μM. Higher concentrations were progressively more inhibitory and at concentrations ⩾ 250 μM the extent of block amounted to ~35% (see also Table 1). Half-maximal reduction of the Ca^{2+} current was attained at ~5 μM ACh. ACh was slightly more effective in metabolically intact B-cells (perforated patch recordings) and the degree of inhibition amounted to ~50% at a

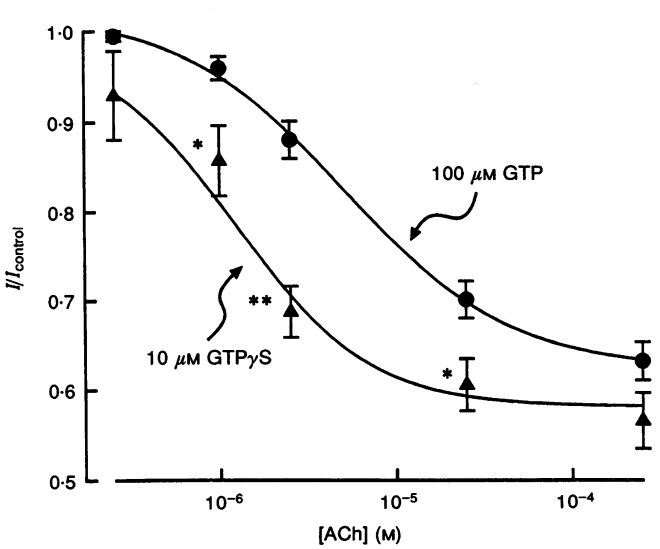

Figure 2. Concentration dependence of the inhibitory effect of ACh on the Ca^{2+} current

Peak Ca^{2+} currents observed in the presence of 0·25–250 μM ACh are expressed as a fraction of that observed under control conditions ($I/I_{control}$). The concentration dependence was estimated from experiments similar to those presented in Fig. 1. Because of the problem of rundown of the Ca^{2+} currents, it was usually not feasible to try more than one concentration in each cell. The cells were dialysed either with GTP (100 μM; ●) or GTPγS (10 μM; ▲). Inhibition with respect to the controls was statistically significant at concentrations ⩾ 2·5 μM. At 1, 2·5 and 25 μM, ACh was more effective when the pipette solution contained GTPγS instead of standard GTP: $*P < 0.05$ and $**P < 0.01$ for the difference between the experiments with GTP and GTPγS. Each point represents the mean ± S.E.M. of 5–33 cells.

Table 1. Effect of ACh on voltage-dependent Ca^{2+} current in mouse pancreatic B-cells

Experimental condition	Percentage inhibition by 250 μM ACh
Conventional whole-cell recordings	
Control	36·8 ± 2·1 (33)
Pretreatment with pertussis toxin	33·8 ± 4·9 (9)
Pretreatment with cholera toxin	37·0 ± 5·9 (6)
In the presence of nifedipine (50 μM)	26·9 ± 8·3 (8)
In the presence of Bay K 8644 (1 μM)	35·4 ± 3·5 (11)
Ba^{2+} (10 mM) as charge carrier	29·0 ± 7·2 (7)
Ins(2,4,5)P_3 (100 μM) in the pipette (> 5 min infusion)	32·4 ± 6·9 (6)
Heparin (1 mg ml^{-1}) in the pipette (> 5 min infusion)	33·1 ± 2·5 (6)
Thapsigargin pretreatment (0·5 μM)	32·7 ± 2·1 (6)
Perforated patch whole-cell recording	
Control	51·8 ± 6·2 (6)*
Thapsigargin pretreatment (0·5 μM)	42·5 ± 5·0 (6)

* $P < 0·05$ *versus* control in conventional whole-cell recording. Numbers in parentheses are n the number of cells.

concentration of 250 μM (Table 1). As was the case in standard whole-cell recordings, the effect in the intact cells was readily and rapidly reversible (not shown).

Voltage dependence of the inhibitory effect of ACh

The voltage dependence of the ACh action was evaluated by comparing the current (I)–voltage (V) relationships recorded in the absence and presence of ACh. Inward Ca^{2+} currents were first observed during depolarizations to −30 mV and their amplitude increased with the applied voltage up to +10 mV (Fig. 3*A* and *B*). At more positive voltages, the peak amplitude decreased as a result of the reduced driving force. The percentage block at different membrane potentials is summarized in Fig. 3*C*. Clearly, ACh produces a similar, ~35% block at all voltages.

To characterize the voltage dependence of the block further, we analysed the tail currents seen upon returning to a holding potential of −80 mV. Figure 4*A* and *B* presents whole-cell currents evoked by membrane depolarization to 0 and +120 mV, respectively. In the cell displayed, application of ACh reduced the amplitude of the steady-state currents seen during the depolarizations by ~50% at both voltages. The associated tail currents are shown in Fig. 4*C*. The amplitude of the tail currents observed in the presence of

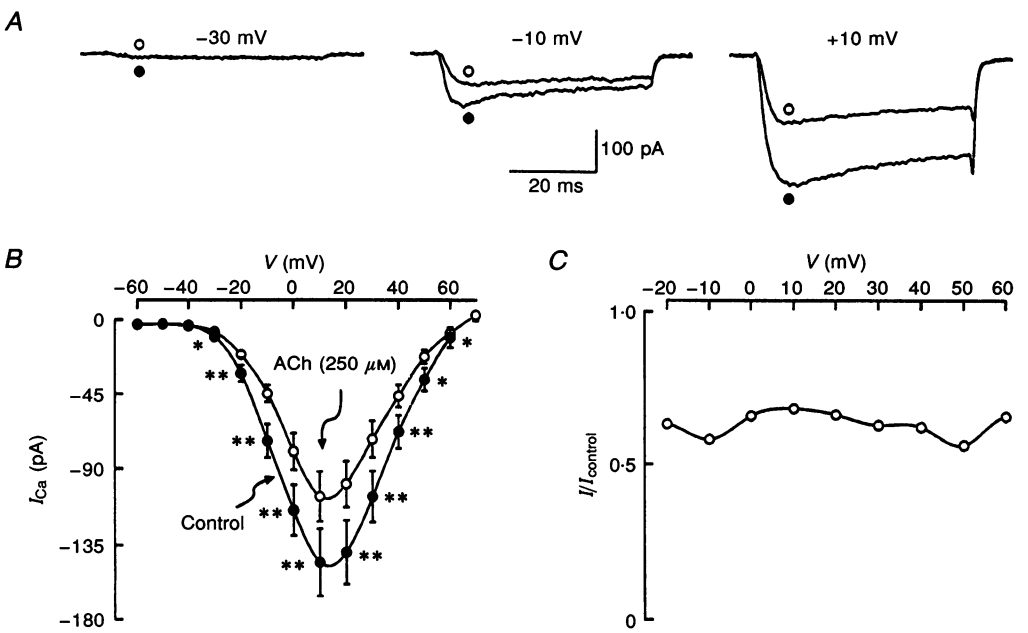

Figure 3. ACh produces a similar inhibition at all membrane potentials

A, Ca^{2+} currents recorded from a B-cell in the absence (●) and in the presence (○) of 250 μM ACh in response to 50 ms depolarizing pulses from a holding potential of −80 mV to the indicated voltages. *B*, current (I)–voltage (V) relationships for peak Ca^{2+} currents recorded in the absence (●) or presence (○) of 250 μM ACh. Because of the rundown of the Ca^{2+} current, the control I–V relationship was obtained by averaging the current amplitudes before addition and following the washout of ACh. Mean values ± S.E.M. of 12 cells. *$P < 0·05$ and **$P < 0·01$ when comparing peak current amplitudes of controls with the corresponding currents in the presence of ACh. *C*, fractional current ($I/I_{control}$) in the presence of ACh as a function of membrane potential (V). The fractional current was calculated using the mean values in *B* and derived by dividing the currents in the presence of ACh (I) with the respective control values ($I_{control}$).

ACh relative to those seen under control conditions amounted to 45%, and 55% after depolarization to 0 and +120 mV, respectively. The data of fifteen different experiments are summarized in Fig. 4*D*. It is clear that the tail currents are similarly reduced at both voltages.

The inhibitory effect of ACh is mediated by activation of G proteins

To test the involvement of G proteins in the inhibition of the Ca^{2+} current by ACh, the cells were infused with different guanine nucleotides. In the presence of GTP (100 μM), ACh produced a dose-dependent and reversible inhibition of the Ca^{2+} current; the extent of inhibition amounted to 10 and 40% in the presence of 2·5 and 250 μM ACh, respectively (Fig. 5*A*). When GTP was replaced by 10 μM GTPγS, a non-hydrolysable activator of G proteins which inhibits the intrinsic GTPase activity of G proteins, ACh produced an *irreversible* inhibition of the Ca^{2+} current (Fig. 5*B*). The larger inhibition observed when 250 μM was applied after 2·5 μM ACh is likely to have resulted from the lower concentration only inhibiting a fraction of the Ca^{2+} channels whilst leaving the remainder unaffected. This is supported by the observation that the higher concentration resulted in maximal inhibition already during the first application and a second exposure exerted no additive effect (Fig. 5*C*). By contrast, ACh failed to inhibit the Ca^{2+} current when 2 mM GDPβS was included in the pipette solution (Fig. 5*D*). This we attribute to the fact that GDPβS binds strongly to the G protein and acts as a competitive inhibitor of GTP binding to the G_α subunits. The ability of GDPβS to counteract the inhibitory action may explain the observation that ACh is a more potent inhibitor of the Ca^{2+} current in cells dialysed with GTPγS than in cells infused with the standard GTP-containing solution: half-maximal inhibition was observed at ~1 μM (Fig. 2). It seems likely that GDP formed by hydrolysis of added GTP tends to counteract the action of ACh thus shifting the dose–inhibition curve towards higher concentrations. Overnight pretreatment of B-cells with PTX (100–500 ng ml^{-1}) or cholera toxin (500 ng ml^{-1}) both failed to influence the capacity of ACh to inhibit the B-cell Ca^{2+} current (Table 1).

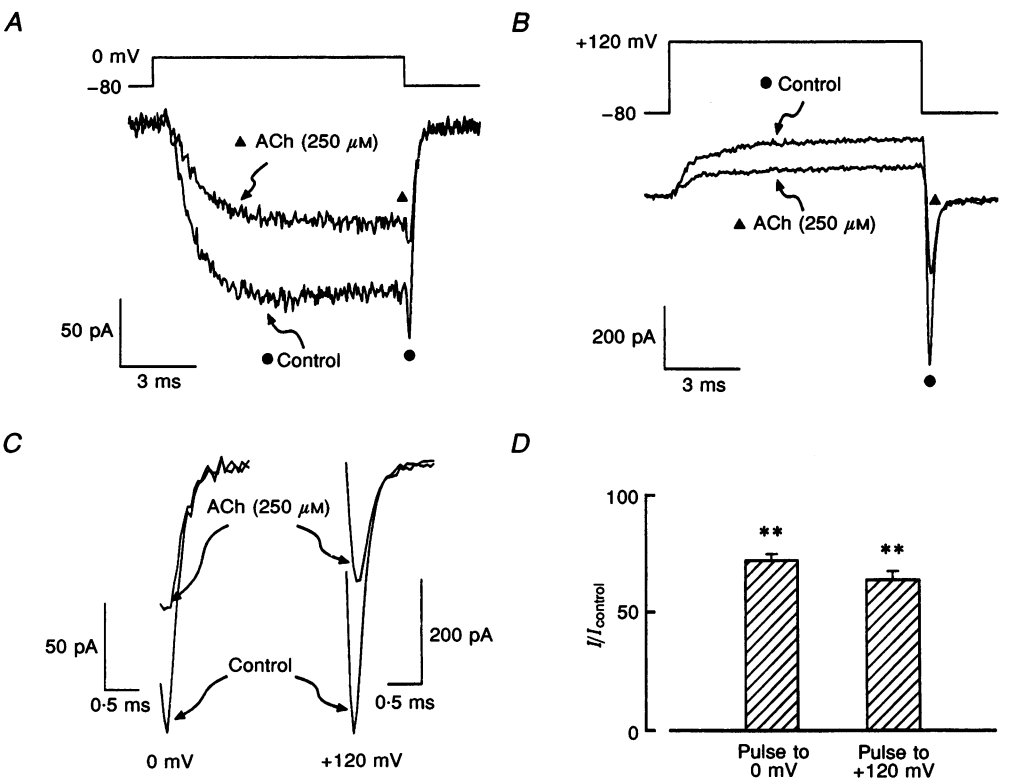

Figure 4. Voltage-independent block of Ca^{2+} current by ACh

Whole-cell currents flowing through L-type Ca^{2+} channels during 10 ms depolarizations from −80 to either 0 mV (*A*) or +120 mV (*B*) were recorded from the same B-cell in the absence (●) or presence (▲) of 250 μM ACh. The tail currents recorded when returning to the holding potential from 0 or +120 mV (as indicated) are shown on an expanded time base in *C* with different scales. *D*, quantification of the inhibitory effects of ACh on the tail currents recorded following depolarizations to 0 and +120 mV. The amplitude of the current recorded in the absence of ACh was taken as unity ($I_{control}$) and the current (I) seen following the addition of ACh is expressed as a fraction hereof ($I/I_{control}$). Mean values ± S.E.M. of 15 separate cells. $^{**}P < 0{\cdot}01$ when compared with the controls.

ACh inhibits L-type Ca^{2+} channels

We next investigated which type of Ca^{2+} channel is affected by ACh. Based on the biophysical properties and pharmacology, we have previously concluded that the Ca^{2+} current in *mouse* pancreatic B-cells flows principally through L-type Ca^{2+} channels (Plant, 1988; Rorsman, Ashcroft & Trube, 1988) but we decided to reinvestigate this using novel and selective inhibitors of different types of Ca^{2+} channel. Consistent with the earlier observations, nifedipine (50 μM) when applied in the presence of 2·6 mM external Ca^{2+} inhibited the peak and integrated Ca^{2+} current by 71 ± 5 and 80 ± 4% ($n = 4$), respectively. The inhibitory action of nifedipine was antagonized by elevation of extracellular Ca^{2+}, and in the presence of 10 mM external Ca^{2+}, 50 μM nifedipine decreased the peak current by only 48 ± 5% ($n = 8$; Fig. 6*A*). The L-type Ca^{2+} channel agonist (−)-Bay K 8644 (1 μM) increased the peak Ca^{2+} current evoked by depolarizations from −80 to +10 mV by 122 ± 15% ($n = 11$; Fig. 6*B*). However, irrespective of whether applied in the presence of nifedipine or (−)-Bay K 8644, ACh produced a similar degree of inhibition and reversibly reduced the peak Ca^{2+} current by 25–35% (Table 1 and Fig. 6*A* and *B*).

The possible contribution of non-L-type Ca^{2+} currents to the B-cell whole-cell Ca^{2+} current was investigated using the neurotoxins ω-conotoxin GVIA (1 μM), ω-agatoxin IVA (0·5 μM) and ω-conotoxin MVIIC (0·5 μM) to specifically inhibit N-type, P-type and Q-type channels, respectively (Fig. 6*C–E*). It is clear that neither the peak Ca^{2+} current, nor the extent of the inhibition produced by ACh was affected by any of these toxins. All experiments were terminated by addition of 100 μM Cd^{2+}. This unspecific Ca^{2+} channel blocker invariably abolished the Ca^{2+} current (> 95% inhibition), thus confirming that the currents we recorded are indeed attributable to Ca^{2+} influx.

The inhibitory effect of ACh is not mediated by intracellular Ca^{2+}

ACh was equally effective as an inhibitor of the whole-cell Ca^{2+} channel current when Ba^{2+} was used as the charge carrier instead of Ca^{2+} (Fig. 7*A* and *B*). Thus, Ca^{2+} influx is not required for the inhibitory action. In a few cells application of ACh slowed Ca^{2+} current activation. However, this was not consistently observed and in most cells, the time course of activation remained the same in the absence and presence of ACh. This has been explained in terms of a portion of the Ca^{2+} channels not being associated with the activated G protein and therefore not accessible for inhibition (Dolphin, 1991). The rapid opening of these channels obscures the slower activation of the Ca^{2+} channels that have been modulated by the neurotransmitter.

Muscarinic receptor stimulation promotes, via generation of $InsP_3$, the mobilization of intracellular Ca^{2+}. Since the B-cell

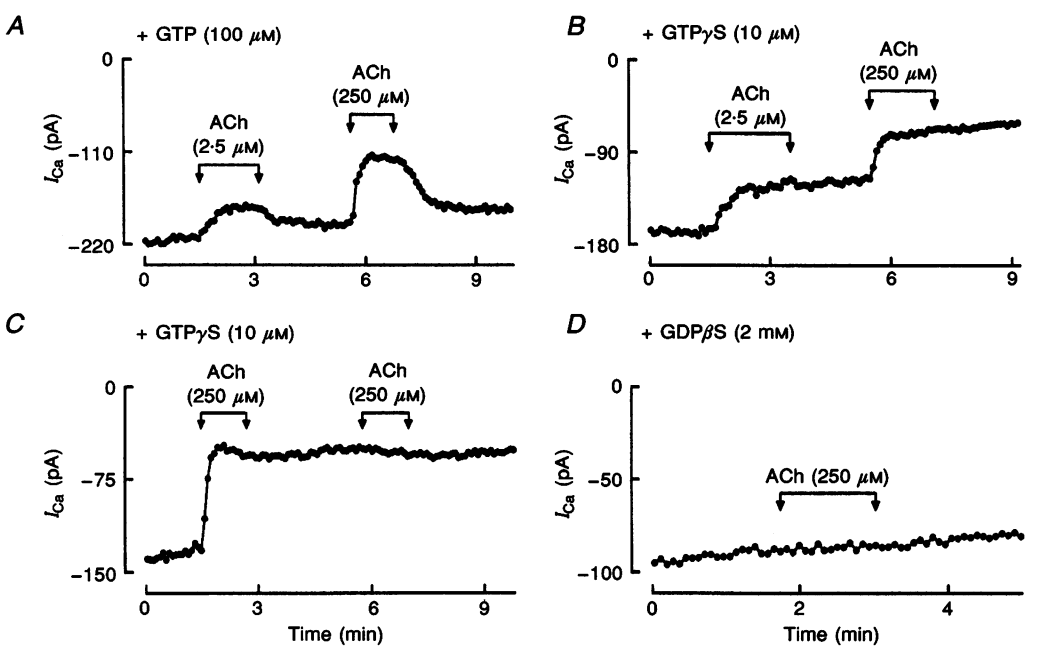

Figure 5. The inhibitory effect of ACh is mediated by a G protein

Effects of ACh on the peak Ca^{2+} currents when the B-cells were dialysed with the standard intracellular solution containing 100 μM GTP (*A*), when GTP was replaced by 10 μM GTPγS (*B–C*) and when the cytoplasmic solution was supplemented with 2 mM GDPβS (*D*). ACh was applied at 2·5 or 250 μM as indicated by the horizontal lines. Data represent the peak Ca^{2+} current amplitude displayed against the time elapsed after the onset of the recording. The depolarizations used to evoke the Ca^{2+} currents went to +10 mV from a holding potential of −80 mV and were applied at 5 s intervals. The traces are representative of results obtained in 8 (*A*), 5 (*B*), 7 (*C*) and 8 cells (*D*).

Ca^{2+} currents are subject to Ca^{2+}-dependent inactivation (Plant, 1988), it may thus be argued that the action of ACh on the whole-cell Ca^{2+} currents was mediated by a rise of $[Ca^{2+}]_i$ through mobilization from intracellular stores. Several pieces of evidence suggest that this is unlikely to be the case (Table 1). First, inclusion of 100 μM Ins(2,4,5)P_3, a poorly metabolizable analogue of Ins(1,4,5)P_3, failed to influence the inhibitory action of ACh. Second, ACh remained effective even when the cell was infused with heparin (1 mg ml^{-1}), an inhibitor of the InsP_3 receptor. Third, pretreatment of the cells with thapsigargin (0·5 μM) did not attenuate the inhibitory effect of ACh. These data argue that an increase in $[Ca^{2+}]_i$ by mobilization from intracellular stores does not mediate the inhibition of Ca^{2+} current by ACh.

To analyse further the correlation between $[Ca^{2+}]_i$ and the peak Ca^{2+} current amplitude, we monitored the Ca^{2+} currents and associated increases in $[Ca^{2+}]_i$ by combining whole-cell voltage-clamp recordings with microfluorimetry. Under conditions where the cells were dialysed with a buffer with a low Ca^{2+}-buffering capacity (no EGTA in the pipette solution), depolarizations from −80 to 0 mV evoked $[Ca^{2+}]_i$ transients from a basal 150 nM to peak values of 400–500 nM (Fig. 8*A*). Application of ACh transiently increased $[Ca^{2+}]_i$ to a plateau concentration of ~400 nM from which it subsequently spontaneously declined despite the continued presence of the neurotransmitter, presumably because of depletion of the intracellular Ca^{2+} stores mobilized by ACh/ InsP_3. The amplitude of the depolarization-evoked $[Ca^{2+}]_i$ transients superimposed on the ACh-induced increase in $[Ca^{2+}]_i$ remained unchanged, however, and amounted to 400–500 nM. These effects of ACh on $[Ca^{2+}]_i$ were correlated with a reduction of the peak Ca^{2+} current. In this series of experiments the percentage inhibition produced by ACh (250 μM) amounted to 34 ± 10% ($n = 4$). The effects of ACh on the peak Ca^{2+} currents reversed upon removal of ACh from the perfusion medium.

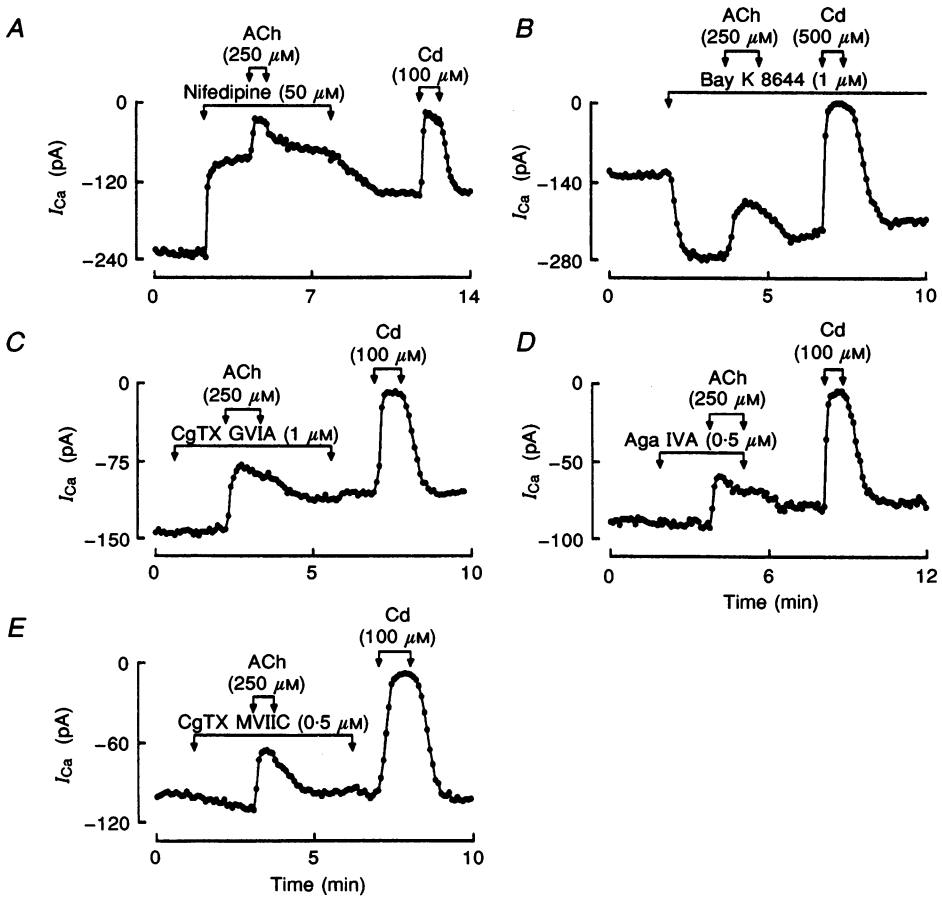

Figure 6. ACh inhibits L-type Ca^{2+} channels

Effects of ACh on Ca^{2+} currents recorded in the presence of 50 μM nifedipine (*A*), 1 μM (−)-Bay K 8644 (*B*), 1 μM ω-conotoxin GVIA (CgTX GVIA; *C*), 0·5 μM ω-agatoxin IVA (Aga IVA; *D*) and 0·5 μM ω-conotoxin MVIIC (CgTX MVIIC; *E*). Measured peak Ca^{2+} currents are displayed against time after establishment of the whole-cell configuration. The currents were evoked at 5 s intervals by depolarizing pulses going from −80 to +10 mV except in *B* where pulses went to 0 mV. Agents were applied as indicated by the horizontal lines above the records. The experiments are representative of results obtained in 8 (*A*), 11 (*B*), 5 (*C*), 3 (*D*) and 3 cells (*E*).

When the same experiments were repeated with a pipette solution containing 10 mM EGTA, i.e. the same as that used for most of the other experiments, the depolarization-evoked Ca^{2+} currents (Fig. 8*B*) were not associated with any $[Ca^{2+}]_i$ transients and application of ACh likewise had no effect on $[Ca^{2+}]_i$. Nevertheless, ACh still reduced the peak Ca^{2+} current amplitude by $34 \pm 8\%$ ($n = 4$) – similar to that obtained with the pipette solution without EGTA. Collectively the data of Table 1 and Fig. 8 indicate that an increase in $[Ca^{2+}]_i$ is not required for the inhibitory action of ACh on the B-cell Ca^{2+} current.

DISCUSSION

Acetylcholine markedly potentiates nutrient-induced insulin secretion. Its action involves the opening of (as yet uncharacterized) Na^+-conducting channels, Ca^{2+} mobilization from intracellular stores and protein kinase C-dependent stimulation of Ca^{2+}-dependent exocytosis. In addition to these stimulatory effects, we demonstrate here that ACh, via activation of G proteins, also exerts a paradoxical inhibitory action on the voltage-dependent Ca^{2+} current. This effect accounts for the recent and surprising observation that ACh decreases $[Ca^{2+}]_i$ in depolarized mouse pancreatic B-cells (Gilon *et al.* 1995). The effects of ACh on the Ca^{2+} current we now describe resemble the inhibitory action evoked by photorelease of caged GTPγS (Ämmälä, Berggren, Bokvist & Rorsman, 1992). It is therefore tempting to speculate that ACh is the physiological agonist utilizing this G protein-dependent pathway of Ca^{2+} channel regulation in the pancreatic B-cell.

The Ca^{2+} current in B-cells flows through L-type Ca^{2+} channels

The resistance to nifedipine of a large component of the Ca^{2+} current is surprising given earlier reports that the whole-cell Ca^{2+} current in mouse B-cells flows principally (if not exclusively) through L-type Ca^{2+} channels which should be blocked by nifedipine (Plant, 1988; Rorsman *et al.* 1988). We propose that this results from the use, in most of our experiments, of a high extracellular Ca^{2+} concentration (10 mM), which counteracts the antagonistic action of nifedipine. Indeed, when the external Ca^{2+} concentration was lowered to the more physiological 2·6 mM, 50 μM nifedipine produced > 80% inhibition of the integrated Ca^{2+} current. Given that single-channel recordings from mouse pancreatic B-cells have failed to document Ca^{2+} channels distinct from the L-type (Rorsman *et al.* 1988; Smith, Ashcroft & Fewtrell, 1992), the strong stimulation of the Ca^{2+} current induced by the L-type Ca^{2+} channel agonist (−)-Bay K 8644 and the lack of effects of ω-conotoxin GVIA, ω-agatoxin IVA or ω-conotoxin MVIIC (specific inhibitors of N-type, P-type and Q-type channels, respectively) it seems reasonable to conclude that the whole-cell Ca^{2+} current in mouse B-cells flows predominantly through L-type Ca^{2+} channels with low sensitivity to nifedipine. An alternative explanation is that ~20% of the B-cell Ca^{2+} current flows through a different type of Ca^{2+} channel (see Hopkins, Satin & Cook, 1991) which is resistant to nifedipine as well as the peptide Ca^{2+} channel antagonist (Magnelli, Pollo, Sher & Carbone, 1995).

ACh modulates L-type Ca^{2+} channels

Our data argue that ACh reduces the whole-cell Ca^{2+} current by inhibiting L-type Ca^{2+} channels. This interpretation is supported by the observations that ACh was equally effective as an inhibitor in the presence or absence of nifedipine and (−)-Bay K 8644. If ACh had blocked Ca^{2+} channels other than L-type, its apparent efficacy as a blocker would have increased following inhibition of the L-type Ca^{2+} channels with nifedipine, and decreased following activation of the L-type Ca^{2+} channels with (−)-Bay K 8644. The fact that ACh produced a similar inhibition under both conditions therefore argues that ACh decreases the whole-cell Ca^{2+} current by inhibiting L-type Ca^{2+} channels. If a fraction of Ca^{2+} current flows through nifedipine-resistant channels as discussed above, these channels must have the same sensitivity to ACh and the same ionic selectivity as otherwise the efficacy of ACh as an inhibitor would be affected by the dihydropyridines and substitution of Ba^{2+} for Ca^{2+} as the charge carrier.

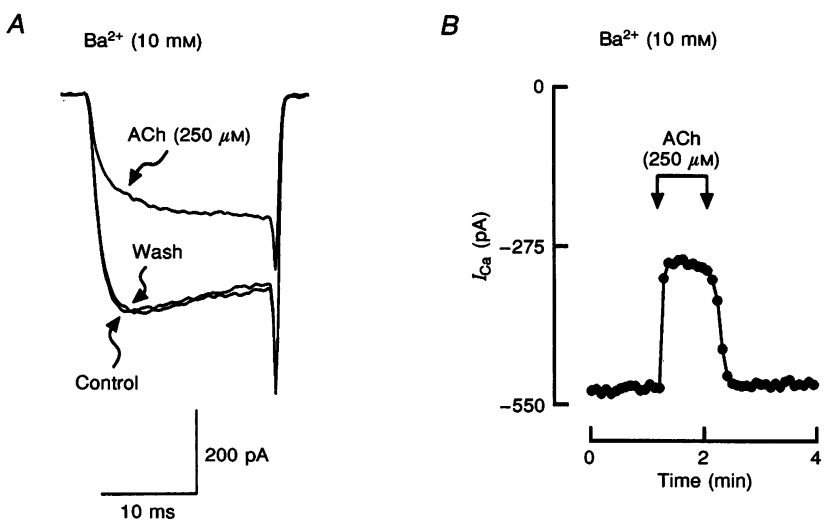

Figure 7. The effect of ACh persists with Ba^{2+} as the charge carrier

A, Ba^{2+} currents evoked by depolarizing pulses from −80 to 0 mV when extracellular Ca^{2+} was replaced by 10 mM Ba^{2+} under control conditions, after application of ACh (250 μM) and following the washout of ACh. Currents were evoked at 5 s intervals. *B*, time course of the inhibitory action of ACh (250 μM) on the peak Ba^{2+} current. The traces are representative of results obtained in 7 cells.

G protein and muscarinic receptor identity

The inhibitory action of ACh on the B-cell Ca^{2+} current is mediated by activation of muscarinic receptors. Five muscarinic receptor subtypes have been cloned so far, which can be subdivided in two categories: the M_1, M_3 and M_5 muscarinic ACh receptors (mAChRs) which activate G proteins of the G_q/G_{11} class; and the M_2 and M_4 mAChRs which are preferentially linked to PTX-sensitive G_i and G_o class G proteins. Whereas activation of the former group of ACh receptors is linked to phosphoinositide breakdown and Ca^{2+} mobilization, members of the latter category initiate a variety of processes such as inhibition of adenylate cyclase and voltage-sensitive Ca^{2+} channels (Hulme, Curtis, Page & Jones, 1993). The observations that the inhibitory effect of ACh on the Ca^{2+} current in the B-cell is suppressed when GDPβS is included in the pipette solution and that it becomes irreversible in the presence of GTPγS indicate the involvement of a G protein. The resistance of the ACh action to pretreatment with pertussis and cholera toxins suggests that it is not mediated via activation of G_i, G_o or G_s, which excludes the participation of M_2 or M_4 ACh receptors in B-cells. The inhibitory effect of ACh on the Ca^{2+} current is therefore most likely mediated by mAChRs of the first category, which are linked to the activation of G_q/G_{11}-containing G proteins. This conclusion is in keeping with that previously deduced from biochemical and functional data (Henquin & Nenquin 1988; Verspohl *et al.* 1990; Boschero *et al.* 1995).

In nerve cells, five different G protein-mediated pathways activated by membrane receptors are associated with inhibition of voltage-dependent Ca^{2+} channels (for a review see Hille, 1994). Only two of these, however, are utilized by muscarinic receptors: (1) a PTX-sensitive mechanism activated by M_4 receptors producing voltage-dependent inhibition of N-type Ca^{2+} channels in neurones (Hille, 1992) and L-type channels in pituitary GH_3 cells (Offermanns *et al.*

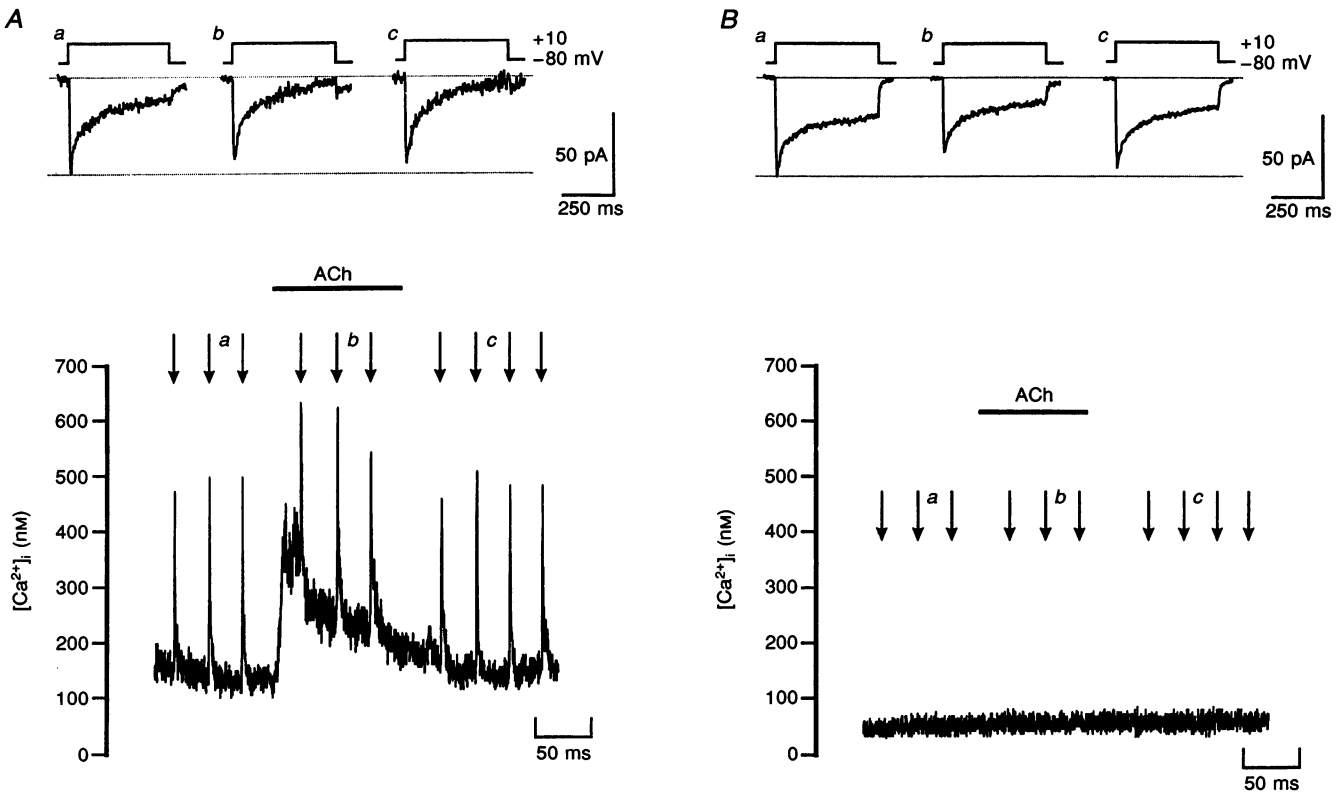

Figure 8. Effects of ACh on $[Ca^{2+}]_i$ in voltage-clamped B-cells with low and high intracellular Ca^{2+} buffering

The B-cell was held at −80 mV and 500 ms membrane depolarizations to 0 mV were applied at 30 s intervals (as indicated by the arrows) to activate the Ca^{2+} channels. *A*, top, Ca^{2+} currents recorded with an EGTA-free pipette solution under control conditions (*a*), in the presence of ACh (*b*) and following the washout of neurotransmitter (*c*). *A*, bottom, depolarization-evoked increases in $[Ca^{2+}]_i$. ACh (250 μM) was applied during the period indicated by the horizontal bar. Note increase in $[Ca^{2+}]_i$ not associated with membrane depolarization. The Ca^{2+} currents selected for display were taken as indicated by the letters *a–c*. *B*, as in *A* but the pipette solution dialysing the cell interior was supplemented with 10 mM EGTA. Note that ACh decreased the peak Ca^{2+} current although no elevation of $[Ca^{2+}]_i$ was seen. In *A* and *B*, the dotted horizontal lines indicate the baseline level and the peak Ca^{2+} current amplitude recorded before addition of ACh. The experiments are representative of 8 experiments carried out with low and high intracellular EGTA levels (4 experiments of each type).

1991), and (2) a PTX-insensitive mechanism that is activated by M_1 receptors and results in the voltage-independent inhibition of N- and L-type Ca^{2+} channels in sympathetic neurons (Bernheim, Mathie & Hille, 1992; Mathie, Bernheim & Hille, 1992). The latter pathway shows the greatest similarity to the one activated by ACh in the B-cell and which we describe in the present study. The presence of M_1 receptors has recently been reported in whole islets, but it is not known whether they are expressed in the B-cell or the other islet cell types (Boschero *et al.* 1995). It seems possible that whereas the latter subclass of muscarinic receptors mediates the inhibitory actions of ACh on the Ca^{2+} current, activation of M_3 receptors (the presence of which has been documented in B-cells; Henquin & Nenquin, 1988) produces the stimulatory effects of the neurotransmitter.

Signal transduction pathway activated by ACh inhibiting the Ca^{2+} current

Ca^{2+} has been reported to mediate bradykinin- and thyrotropin-releasing hormone-induced Ca^{2+} current inhibition in neuronal (Dolphin, McGuirk & Scott, 1989) and GH_3 cells (Kramer, Kaczmarek & Levitan, 1991). However, several features suggest that the G protein-dependent inhibitory action of ACh on the B-cell Ca^{2+} current is not mediated by an elevation of the cytoplasmic Ca^{2+} concentration. First, substituting Ba^{2+} for extracellular Ca^{2+} did not reduce the inhibitory action of ACh. Second, ACh remained effective as an inhibitor of the Ca^{2+} current when $[Ca^{2+}]_i$ was clamped at low concentrations by inclusion of 10 mM EGTA in the pipette solution. Third, depletion of intracellular Ca^{2+} stores by pretreatment with thapsigargin or infusion of the cell with Ins(2,4,5)P_3, a stable analogue of Ins(1,4,5)P_3, did not interfere with the inhibitory action of ACh, and inhibition of the InsP_3 receptors by inclusion of heparin in the cytoplasmic solution was likewise ineffective. The fact that ACh remained inhibitory when the cell was dialysed with Ins(2,4,5)P_3 in the pipette solution also enables us to exclude the possibility that InsP_3 itself, formed in response to the ACh-induced activation of phospholipase C, mediates the action on the Ca^{2+} current.

Inhibition of adenylate cyclase is a major G protein-dependent mechanism by which muscarinic stimulation decreases cardiac L-type current (Jones, 1993). For several reasons such a mechanism of action is unlikely to account for ACh-induced inhibition of the Ca^{2+} current in the B-cell. First, there is no evidence that ACh affects cAMP levels in pancreatic islet cells (Gagerman, Idahl, Meissner & Täljedal, 1978). Second, the effect on the Ca^{2+} current is resistant to PTX, which would not be expected if it involved G_i-mediated inhibition of adenylate cyclase (Birnbaumer, Abramowitz & Brown, 1990; Offermanns *et al.* 1991). Third, inclusion of cAMP in the pipette solution does not prevent GTPγS-induced inhibition of the whole-cell Ca^{2+} current (Ämmälä *et al.* 1992). Last, the suppressor action of ACh is not affected by pharmacological manoeuvres that elevate the cytoplasmic cAMP levels such as cholera toxin (Table 1) and forskolin (P. Gilon, unpublished results).

We can finally exclude the involvement of protein kinase C (PKC) since it is well established that ACh leads to the stimulation of PKC and activators of PKC do not decrease, but rather increase, the Ca^{2+} current (Velasco & Petersen, 1989; Ämmälä *et al.* 1994).

Physiological relevance

The ability of GTPγS to inhibit the B-cell Ca^{2+} current, presumably via activation of G proteins, has previously been documented (Ämmälä *et al.* 1992), but it was not possible to determine the physiological agonist(s) utilizing this pathway. Experiments on insulin-secreting cell lines have shown that hormones and neurotransmitters such as somatostatin (Hsu, Xiang, Rajan, Kunze & Boyd, 1991), galanin (Homaidan, Sharp & Nowak, 1991) and adrenaline (Keahey, Boyd & Kunze, 1989) inhibit the voltage-gated Ca^{2+} current in these cells via a PTX-sensitive pathway. None of these effects could be demonstrated in mouse pancreatic B-cells (Renström, Ding, Bokvist & Rorsman, 1996). The present study is the first report of inhibition of voltage-dependent Ca^{2+} channels by a neurotransmitter in non-tumorous B-cells. Unexpectedly and paradoxically, this inhibitory action is exerted by ACh, which otherwise is regarded as a potentiator of insulin secretion.

The inhibitory action of ACh on the Ca^{2+} current is observable at concentrations within the micromolar range comparable to those that stimulate electrical activity and mobilize intracellular Ca^{2+} (Henquin *et al.* 1988; Gilon *et al.* 1995; Yada *et al.* 1995). It therefore seems likely that these three processes operate in parallel and that the overall (secretory) response to ACh reflects the balance between the opposing effects on $[Ca^{2+}]_i$. The inhibitory action we now describe is likely to account for the previous observations that high concentrations of ACh or carbamyl-choline reduce the upstroke velocity of the B-cell action potentials (Sánchez-Andrés, Ripoll & Soria, 1988). It may be speculated that the ability of ACh to reduce the Ca^{2+} current serves to protect B-cells from being overloaded with Ca^{2+}. This mechanism is likely to co-operate with the $[Ca^{2+}]_i$-lowering action resulting from acceleration of Ca^{2+} extrusion due to ACh/diacylglycerol-induced activation of PKC (Berggren, Arkhammar & Nilsson, 1989). Finally, it should be emphasized that the reduction of Ca^{2+} influx and the stimulation of Ca^{2+} extrusion by ACh does not result in a decrease in insulin secretion. In fact, ACh remains stimulatory up to concentrations as high as $\geqslant 100$ μM (Garcia, Hermans & Henquin 1988) and capacitance measurements of exocytosis indicate that ACh (250 μM) produces a 3-fold PKC-dependent potentiation of Ca^{2+}-dependent exocytosis despite the concomitant inhibition of the Ca^{2+} current (E. Renström, K. Bokvist, J. Gromada & P. Rorsman, unpublished data).

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