Depolarization of pancreatic β-cells is critical for stimulation of insulin secretion by acetylcholine but remains unexplained. Using voltage-clamped β-cells, we identified a small inward current produced by acetylcholine, which was suppressed by atropine or external Na⁺ omission, but was not mimicked by nicotine, and was insensitive to nicotinic antagonists, tetrodotoxin, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DiDS), thapsigargin pretreatment, and external Ca²⁺ and K⁺ removal. This suggests that muscarinic receptor stimulation activates voltage-insensitive Na⁺ channels distinct from store-operated channels. No outward Na⁺ current was produced by acetylcholine when the electrochemical Na⁺ gradient was reversed, indicating that the channels are inward rectifiers. No outward K⁺ current occurred either, and the reversal potential of the current activated by acetylcholine in the presence of Na⁺ and K⁺ was close to that expected for a Na⁺-selective membrane, suggesting that the channels opened by acetylcholine are specific for Na⁺. Overnight pretreatment with pertussis toxin or the addition of guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) or guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) instead of GTP to the pipette solution did not alter this current, excluding involvement of G proteins. Injection of a current of a similar amplitude to that induced by acetylcholine elicited electrical activity in β-cells perfused with a subthreshold glucose concentration. These results demonstrate that muscarinic receptor activation in pancreatic β-cells triggers, by a G protein-independent mechanism, a selective Na⁺ current that explains the plasma membrane depolarization.

During the feeding periods, the increase in glycemia is limited in time and amplitude by the hypoglycemic action of insulin. Blood glucose itself is the main stimulator of insulin secretion by pancreatic β-cells. This stimulation involves two complementary pathways. Glucose generates a triggering signal, a rise in cytosolic Ca²⁺ concentration ([Ca²⁺]cyt), through the following sequence of events: the acceleration of cell metabolism increases the ATP/ADP ratio, which closes ATP-sensitive K⁺ (K⁺-ATP) channels in the plasma membrane; the resulting decrease in K⁺ conductance leads to membrane depolarization, opening of voltage-dependent Ca²⁺ channels, and Ca²⁺ influx (1–5). Glucose also produces amplifying signals that increase the efficacy of Ca²⁺ on exocytosis (6, 7).

Besides glucose, physiological agents such as hormones and neurotransmitters also modulate insulin secretion. A rich parasympathetic and sympathetic innervation enters the islets and ends close to the endocrine cells, allowing a fine neural tuning of the islet function (8, 9). Acetylcholine (ACH) is released by parasympathetic nerve endings during the preabsorptive phase of feeding to enhance insulin secretion prior to the rise in plasma glucose and during the absorptive phase (10). By binding to muscarinic receptors of the M₄ type, ACh triggers changes in phospholipid metabolism leading to formation of diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate, which mobilizes Ca²⁺ from intracellular Ca²⁺ stores. The resulting fall of the Ca²⁺ concentration in the endoplasmic reticulum activates a modest Ca²⁺ influx, through voltage-independent Ca²⁺ channels, which is commonly referred to as a capacitative Ca²⁺ entry. In addition, ACh depolarizes the plasma membrane of β-cells. This depolarization is small and does not cause Ca²⁺ influx in unstimulated β-cells. However, in the presence of stimulatory (depolarizing) concentrations of glucose, this additional depolarization by ACh enhances Ca²⁺ influx through voltage-dependent Ca²⁺ channels, leading to a sustained [Ca²⁺]cyt elevation (10).

Although central for the increase in insulin secretion by ACh (10, 11), the depolarization has never been conclusively explained. Several observations suggest that a Na⁺ current is involved. Thus, the depolarization of β-cells by ACh is abrogated by omission of extracellular Na⁺ (12) and accompanied by increases in total Na⁺ content (13), Na⁺ uptake (12, 14), and free cytosolic Na⁺ concentration ([Na⁺]cyt) (15). These arguments, however, remain indirect and conflict with the general concept that nicotinic rather than muscarinic receptors mediate cholinergic effects on Na⁺ conductance. In the present study, we used membrane potential recordings with microelectrode and both conventional and perforated whole cell modes of the patch-clamp technique to identify and characterize the current by which ACh depolarizes the plasma membrane of mouse β-cells. Our study provides the first direct electrophysi-
Muscarinic Activation of Na\(^+\) Current in \(\beta\)-Cells

**Experimental Procedures**

Preparation of Cells—The pancreas from NMRI mice killed by cervical dislocation was aseptically digested with collagenase in a bicarbonate-buffered solution containing 120 mmol/liter NaCl, 4.8 mmol/liter KCl, 2.5 mmol/liter CaCl\(_2\), 1.2 mmol/liter MgCl\(_2\), 24 mmol/liter NaHCO\(_3\), 5 mmol/liter Hepes, 10 mmol/liter glucose, and 1 mmol/liter Mg-atelivine serum albumin (fraction V; Roche Molecular Biochemicals) and gassed with O\(_2\)/CO\(_2\) (94:6%) to have a pH of 7.4. The islets were handpicked under a stereomicroscope. Single cells were obtained by incubating the islets for 5 min in a Ca\(^{2+}\)-free medium containing 138 mmol/liter NaCl, 5.6 mmol/liter KCl, 1.2 mmol/liter MgCl\(_2\), 5 mmol/liter Hepes, 3 mmol/liter glucose, and 1 mmol/liter EGTA (pH 7.4). After a brief centrifugation, this solution was replaced by culture medium, and the islets were disrupted by gentle pipetting through a siliconized glass pipette. The cells were plated on 22-mm-diameter glass coverslips. Intact islets and single islet cells were cultured for, respectively, 1 and 1–3 days in RPMI 1640 culture medium (Invitrogen) containing 10% heat-inactivated fetal calf serum and 10 mmol/liter glucose. All of the solutions for tissue preparation and culture medium were supplemented with penicillin and 100 mmol/liter streptomycin.

Electrophysiological Recordings—The membrane potential of a single \(\beta\)-cell within an islet was continuously recorded at 37 °C with a high resistance (~200 M\(\Omega\)) intracellular microelectrode (16). \(\beta\)-Cells were identified by the typical electrical activity that they display in the presence of 10 mmol/liter glucose.

Two criteria defined previously (17) were used to identify single \(\beta\)-cells: a cell capacitance above 5 pF and the presence of a voltage-dependent Na\(^+\) current that is inactivated at a holding potential of ~70 mV but can be activated after a hyperpolarizing pulse to ~140 mV. Patch-clamp measurements were carried out in both conventional and perforated whole cell modes, using an EPC-9 patch-clamp amplifier (Heka Electronics, Lambrecht/Pfalz, Germany) and the software Pulse- \(\gamma\)-injection (Heka Electronics, Lambrecht/Pfalz, Germany). The voltage clamp was considered satisfactory when the resistance of the microelectrode was above 5 M\(\Omega\). The typical series resistance correction was ~35–40 nS.

In conventional whole cell recordings of \(I_{\text{Na,ACh}}\), the pipette solution contained 125 mmol/liter CaCl\(_2\), 30 mmol/liter KOH, 1 mmol/liter MgCl\(_2\), 10 mmol/liter EGTA, 3 mmol/liter MgATP, 0.1 mmol/liter Na\(_2\)GTP, and 5 mmol/liter Hepes (pH 7.15) (Int Sol G). When specified, GTP-γ-S or GDP-β-S was substituted for GTP in the pipette solution. For conventional whole cell mode, the current was applied 5 min after the rupture of the plasma membrane.

The standard extracellular solution used to monitor \(I_{\text{Na,ACh}}\) contained 120 mmol/liter NaCl, 4.8 mmol/liter KCl, 2.5 mmol/liter CaCl\(_2\), 1.2 mmol/liter MgCl\(_2\), 24 mmol/liter NaHCO\(_3\), 5 mmol/liter Hepes, 10 mmol/liter glucose, and 1 mmol/liter EGTA (pH 7.4), and 10 mmol/liter glucose (Ext Sol A). Ca\(^{2+}\)-free solution was prepared by substituting MgCl\(_2\) for CaCl\(_2\) of external solution A and the calcium ionophore A23187 (10 μmol/liter EGTA (Ext Sol B). When needed, Na\(^{+}\)-free solution was prepared by substituting NaCl for KCl for KCl of external solution A (Ext Sol C). Na\(^{+}\)-free/K\(^{+}\)-rich (Ext Sol F) solution contained 135 mmol/liter N-methyl-D-glucamine, 4.8 mmol/liter KCl, 2.5 mmol/liter CaCl\(_2\), 1.2 mmol/liter MgCl\(_2\), 5 mmol/liter Hepes (pH adjusted to 7.4 with 131 mmol/liter HCl), and 10 mmol/liter glucose (Ext Sol D). When needed, Na\(^{+}\)-free solution was prepared by substituting N-methyl-D-glucamine for KCl of external solution D (pH adjusted to 7.4 with 136 mmol/liter HCl)) (Ext Sol E). For experiments during which the equilibrium potential for Na\(^+\) was fixed at ~60 mV, the external solution contained 11 mmol/liter NaCl, 10 mmol/liter KCl, 180 mmol/liter N-methyl-D-glucamine, 2.5 mmol/liter CaCl\(_2\), 1.2 mmol/liter MgCl\(_2\), 5 mmol/liter Hepes (pH 7.4), 0.1 mmol/liter CdCl\(_2\), 0.25 mmol/liter tolbutamide, and 10 mmol/liter glucose (Ext Sol F). For experiments during which the equilibrium potential for Na\(^+\) was fixed at ~20 mV, the external solution contained 53 mmol/liter NaCl, 10 mmol/liter KCl, 100 mmol/liter N-methyl-D-glucamine, 2.5 mmol/liter CaCl\(_2\), 1.2 mmol/liter MgCl\(_2\), 5 mmol/liter Hepes (pH 7.4), 0.1 mmol/liter CdCl\(_2\), 0.25 mmol/liter tolbutamide (pH adjusted to 7.4 with 96 mmol/liter HCl), and 10 mmol/liter glucose (Ext Sol G). For recordings of \(I_{\text{Na}}\), the external solution was supplemented with 2 mmol/liter EGTA (pH adjusted to 7.4 with 172 mmol/liter HCl), and 10 mmol/liter glucose (Ext Sol H).

Thapsigargin was obtained from Alomone Labs (Jerusalem, Israel). Unless otherwise stated, all of the other chemicals were from Sigma.

**Muscarinic Receptor Activation Induces an Inward Current in \(\beta\)-Cells**—The effect of ACh on the whole cell current was first studied in single \(\beta\)-cells held hyperpolarized at ~80 mV by the conventional whole cell configuration of the patch-clamp technique. Addition of ACh induced a sustained and reversible inward current, the amplitude of which increased with the concentration of the neurotransmitter (Fig. 2, A–D) to reach a maximum of 0.77 ± 0.15 pA/pF (n = 5) at 100 μmol/liter ACh. The half-maximal effective concentration (EC\(_{50}\)) estimation was performed by fitting the data to a conventional function at 2.5 μmol/liter ACh (Fig. 2D). The kinetics of activation of the current by ACh could not be established reliably because the characteristics of our perfusion system (volume chamber of ~0.8 ml and flow rate of ~0.5 ml/min) preclude fast solution exchange. However, it was repeatedly noted that the current activated by ACh developed rapidly, within ~1 s (Figs. 2B and 3C), in cells that were located very close to the inflow of solution and more slowly...
FIG. 1. Effects of ACh on the membrane potential of a mouse β-cell. An isolated islet was perfused with a medium containing 10 mmol/liter glucose (G10) and stimulated with 1 μmol/liter ACh as indicated. This recording is representative of results obtained in six islets.

FIG. 2. ACh induces an inward current by activating muscarinic receptors in mouse β-cells. Single β-cells were voltage-clamped at −80 mV using the conventional (A–D) or the perforated (E) whole cell mode of the patch-clamp technique. The composition of the external (Ext Sol) and pipette solutions (Int Sol) used is described under “Experimental Procedures.” ACh and atropine were applied when indicated by the arrows. A–D, ACh induced a concentration-dependent inward current that was reversed or blocked by atropine. A–C are representative of three (A), three (B), and four (C) experiments. D shows the concentration-dependence of ACh-induced inward current. The values are the means ± S.E. of the amplitude of the current recorded in three to five cells for each ACh concentration. Fitting data points to a sigmoidal function yielded a half-maximal effective concentration (EC50) of 2.5 μmol/liter. E, pretreatment of intact β-cells by thapsigargin (30 min, 1 μmol/liter) did not prevent ACh from activating an inward current. This trace is representative of five experiments.

The current elicited by ACh was completely suppressed or prevented by the muscarinic receptor antagonist, atropine (Fig. 2, A–C), but was not mimicked by 10 μmol/liter nicotine (n = 5, not shown), and was insensitive to nicotine or nicotinic agonists. Thus, in the presence of 10 μmol/liter nicotine, 0.1 μmol/liter α-bungarotoxin, or 100 μmol/liter hexamethionium, 100 μmol/liter ACh elicited a current that was, respectively, 90 ± 8% (n = 11), 111 ± 11% (n = 6), or 107 ± 6% (n = 7) of the current activated by ACh in the absence of nicotinic agents. These experiments show that the ACh-induced inward current in β-cells results from activation of muscarinic, but not nicotinic, receptors.

In another series of experiments, β-cells were voltage-clamped in the perforated whole cell mode and treated with 1 μmol/liter thapsigargin, which completely emptied the endoplasmic reticulum in Ca2+, as indicated by the suppression of Ca2+ mobilization by ACh (n = 5; not shown). Subsequent application of ACh elicited a current of the same amplitude (0.77 ± 0.09 pA/pF, n = 11) as that measured in the whole cell configuration (Fig. 2E). The ACh-induced inward current, therefore, is not a store-operated current.

Characteristics of the Current Activated by ACh in β-Cells—The ionic specificity of the current was first evaluated in the standard whole cell mode by removing Ca2+, Na+, or K+ from the bath or pipette solutions. Addition of ACh to a Ca2+-free medium elicited an inward current, indicating that the latter was not carried by Ca2+ (Fig. 3A). Omission of K+ from the perfusion medium did not prevent the current elicited by ACh, indicating that the current does not involve changes in Na+ pump activity (Fig. 3B). By contrast, omission of extracellular Na+ abrogated the current, which suggests that it is carried by Na+ (Fig. 3C). The current was nevertheless insensitive to tetrodotoxin, indicating that voltage-gated Na+ channels are not involved (Fig. 3D). When the electrochemical gradient for Na+ was reversed (Na+-rich pipette solution and Na+-free bath medium) to permit an outward current, ACh was ineffective (Fig. 3E), suggesting that Na+ flows only in the inward direction.

A nonspecific cationic current carrying both Na+ and K+ could also depolarize the plasma membrane because it usually has a reversal potential close to 0 mV. The experiments performed above did not allow us to exclude the possibility that ACh activates such a current because either the membrane was clamped close to the equilibrium potential of K+ (Fig. 3C) or no K+ was present in the pipette and bath solutions (Fig. 3E). To address that question, two series of experiments were thus performed. In the first series, Na+ was omitted from both pipette and bath solutions, whereas K+ was present at a high concentration in the pipette solution only (Fig. 3F). Under these conditions where the equilibrium potential for K+ was infinitely negative and no Na+ current could occur, ACh did not activate any outward current. In the second series of experiments, the Na+ versus K+ specificity of the current activated by ACh was evaluated by measuring its reversal potential with pipette and external solutions selected to have very different equilibrium potentials for Na+ and K+, i.e. −60 or −20 mV for Na+ and infinitely positive potentials for K+. These experi-
The current activated by ACh in mouse β-cells is rectifying inwardly. The reversal potential for Na⁺ was fixed at −60 mV (open squares) or −20 mV (closed circles) by using external (Ext Sol) and pipette solutions (Int Sol) with appropriate concentrations of Na⁺ (see “Experimental Procedures” for compositions). Single β-cells were voltage-clamped in conventional whole cell mode at various potentials (−130, −100, −60, −30, and 0 mV when ENa was set at −60 mV and −100, −60, −20, and 0 mV when ENa was set at −20 mV) around the reversal potential for Na⁺. Each point shows the mean ± S.E. of the current amplitude elicited by 100 μmol/liter of ACh at each potential in three to five cells.

An increase in Cl⁻ permeability is expected to depolarize the plasma membrane because the equilibrium potential for Cl⁻ in β-cells has been estimated to be above the threshold for activation of voltage-dependent Ca²⁺ channels (19, 20). Activation of a Cl⁻ current by ACh has not been directly tested by omitting Cl⁻ from the medium. However, this possibility can also be discarded for two reasons. First, ACh did not elicit any current when the membrane was clamped at a potential different from the equilibrium potential of Cl⁻ and under conditions where no Na⁺ current occurred (e.g. at holding potentials of −80, −80, and −60 mV in Figs. 3 (C and F) and 4 (open squares), when EC1 was at −6, 0, and −14 mV, respectively). Second, I_{Na-ACh} was unaffected by DIDS, a blocker of the volume-activated current (21) that carries Cl⁻ and possibly other ions in β-cells (22).

Thus, in the presence of 100 μmol/liter DIDS, 100 μmol/liter ACh elicited a current that was 112 ± 10% (n = 14) of
Activation of $I_{\text{Na-ACh}}$ in mouse $\beta$-cells does not involve G proteins—Muscarinic effects of ACh in $\beta$-cells are known or assumed to be transduced by a G protein, and it has been reported that in neurons and cardiomyocytes, several muscarinic receptor subtypes mediate ionic channels, such as Ca$^{2+}$ and K$^+$ channels, through pertussis toxin-sensitive G proteins of the $G_i$ or $G_o$ class (10). However, after permanent inactivation of $G_i$ or $G_o$ proteins by overnight pretreatment of $\beta$-cells with pertussis toxin (250 ng/ml), the amplitude of $I_{\text{Na-ACh}}$ was 109 ± 7% ($n=6$) of that observed in untreated cells. To test the possible involvement of all kinds of G proteins in the activation of $I_{\text{Na-ACh}}$, GTP in the pipette solution (control conditions) was replaced by GTP-γ-S or GDP-β-S, which are, respectively, non-hydrolyzable activator and inhibitor of G proteins. Fig. 5 shows the maximum inward current ($I_{\text{Na-ACh}}$) elicited by a 1-min application of 100 μmol/liter ACh to $\beta$-cells voltage-clamped at −80 mV and dialyzed for 5 min with a solution containing 100 μmol/liter GTP, 10 μmol/liter GTP-γ-S, or 4 μmol/liter GDP-β-S. In all conditions, $I_{\text{Na-ACh}}$ was reversible upon washout of the neurotransmitter, and its amplitude was similar with the three nucleotides, suggesting that activation of $I_{\text{Na-ACh}}$ does not involve G proteins.

To ascertain that our experimental conditions were adequate to identify the involvement of a G protein, we tested the GTP analogues on the ACh-induced inhibition of voltage-dependent Ca$^{2+}$ current in pancreatic $\beta$-cells (23). Fig. 6A shows representative whole cell Ca$^{2+}$ current traces recorded with a pipette solution containing 100 μmol/liter GTP. The current ($I_{\text{Ca}}$) was evoked by 25-ms depolarizing pulses from −80 to 10 mV before (control), during (ACh), and after (wash) application of 100 μmol/liter ACh to the bath. Fig. 6B summarizes the changes of the normalized peak Ca$^{2+}$ current produced by ACh in the presence of different guanine nucleotides in the pipette solution. With 100 μmol/liter GTP in the pipette solution (control), ACh reversibly inhibited the current. This inhibitory effect was abolished when the pipette solution contained 4 mmol/liter GDP-β-S and became irreversible when 10 μmol/liter GTP-γ-S was included in the pipette. The spontaneous decrease in the current amplitude recorded with GDP-β-S reflects rundown (23). These control experiments thus show that the guanine nucleotides are effective in our recording conditions.

Impact of a Depolarizing Current Equivalent to $I_{\text{Na-ACh}}$ on the $\beta$-Cell Membrane Potential—Because $I_{\text{Na-ACh}}$ is small, it was important to verify that a current of a similar amplitude was sufficient to elicit electrical activity. These experiments were performed in $\beta$-cells perfused at 34–36 °C with 6 mmol/liter glucose, a concentration that is subthreshold in islets (24). A depolarizing current corresponding to the average $I_{\text{Na-ACh}}$ induced by 100 μmol/liter ACh (0.77 pA/pF) and adjusted to cell size (0.77 pA × capacitance of the tested cell) was injected in the current-clamp mode. Such a current elicited an electrical activity in all tested cells, and its removal was accompanied by an immediate repolarization (Fig. 7A). In other experiments shown in Fig. 7B, the depolarizing effect of 1 μmol/liter ACh was first tested and compared with that of depolarizing currents corresponding to $I_{\text{Na-ACh}}$ elicited by 1 (0.23 pA/pF), 10 (0.61 pA/pF), and 100 μmol/liter ACh (0.77 pA/pF). The addition of 1 μmol/liter ACh triggered electrical activity with action potentials that ceased upon washout of the neurotransmitter. Subsequent injection of a current with an amplitude similar to that of $I_{\text{Na-ACh}}$ induced by 1 μmol/liter ACh (a in Fig. 7B) elicited electrical activity similar to that produced by 1 μmol/
Acetylcholine Activates an Inward Na⁺ Current in β-Cells

Our data demonstrate that ACh activates an inward current that is attributed to Na⁺ influx because of its suppression either when Na⁺ was removed from the extracellular medium or when the membrane potential of the cell was close to the equilibrium potential of Na⁺. On the other hand, no contribution of Ca²⁺, K⁺, or Cl⁻ to the current could be obtained. Thus, the ACh-induced current was not affected by removal of extracellular Ca²⁺. When no Na⁺ current could occur, no inward or outward current was evoked by ACh, even when the equilibrium potential of K⁺ was infinitely negative or positive or when the membrane potential was clamped away from the equilibrium potential of Cl⁻. The current elicited by ACh was also insensitive to DIDS, a blocker of the Cl⁻-mediated, volume-activated current in β-cells (22).

Activation of this Na⁺ current by ACh may explain the increase in total Na⁺ content (13, 22) Na⁺ uptake (12, 14), and [Na⁺], (15) that the neurotransmitter induces in islet cells and the abrogation of all of these effects in a Na⁺-free medium. The amplitude of the Na⁺ current elicited by ACh is small but is sufficient to account for the 15-mmol/liter increase in [Na⁺], occurring in β-cells after 15 min of stimulation with 100 μmol/liter ACh (15, 25). Indeed, 100 μmol/liter ACh activated a mean inward current of 0.77 pA/pF, which corresponds to 6.15 pA for an average β-cell capacitance of 7.9 ± 0.08 pF (estimated from 644 β-cells tested in the present study). Assuming that the current remains stable over 15 min, the current charge amounts to 5537 pC, which corresponds to ~57 fmol of Na⁺.

For an intracellular space of 620 fl/β-cell (26), this would result in a [Na⁺], increase by 92 mmol/liter, which is well above the 15 mmol/liter measured. Activation of the Na⁺ pump obviously tends to correct the [Na⁺], rise. The reverse mechanism, an inhibition of the Na⁺ pump, has been proposed to explain the increase in [Na⁺], produced by ACh in sheep Purkinje fibers (27). The explanation does not hold for β-cells in which ACh still increases [Na⁺], after blockade of the pump by ouabain (15). This is fully compatible with the persistence of the ACh-induced inward current in a K⁺-free medium, another situation where the Na⁺ pump is blocked.

Nicotinic receptors, which are nonselective cationic channels (28, 29), classically mediate cholinergetic effects on Na⁺ currents. Such channels are clearly not responsible for the ACh-induced inward current in β-cells because the muscarinic antagonist atropine completely prevented the current and the rise in [Na⁺], (15), whereas the ACh-activated current was not mimicked by nicotine and was insensitive to nicotinic antagonists. Activation of a Na⁺ conductance by muscarinic receptors is unusual but has occasionally been reported in cardiac myocytes (30, 31), smooth muscle cells of the gastrointestinal tract (32, 33), chromaffin cells (34), and Chinese hamster ovary cells expressing M₃ receptors (35).

The channels activated by ACh in β-cells have not been identified, but several of their properties could be established. Voltage-dependent Na⁺ channels are present in mouse β-cells, but they are inactivated at the holding potential of ~80 mV that we used (36). We can discard the possibility that such channels mediate the effect of ACh because the Na⁺ current evoked by the neurotransmitter did not require any voltage change and was insensitive to tetrodotoxin, as are the ACh-induced increases in Na⁺ uptake (12) and [Na⁺], (15). Our results also indicate that the current is not carried by a nonselective cationic channel allowing flow of both Na⁺ and K⁺ or Ca²⁺. The Na⁺ channels activated by ACh display inward rectifying properties as shown by their inability to carry an inward current.
Mechanisms of Activation of \( I_{Na-ACh} \) in other cells (37, 38), lowering the \( Ca^{2+} \) content of the endoplasmic reticulum in \( \beta \)-cells activates conductances for \( Ca^{2+} \) and perhaps other ionic species including \( Na^{+} \) (25, 39). Even if such a mechanism slightly contributes to the current, it is not responsible for \( I_{Na-ACh} \), because \( ACh \) still activated the inward current after emptying of the endoplasmic reticulum \( Ca^{2+} \) stores by thapsigargin. This is consistent with our previous report that thapsigargin and cyclopiazonic acid, which empty the endoplasmic reticulum in \( Ca^{2+} \) more efficiently than does \( ACh \), did not mimic or prevent the \( [Na^{+}] \) rise elicited by \( ACh \) (25). The fact that neither pretreatment with thapsigargin nor inclusion of a high concentration of \( EGTA \) in the pipette solution prevented \( ACh \) from inducing an inward current also excludes the possibility that activation of \( I_{Na-ACh} \) is secondary to a rise in \( [Ca^{2+}] \).

Although it is classically admitted that muscarinic receptors are coupled to G proteins (40), activation of \( I_{Na-ACh} \) was unaffected by inactivation of \( G_{i/0} \) or \( G_{q/1} \) proteins by pertussis toxin pretreatment or influsing \( \beta \)-cells with \( GTP\gamma-S \) or GDP-\( \beta \)-S, two guanine nucleotide analogues that, respectively, activate and inhibit G proteins. However, both analogues were effective in our experimental conditions as shown by their modulation of ACh-induced inhibition of the voltage-dependent \( Ca^{2+} \) current (23). These observations unexpectedly indicate that activation of \( I_{Na-ACh} \) does not involve G proteins in \( \beta \)-cells. There is growing evidence that various seven-transmembrane metabotropic receptors can also activate transduction systems without the involvement of G proteins (41, 42). In particular, muscarinic agonists have been found to activate, in a G protein-independent way, a \( Na^{+} \) current in ventricular myocytes (31), a cationic current in \( CA3 \) pyramidal cells (43), and a \( K^{+} \) current in aortic endothelial cells (44, 45). The transduction mechanisms have not been identified, but direct or indirect (via adaptor proteins) interactions between the receptor and effector proteins have tentatively been proposed. Activation of cationic conductance by a Src tyrosine kinase in \( CA3 \) pyramidal neurons (41) and facilitation of the stimulation of inositol 1,4,5-trisphosphate receptors by the protein Homer (42) are two examples of G protein-independent events linked to activation of the glutamate receptor.

Role of \( I_{Na-ACh} \) in the Control of \( \beta \)-Cell Membrane Potential by \( ACh \) —Apart from the increase in \( Na^{+} \) conductance, all of the plausible mechanisms by which \( ACh \) might depolarize the \( \beta \)-cell membrane can be excluded. First, \( ACh \) does not decrease the \( \beta \)-cell membrane \( K^{+} \) conductance. Unlike glucose and sulfonlureas, \( ACh \) does not inhibit the efflux of \( 86Rb^{+} \) (a tracer of \( K^{+} \)) (12, 46) and does not reduce \( K^{+}-\)ATP (17) or other \( K^{+} \) currents through voltage-dependent \( Ca^{2+} \) channels. On the contrary, we confirm that activation of \( INa-ACh \) is secondary to a rise in \( [Ca^{2+}] \), that high concentrations of the neurotransmitter rather than the depolarization induced by \( ACh \) (25, 39) abrogate extracellular \( Na^{+} \) loss but insensitive to tetrodotoxin (12). We further show here that the mechanism of \( I_{Na-ACh} \) is sufficient to explain the effects of \( ACh \) on the membrane potential. Thus, injection of a current with an amplitude similar to that activated by 1 \( \mu \)mol/liter of \( ACh \) (i.e. 0.23 ± 0.02 pA/pF) elicited electrical activity in cells perfused with a subthreshold glucose concentration. Because the effect of a given current augments with the resistance of the membrane and because the latter increases with the glucose concentration (closure of \( K^{+}-\)ATP channels), it can be anticipated that even smaller currents could depolarize the plasma membrane in the presence of stimulating glucose concentrations.

The subsequent activation of voltage-dependent \( Ca^{2+} \) channels eventually leads to a sustained increase in \( [Ca^{2+}] \) that largely contributes to the insulin-releasing action of \( ACh \) (10).

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*Muscarinic Activation of Na⁺ Current in β-Cells*
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