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Muscarinic Agonists Activate Ca^{2+} Store-operated and -independent Ionic Currents in Insulin-secreting HIT-T15 Cells and Mouse Pancreatic β-Cells

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Abstract. The neurotransmitter acetylcholine, a muscarinic receptor agonist, augments glucose-induced insulin secretion from pancreatic β-cells by depolarizing the membrane to enhance voltage-gated Ca^{2+} influx. To clarify the electrical events involved in this process, we measured ionic currents from a clonal β-cell line (HIT-T15) and mouse pancreatic β-cells. In whole-cell recordings, the muscarinic agonist carbachol (CCh) dose-dependently and reversibly activated a voltage-independent, nonselective current (whole-cell conductance 24 pS/pF, reversal potential ~ -15 mV). The current, which we refer to as I_{musc}, was blocked by atropine, a muscarinic receptor antagonist, and SKF 96365, a nonspecific ion channel blocker. The magnitude of the current decreased by 52% when extracellular Na^+ was removed, but was not affected by changes in extracellular Ca^{2+}, confirming that I_{musc} is a nonselective current. To determine if I_{musc} activates following release of Ca^{2+} from an intracellular store, we blocked intracellular IP_3 receptors with heparin. Carbachol still activated a current in the presence of heparin, demonstrating the presence of a Ca^{2+} store-independent, muscarinic agonist-activated ionic current in HIT cells. However, the store-independent current was smaller and had a more positive reversal potential ($\sim$0 mV) than the current activated by CCh under control conditions. This result indicates that heparin had blocked a component of I_{musc}, which likely activates following release of stored Ca^{2+}. Depleting IP_3-sensitive calcium stores with thapsigargin also activated a non-selective, SKF 96365-blockable current in HIT cells. The properties of this putative store-operated current were similar to the component of I_{musc} that was blocked by heparin, being voltage-independent and reversing near -30 mV. We conclude that I_{musc} consists of store-operated and store-independent components, both of which may contribute to the depolarizing action of muscarinic agonists on pancreatic β-cells.

Key words: Stimulus-secretion coupling — Islet of Langerhans — Endoplasmic reticulum — Acetylcholine — Patch clamp — Diabetes mellitus

Introduction

Insulin secretion from pancreatic β-cells is controlled primarily by the plasma glucose concentration, as well as by circulating hormones and locally released neurotransmitters (Ahrén, 2000). Release of acetylcholine from parasympathetic nerve terminals during a meal stimulates a rise in plasma insulin prior to nutrient absorption that is critical for normal postprandial glucose tolerance (Steffens, 1976; Teff & Engelman, 1996; Ahrén, 2000). The neurotransmitter may also contribute to controlling the kinetics of insulin release during the phase of nutrient absorption (D'Alessio et al., 2001).

Both in vivo and in vitro, the secretory effects of acetylcholine are critically dependent on the prevailing glucose level. Little or no stimulation of insulin secretion is observed under hypoglycemic conditions, and the agent becomes more effective as the concentration of the sugar is increased in the stimulatory range (Bloom & Edwards, 1985; Garcia, Hermans & Henquin, 1988; Persaud et al., 1989). Here we focus on the electrical events involved in cholinergic potentiation of glucose-induced insulin secretion from pancreatic β-cells.

Elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) is the principal trigger for insulin secretion. With few exceptions, $[Ca^{2+}]_i$ in the β-cell is controlled by the membrane potential, which determines the rate of

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Ca^{2+} influx through voltage-gated Ca^{2+} channels (Gilon & Henquin, 1992). At substimulatory glucose levels, the activity of adenosine triphosphate-sensitive K^+ (K_{ATP}) channels keeps the β-cell membrane hyperpolarized, and $[Ca^{2+}]_i$ is low (Cook & Hales, 1984; Aguilar-Bryan, Bryan & Nakazaki, 2001). As the glucose concentration rises, K_{ATP} channels close due to increases in the cytoplasmic ATP/ADP ratio, resulting in membrane depolarization (Kohler et al., 1998) and induction of an oscillatory electrical activity known as bursting (Dean & Matthews, 1968). Calcium influx through voltage-gated Ca^{2+} channels during the depolarized phases of the bursts drive oscillations of $[Ca^{2+}]_i$ and insulin secretion that are in phase with the bursting electrical activity (Santos et al., 1991; Gilon, Shepherd & Henquin, 1993).

Cholinergic agonists enhance insulin secretion, at least in part, by augmenting glucose-induced membrane depolarization. This process involves an initial interaction with M_3 subtype muscarinic receptors on the β-cell membrane (Henquin & Nenquin, 1988; lismaa et al., 2000), resulting in membrane phospholipid turnover to produce inositol 1,4,5-trisphospate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular stores, producing a transient rise in $[Ca^{2+}]_i$, and DAG activates protein kinase C (PKC). At low glucose levels, activation of this pathway produces only a small membrane depolarization, which is insufficient to stimulate voltage-dependent Ca^{2+} influx (Henquin et al., 1988). At stimulatory glucose concentrations, cholinergic agonists convert the burst pattern to one of higher frequency with depolarized silent phases (Satin & Kinard, 1998; Mears & Atwater, 2000). The additional depolarization produced by cholinergic agonists increases voltage-gated Ca^{2+} influx to produce a sustained augmentation of insulin secretion. This effect is further enhanced by cholinergic-generated amplifying signals such as PKC, which increase the sensitivity of the exocytotic machinery to Ca^{2+} (Ammala et al., 1994; Gilon & Henquin, 2001).

Sodium-carrying currents appear to play a prominent role in cholinergic modulation of β-cell electrical activity, as the depolarizing effect is inhibited by removal of extracellular Na^+ (Henquin et al., 1988). Also, cholinergic stimulation is accompanied by increased Na^+ uptake in islets and increased intracellular Na^+ concentration in β-cells (Gagerman, Sehlin & Taljedal, 1980; Gilon & Henquin, 1993). Recently, Rolland and co-workers (2002b) provided the first direct evidence for activation of Na^+ currents by acetylcholine in mouse β-cells. The small, inwardly rectifying current (called $I_{Na,Ach}$) was inhibited by the muscarinic receptor antagonist atropine, but was not dependent on activation of G-proteins or release of Ca^{2+} from intracellular stores. This seminal report suggests that muscarinic agonists could depolarize the β-cell, at least in part, by mechanisms that do not depend on changes in phospholipid metabolism.

Store-operated currents (SOCs), which activate following depletion of Ca^{2+} from IP_3-sensitive intracellular stores (Parekh & Penner, 1997), provide another mechanism by which cholinergic agonists could affect β-cell membrane potential (Bertram et al., 1995). Worley and co-workers (1994a, b) showed that removal of extracellular Ca^{2+}, to passively deplete intracellular Ca^{2+} stores, led to activation of a voltage-independent, nonselective current in mouse β-cells. The current, referred to as Ca^{2+}-release-activated non-selective current (I_{CRAN}), was also activated by maitotoxin and inhibited by SKF 96365 (Roe et al., 1998). Additionally, depletion of Ca^{2+} stores induces a sustained, voltage-independent Ca^{2+} entry in β-cells (Liu & Gylfe, 1997; Miura, Henquin & Gilon, 1997) and also enhances glucose-induced electrical activity (Worley et al., 1994b; Mears et al., 1997). Finally, several members of the transient receptor potential channel (TRP) family of genes, whose products have been implicated as candidates for SOC channels, are expressed in mouse β-cells and clonal β-cell lines (Sakura & Ashcroft, 1997; Roe et al., 1998; Qian et al., 2002). However, to date there is no direct evidence that store-operated currents are activated by cholinergic agents or sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) antagonists in β-cells.

In this paper, we present evidence that muscarinic agonists activate a multi-component, nonselective ionic current in insulin-secreting HIT cells and mouse pancreatic β-cells. One component is activated by the SERCA antagonist thapsigargin and blocked by the IP_3 receptor antagonist heparin, suggesting that it is a store-operated current. The second component, with more positive reversal potential than the first, is insensitive to block of IP_3-dependent Ca^{2+} release or inhibition of G-proteins. These data provide the first direct measurements of SOCs activated by cholinergic agonists in the β-cell, and suggest that both store-operated and store-independent ionic currents could be involved in cholinergic potentiation of β-cell electrical activity and insulin secretion.

Materials and Methods

Cell Preparation and Culture

Hamster Insulin Tumor (HIT-T15) cells were obtained from American Type Culture Collection (Manassas, VA) and kept in an air-CO_2 incubator at 37°C. The tissue culture medium was Ham's F12-K, containing 7 mM glucose, and was supplemented with 10% horse serum and 2.5% fetal bovine serum (GIBCO, Grand Island, NY). The cells were subcultured weekly using 0.05% trypsin solution to detach the cells from the tissue culture flask. During subculture, aliquots of cells ($3 \times 10^4/cm^3$) were plated onto clean glass coverslips for use in experiments 1–3 days later. Cells were used from passages 61–70.

Some experiments were performed on mouse β-cells in primary culture. Briefly, 2–6 month old NIH Swiss mice were sacrificed by cervical dislocation and the abdominal wall was opened. The pancreas was distended by injection of 6 ml of Hank's Balanced Salt Solution containing 0.23 mg/ml liberase enzyme blend (Roche Biochemicals, Indianapolis, IN) through a cannula inserted in the common bile duct. The pancreas was then removed and incubated in a water bath at 37°C for 20 minutes. Following this digestion procedure, clean islets of Langerhans were collected by hand and dispersed into single cells by shaking in a Ca^{2+}-free medium supplemented with 30 mg/ml dispase (Boehringer Mannheim, Indianapolis, IN). The resulting cell suspension was washed in tissue culture medium (RPMI 1640 with 11 mM glucose, 10% fetal bovine serum), after which the cells were plated onto glass coverslips and used in experiments 1–4 days later. In electrophysiological recordings, β-cells were identified based on size, being larger than the glucagon and somatostatin-containing cells of the islet (He et al., 1998).

Electrophysiological Recordings

Glass coverslips containing HIT cells or mouse pancreatic β-cells were used to form the bottom of a sample chamber that was placed on the stage of an inverted microscope. The cells were continuously superfused at room temperature with a HEPES-buffered Krebs solution (in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 3 $CaCl_2$, 5 $NaHCO_3$, 5 HEPES, 11 glucose, pH 7.4 with NaOH). The solution was supplemented with 200 nM glyburide to ensure that K_{ATP} channels were blocked throughout the experiment. In some experiments, Na^+ was replaced with equimolar N-methyl-D-glucamine and bicarbonate was omitted. Whole-cell patch pipettes were pulled from thin-walled capillary glass and had resistances of 3–5 MΩ when filled with the following solution (mM): 100 gluconic acid, 30 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 Hepes, 3 Mg-ATP, 0.5 Li-GTP, pH 7.2 with KOH. The calculated free Ca^{2+} concentration of the pipette solution was 100 nM, and was confirmed by ratiometric measurement using fura-2 pentapotassium (25 μM) as a fluorescent Ca^{2+} indicator. In some experiments, sodium heparin (1 mg/ml) was included in the pipette solution to block intracellular IP_3 receptors.

Pipettes were sealed onto the membranes of individual cells and the whole-cell configuration was obtained by applying gentle suction. The membrane conductance was allowed to stabilize for three minutes, after which membrane current was recorded under voltage-clamp conditions using a computer-controlled amplifier (EPC-9, Heka Elektronik, Lambrecht, Germany). Voltage commands were applied and digital current records were acquired using Pulse v8.5 software (Heka Elektronik). The membrane potential was clamped at a baseline level of −70 mV. Families of brief (40 ms) voltage pulses to levels between −100 mV and −40 mV were applied every 20 seconds. The current responses to these voltage pulses were filtered at 3 kHz and sampled at 5 kHz. Depolarized potentials were avoided to prevent the activation of large voltage-gated Ca^{2+} and K^+ currents known to be present in HIT cells and mouse pancreatic β-cells. During off-line analysis, membrane current-voltage (*I-V*) curves were calculated for each family of pulses by averaging the measured current over the last half of each record and plotting versus membrane potential. Currents activated by cholinergic agonists were detected as changes in current at the baseline holding potential and as changes in the membrane conductance after adding 1–100 μM carbamylcholine chloride (carbachol), a cholinesterase-resistant acetylcholine analog, to the perfusate. The series resistance in these experiments was always less than 20 MΩ and was not compensated.

For perforated-patch experiments, pipettes were filled with a solution containing (mM): 100 gluconic acid, 20 KCl, 1 $MgCl_2$, 5 Hepes, pH 7.35 (KOH), supplemented with 240 μg/ml amphotericin B. Seals were formed onto the membranes of individual cells and whole-cell currents were measured after the membrane under the pipette had been perforated to a stable access resistance less than 25 MΩ (generally 10–15 minutes).

Data Presentation and Statistical Analysis

Results are presented as mean ± SEM. Statistical significance was analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc testing where appropriate. Linear regression was carried out using Origin 6.1 software (OriginLab, Northampton, MA).

Results

We used the tight-seal, whole-cell configuration of the patch-clamp technique to measure the effects of muscarinic receptor activation on membrane currents in HIT cells. In control experiments ($n = 18$) we found that the membrane conductance of HIT cells varies spontaneously during the first 60–90 seconds following formation of the whole-cell configuration, and then remains stable for at least 12 minutes (*data not shown*). For this reason, we started each experiment after the membrane conductance had been allowed to stabilize for three minutes, and limited the duration to nine minutes. Figure 1*A* shows representative current traces recorded from individual HIT cells in response to hyperpolarizing voltage pulses, before, during and after exposure to carbachol (CCh). Addition of either 3 μM or 30 μM CCh induced a negative shift in the current measured at the baseline holding potential (−70 mV) and an increase in the size of the current responses to voltage pulses (traces *i* and *ii*). These changes developed within 90 seconds of addition of CCh, were sustained for the remainder of the exposure period (3–5 minutes), and reversed partially within two minutes of removal of the agonist. The effects were not observed in the presence of the muscarinic receptor antagonist atropine (trace *iii*). Figure 1*B* demonstrates how the effects of CCh were analyzed in individual cells. Membrane current-voltage (*I-V*) curves were calculated from the current responses to applied voltage pulses, before and after addition of CCh. To account for differences in cell size, the current magnitudes were normalized to cell capacitance. In the experiment illustrated in Fig. 1*B*, the current measured in the presence of CCh was more negative at all applied potentials (depolarized potentials were avoided to prevent activation of voltage-dependent channels). The CCh-induced changes in membrane current were nearly completely reversed by SKF 96365, a nonselective ion channel antagonist. The *I-V* curve of the current activated by CCh was calculated by subtracting the membrane *I-V* curve measured prior to addition of CCh from that recorded after the agonist was applied. The current was inward and linear in the −100 to −40 mV range of membrane potential. The whole-cell conductance of the CCh-

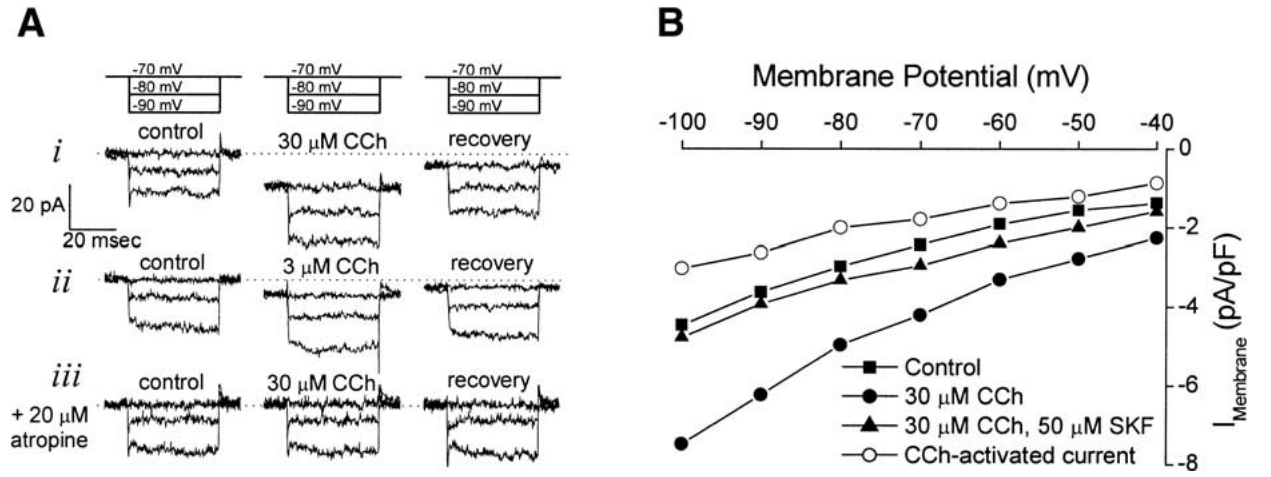

Fig. 1. Carbachol-activated current in HIT cells. (*A*) Effect of carbachol (CCh) on voltage-clamp currents recorded from HIT cells (pulse protocol illustrated at top). Traces *i*, *ii* and *iii* recorded from different cells. Scale bars are the same for all traces. Dashed lines extend control baseline. (*B*) Normalized membrane *I-V* curves calculated from current responses of a single HIT cell to voltage pulses under the indicated conditions. The control curve (*squares*) was subtracted from the curve measured in the presence of CCh (*filled circles*) to calculate the CCh-activated current (*empty circles*).

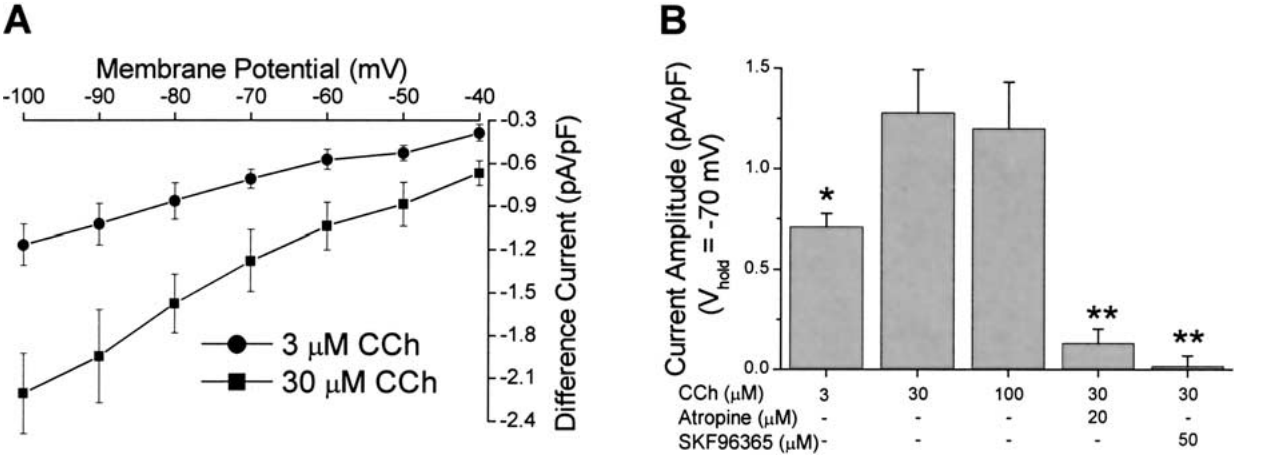

Fig. 2. Properties of carbachol (CCh)-activated current in HIT cells. (*A*) Average *I-V* curves of the current induced by 3 μM (*circles*, $n = 10$) and 30 μM CCh (*squares*, $n = 6$) in HIT cells. (*B*) Average magnitude of the current induced by CCh at −70 mV under the indicated conditions. (*$P < 0.05$; **$P < 0.01$, compared to 30 μM CCh alone).

activated current was calculated from the slope of a straight line to the *I-V* curve (35 pS/pF for this cell).

Figure *2A* shows the average *I-V* curves for the current activated by 3 μM and 30 μM CCh in HIT cells. The whole-cell conductances from linear fits to the data were 24 pS/pF and 11.9 pS/pF for 30 μM and 3 μM CCh, respectively. Although the direction of the current was always inward at the applied membrane potentials, extrapolation of the linear fit to the *I-V* curve for 30 μM CCh suggested a reversal potential of −15 mV, consistent with a nonselective ionic current. The extrapolated reversal potential of the current activated by 3 μM CCh was similar (−12 mV). Figure 2*B* confirms that the current was activated dose-dependently by CCh, was not observed in the presence of atropine ($P < 0.01$), and was blocked by SKF 96365 ($P < 0.01$). Unfortunately, we found that SKF 96365 also reduced the membrane conductance in the absence of cholinergic agonists and, in accord with a previous report (Fasolato, Pizzo & Pozzan, 1990), blocked the voltage-gated Ca^{2+} and K^+ channels that are crucial for generation of electrical activity in β-cells (*data not shown*). SKF 96365 is therefore not an ideal agent for determining the role of I_{musc} in β-cell electrical activity.

Together, the results of Figs. 1 and 2 demonstrate that CCh activates a small, voltage-independent current in HIT cells. The dose-dependence of activation of the current, its reversibility and its susceptibility to block by atropine indicate that the current develops subsequently to activation of muscarinic receptors. We refer to this muscarinic agonist-activated current as I_{musc}.

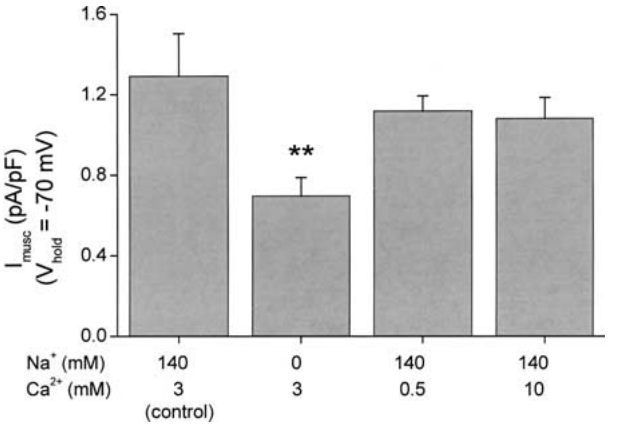

Fig. 3. Effects of ion substitution on I_{musc}. Average magnitude of I_{musc} (measured as the change in current at −70 mV holding potential induced by 30 μM carbachol) in HIT cells exposed to extracellular Na^+ and Ca^{2+} concentrations as indicated. Na^+ was replaced with N-methyl-D-glucamine. When extracellular Ca^{2+} was changed, osmolarity was balanced with sucrose. (**$P < 0.05$ compared to column at left).

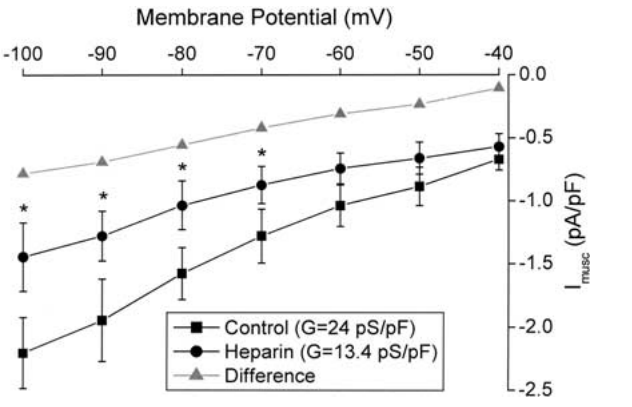

Fig. 4. Components of I_{musc} in HIT cells. *I-V* curves of the current activated by 30 μM carbachol (CCh) in HIT cells without (*squares*, $n = 6$) or with (*circles*, $n = 8$) 1 mg/ml heparin in the pipette solution. The current measured in the presence of heparin was smaller and had more positive extrapolated reversal potential than control. (*,$P < 0.05$ compared to filled circles). The difference current (*gray triangles*) represents the current blocked by heparin, and was calculated by subtracting the *I-V* curve measured with heparin from the control *I-V* curve.

Figure 3 shows the effects of ion substitution on I_{musc} in HIT cells. The magnitude of the current (measured at −70 mV) was reduced by 52% when Na^+ was removed from the extracellular solution ($P < 0.05$). The reversal potential of I_{musc} also appeared to be more negative in the absence of Na^+, but this change did not reach statistical significance (*data not shown*). Figure 3 also shows that increasing or decreasing the extracellular Ca^{2+} concentration did not affect the magnitude of I_{musc}. These results support the assertion that I_{musc} is a nonselective current, and indicate that Na^+ is likely the principal charge carrier with physiologic ion gradients. The remaining inward current observed in the absence of Na^+ suggests a finite permeability to Ca^{2+}, Cl^- and/or NMDG, as has been reported for other nonselective ion channels (Nakagawa et al., 1998; Bo et al., 2003).

We next examined the mechanism of activation of I_{musc}. To determine if I_{musc} is a store-operated current, we measured whole-cell currents from HIT cells with heparin in the pipette solution to block IP_3-induced Ca^{2+} release. We found that CCh consistently activated a current with heparin in the pipette (8 of 10 cells). However, Fig. 4 shows that when I_{musc} was measured in the presence of heparin, the whole-cell conductance was smaller (13.4 pS/pF) and the extrapolated reversal potential was more positive (∼0 mV) than under control conditions. This observation indicates that heparin blocked a component of I_{musc}. The *I-V* curve of the heparin-blockable component was determined as the difference between the *I-V* curves of I_{musc} measured with and without the IP_3 receptor antagonist (Fig. 4). By extrapolating a straight-line fit to the data, we estimated the reversal potential of this current to be −31 mV. The results of Fig. 4 suggest that the nonselective current I_{musc} consists of a component that depends on IP_3-mediated Ca^{2+} release from intracellular stores (heparin-blockable) and a component that is Ca^{2+}-store independent (heparin-insensitive).

Rolland and co-workers (2002b) recently described a muscarinic-activated Na^+ current in mouse β-cells that is both G-protein and Ca^{2+} store-independent. To evaluate the role of G-proteins in activation of the store-independent component of I_{musc}, we repeated the experiment of Fig. 4 with GTP in the pipette solution replaced with the non hydrolyzable G-protein inhibitor guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S). Carbachol (30 μM) activated the store-independent current in 6 of 7 cells dialyzed with 1 mg/ml heparin and 4 mM GDP-β-S (*data not shown*). The magnitude of the current measured in the presence of GDP-β-S (0.81 ± 0.09 pS/pF) was not significantly different from that measured under control conditions (0.87 ± 0.15 pS/pF). We conclude that CCh activates the store-independent component of I_{musc} via a G-protein-independent mechanism.

We next examined whether depletion of intracellular Ca^{2+} stores with thapsigargin (Tg) would result in activation of a current similar to the heparin-sensitive component of I_{musc}. Using the same protocol as for CCh-activated currents, we found that Tg also activated a small inward current in HIT cells. Like I_{musc}, the Tg-activated current was blocked by SKF 96365 (Fig. 5, inset). The Tg-activated current developed fully within 3 minutes of exposure to the agent, and was generally not reversible following its withdrawal. Figure 5 shows that the Tg-activated current had a whole-cell conductance of 14.1 pS/pF and an extrapolated reversal potential of −32 mV ($n = 6$). Also shown for comparison is the component

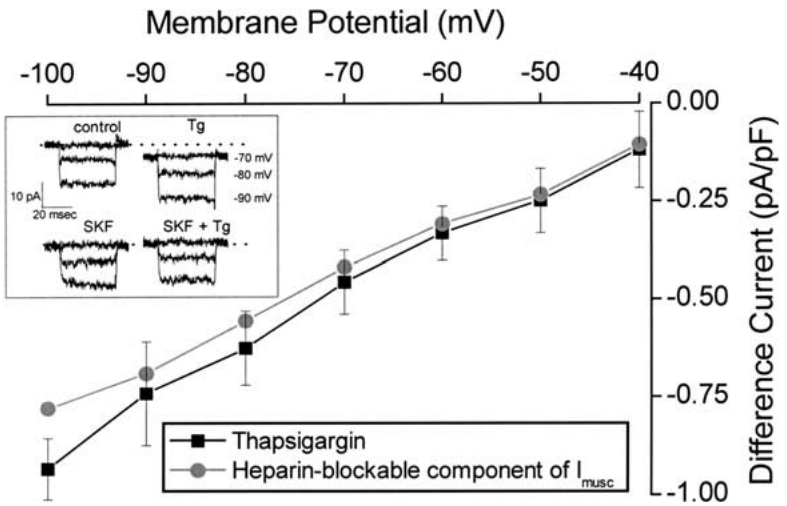

Fig. 5. Thapsigargin-activated current in HIT cells. *I-V* curve of current activated by 1 μM thapsigargin (Tg) in HIT cells (*squares*, $n = 6$). Shown in open circles is the heparin-blockable component of I_{musc} (difference current from Fig. 4). *Inset:* representative current traces from two individual experiments. Tg changed the current at the baseline holding potential and increased the size of current responses to voltage pulses (*top*), but not when SKF 96365 was present (*bottom*). Scale and voltage pulse levels (*top right*) apply to all traces. Dashed lines extend the control baseline.

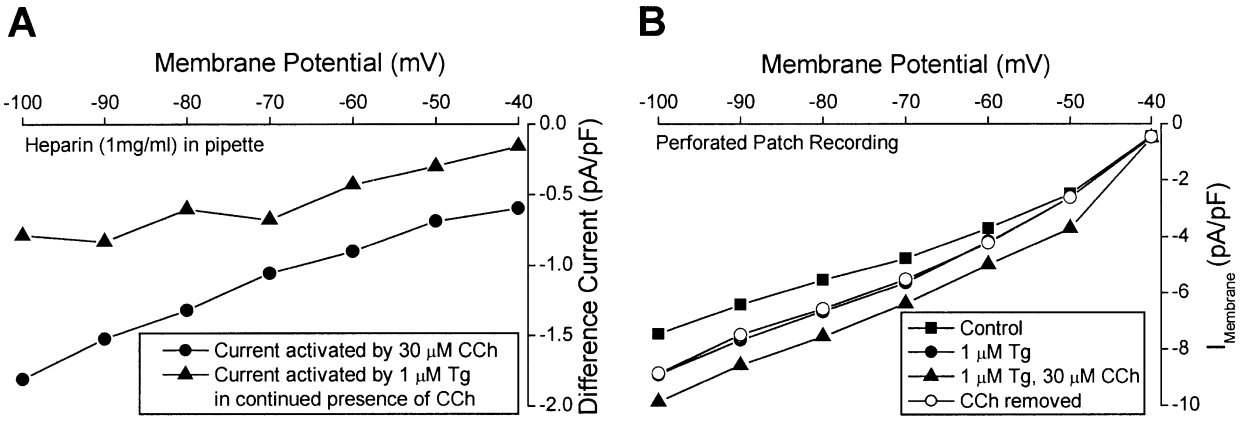

Fig. 6. Sequential activation of two components of I_{musc}. (*A*). Whole-cell currents were measured from a HIT cell dialyzed with 1 mg/ml heparin. The cell was first exposed to 30 μM carbachol (CCh), which activated a current (*circles*, calculated by subtracting the membrane *I-V* curve measured before addition of CCh from that measured after the agonist was added). Thapsigargin (Tg, 1 μM) was then applied in the continued presence of CCh, resulting in activation of a current with more negative reversal potential than that activated by CCh (*triangles*, calculated by subtracting the membrane *I-V* curve measured before addition of Tg from that recorded after the agent was applied). (*B*) Membrane *I-V* curves recorded from a HIT cell in the perforated-patch configuration, under control conditions (*squares*), after addition of Tg (*filled circles*), following addition of CCh in the presence of Tg (*triangles*) and after removal of CCh (*empty circles*). CCh reversibly activated a current after the Ca^{2+} stores had been emptied by Tg. Results are representative of four (part *A*) and three (part *B*) experiments with each protocol.

of I_{musc} that was blocked by heparin (the difference current from Fig. 4). Importantly, the Tg-activated current had essentially the same reversal potential (−32 mV) as the heparin-sensitive component of I_{musc}, suggesting that they are likely the same current. These results provide the first direct electrophysiological measurements of a current activated by Tg in insulin secreting cells. The similarity of this putative store-operated current to the component of I_{musc} blocked by heparin provides evidence that the latter current is a store-operated current.

To provide further evidence that I_{musc} consists of both store-operated and store-independent components, we performed experiments to activate both components sequentially. In the experiment of Fig. 6*A*, a HIT cell was dialyzed with heparin to block IP_3-dependent Ca^{2+} release. Membrane *I-V* curves (*not shown*) were measured before and after addition of 30 μM CCh, and were subtracted to demonstrate that the agonist activated the store-independent component of I_{musc} (Fig. 6*A*, circles, $V_{rev} \sim +5$ mV). We then applied Tg in the continued presence of CCh to empty the IP_3-sensitive Ca^{2+} store in the heparin-dialyzed cell. Subtracting the membrane *I-V* curves measured before and after addition of Tg confirmed that the SERCA antagonist activated the store-operated component of I_{musc} (Fig. 6*A*, triangles, $V_{rev} \sim -28$ mV). In four experiments, neither the magnitude nor the reversal potential of the Tg-activated current were affected by heparin in the pipette (*data not shown*). In Fig. 6*B*, an experiment using the perforated-patch configuration, Tg was first applied to

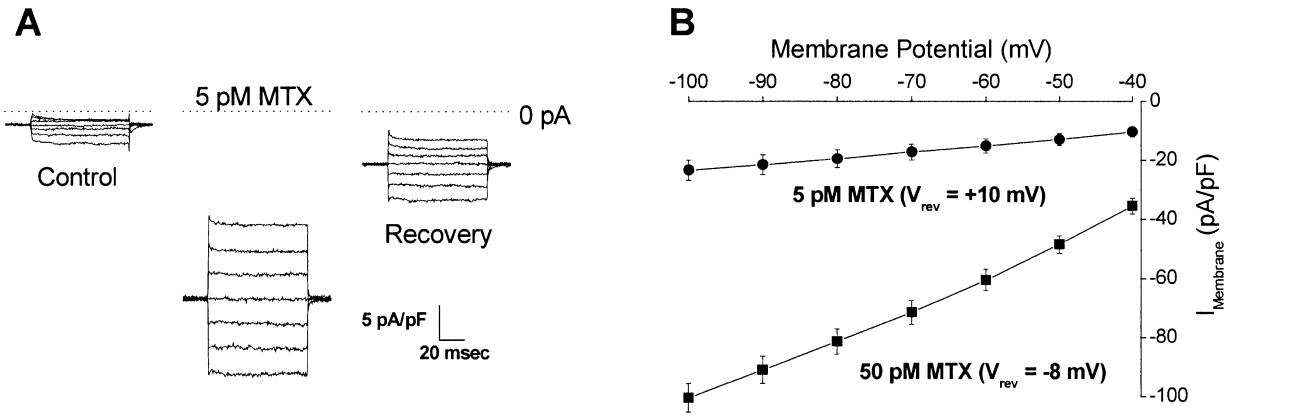

Fig. 7. Maitotoxin-activated current in HIT cells. (*A*) Current traces recorded from a HIT cell in response to voltage pulses from the holding potential (−70 mV) to potentials between −100 mV and −30 mV. Scale applies to all traces, and dashed lines extend control baseline. (*B*) Average *I-V* curves of current activated by 5 pM (*circles*, $n = 4$) and 50 pM maitotoxin, (*squares*, $n = 5$) in HIT cells. Reversal potentials were estimated by extrapolating linear fits to the curves.

activate the store-operated component of I_{musc}. As expected, the agent shifted the membrane *I-V* curve of the HIT cell downward. Carbachol was then applied and reversibly activated additional current. The additional current is most likely the store-independent component and not additional store-operated current from further emptying of Ca^{2+} stores, since such an effect would be irreversible in the presence of Tg. Furthermore, the extrapolated reversal potential of the CCh-activated current in Tg-treated cells ($n = 3$) was +1.8 mV (*not shown*), consistent with the heparin-insensitive component of I_{musc}. Together, the experiments of Fig. 6 provide further evidence that a muscarinic-activated, store-independent ionic current and a biophysically distinct store-operated current are both present in HIT cells and can be individually activated under the appropriate conditions.

The dinoflagellate toxin maitotoxin (MTX) activates non-selective ion channels and stimulates Ca^{2+} influx in pancreatic β-cells (Soergel et al., 1992; Worley et al., 1994a; Leech & Habener, 1997; 1998; Roe et al., 1998). The effect of 5 pM MTX on whole-cell current traces recorded from a HIT cell is shown in Fig. 7*A*. The toxin activated a large current that was partially reversible within five minutes of removal of MTX. The magnitude of the current activated by 5 pM MTX at −70 mV (17.2 ± 2.7 pA/pF) was significantly reduced by 25 μM SKF 96365 (4.9 ± 2.9 pA/pF, $P < 0.001$) (*data not shown*). Figure 7*B* shows *I-V* curves of the MTX-activated current in HIT cells. The extrapolated reversal potential of the current was dose-dependent, being +10 mV at 5 pM MTX and −8 mV at 50 pM. The voltage-dependent reversal potential indicates that MTX activates multiple currents with different sensitivities for the toxin. The current activated by low concentrations of the toxin has a more positive reversal potential than either component of I_{musc}, indicating that it is a different current. Although we can not rule out that MTX also activates one or both components of I_{musc}, our results indicate that, at least in HIT cells, the agent is not selective for either component of the current.

Finally, we have examined whether muscarinic agonists also activate a multi-component ionic current in mouse pancreatic β-cells. Figure 8*A* shows the effect of CCh on membrane *I-V* curves measured from a mouse β-cell, with representative current traces shown in the inset. The agonist caused a negative shift in the current at all applied potentials, which partially reversed within one minute of removal of the agonist. The CCh-induced difference current is also shown in Fig. 8*A*. As in HIT cells, the current was fairly linear at the measured potentials. To determine if I_{musc} in β-cells also has multiple components, we compared the magnitude of the current induced by CCh with and without heparin in the whole-cell pipette solution. Figure 8*B* shows that heparin reduced the average magnitude of the current from 1.3 pA/pF to 0.6 pA/pF ($P < 0.025$). This result indicates that I_{musc} in native mouse pancreatic β-cells also has a Ca^{2+} store-dependent component.

Discussion

Cholinergic Agonists Activate a Nonselective Current in Pancreatic β-Cells

Acetylcholine and other muscarinic receptor agonists potentiate insulin secretion both by enhancing glucose-induced β-cell electrical activity, which increases the rate of Ca^{2+} influx through voltage-gated Ca^{2+} channels, and by increasing the efficiency of Ca^{2+} in inducing exocytosis (Gilon & Henquin, 2001). In studies of potential mechanisms of cholinergic modulation of β-cell electrical activity, the agonists have been shown to activate or inhibit K_{ATP} channels (Nakano et al., 2002; Rolland, Henquin & Gilon,

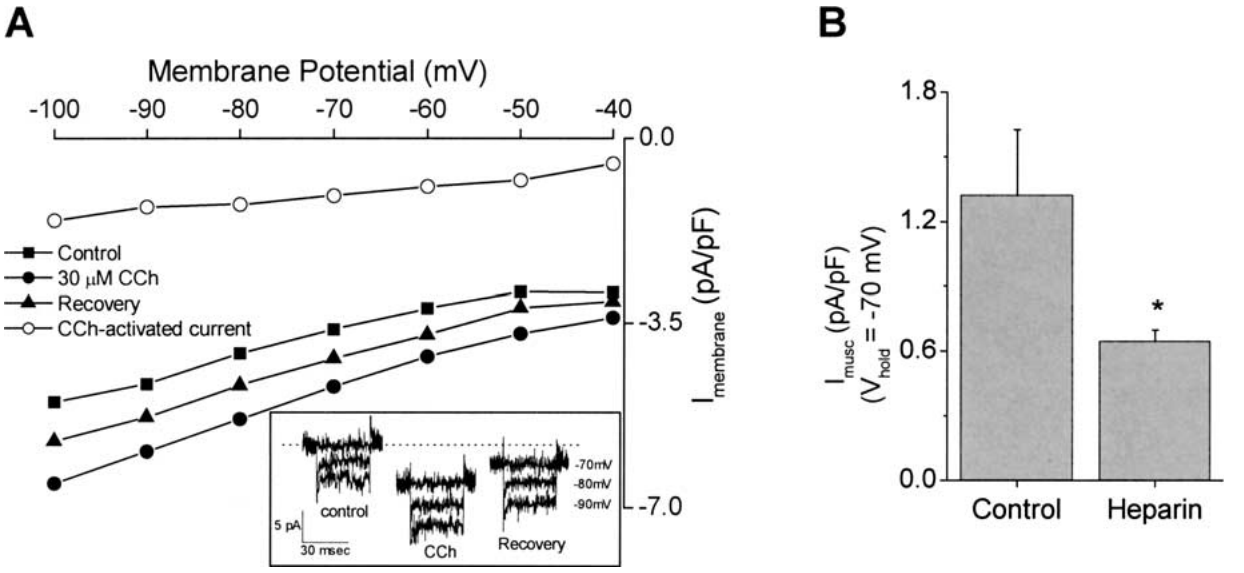

Fig. 8. I_{musc} in mouse β-cells. (*A*) *I-V* curves recorded in whole-cell mode from a mouse β-cell before (*squares*), during (*filled circles*) and after (*triangles*) exposure to carbachol (CCh). Open circles show the CCh-activated current (squares subtracted from filled circles). *Inset* shows actual current responses to representative voltage pulses (*dashed line* is extension of control baseline). (*B*) Average magnitude of the current activated by 30 μM CCh in mouse β-cells (measured as a negative shift in current at −70 mV), with or without 1 mg/ml heparin in the pipette solution (*, $P < 0.025$).

2002a), activate or inhibit voltage-gated Ca^{2+} channels (Gilon et al., 1997; Love et al., 1998), and most recently, activate a small, voltage-independent Na^+ channel (Rolland et al., 2002b). Here we present the first direct evidence that cholinergic agonists simultaneously activate store-operated and store-independent nonselective currents in insulin-secreting cells. We have identified and characterized these currents in an insulin-secreting cell line (HIT), but have also verified the existence of both components in cultured mouse β-cells.

The acetylcholine analog carbachol (CCh) dose-dependently and reversibly activated a small current in both HIT cells and mouse β-cells, and this effect was potently suppressed by the muscarinic receptor atropine. In these experiments we intentionally kept the membrane potential at relatively hyperpolarized levels to prevent activation of voltage-gated Ca^{2+} and K^+ currents, since we found that even small changes in the amplitudes of these large currents (caused by run-down or muscarinic agonist-induced modulation) introduce errors into the subtractive technique used to identify I_{musc}. As a result, we could not directly measure the reversal potential of the current. However, extrapolation of the linear *I-V* curve of I_{musc} allowed us to estimate a reversal potential of −15 mV, indicating that I_{musc} is a nonselective ionic current.

I_{musc} Has a Store-operated Component

We found that the magnitude of I_{musc} is similar when measured in either the perforated patch or conventional whole-cell recording configuration (*data not shown*). We therefore chose to characterize I_{musc} using predominantly the whole-cell mode, since this configuration allowed us to tightly control $[Ca^{2+}]_i$ and to dialyze cells with heparin, a membrane-impermeable IP_3 receptor antagonist.

When I_{musc} was measured in HIT cells dialyzed cells with 1 mg/ml heparin, a concentration that effectively inhibits IP_3-induced Ca^{2+} release in permeablized cells (Hofer & Machen, 1993), the current was smaller and had a more positive extrapolated reversal potential than under control conditions. These changes did not likely result from negatively charged heparin molecules binding to the inner surface of the plasma membrane to alter the membrane potential or Na^+ equilibrium potential, since the thapsigargin-activated current was not similarly affected by the presence of heparin (Fig. 6). Given that heparin is an IP_3 receptor antagonist, the agent most likely blocked a component of I_{musc} by preventing CCh from releasing Ca^{2+} from IP_3-sensitive stores. However, we cannot rule out that heparin acted by a different mechanism, such as direct interaction with ion channels. We therefore found it important to demonstrate that thapsigargin (Tg), an agent that has been used extensively to elicit receptor-independent depletion of IP_3-sensitive Ca^{2+} stores, activates a current with similar properties to the heparin-sensitive component of I_{musc}. Together, we have shown that one component of I_{musc} is affected by three agents (CCh, heparin and Tg) that are known to act on IP_3-sensitive Ca^{2+} stores, suggesting that release of stored Ca^{2+} is the activating signal for the current. Since $[Ca^{2+}]_i$ was

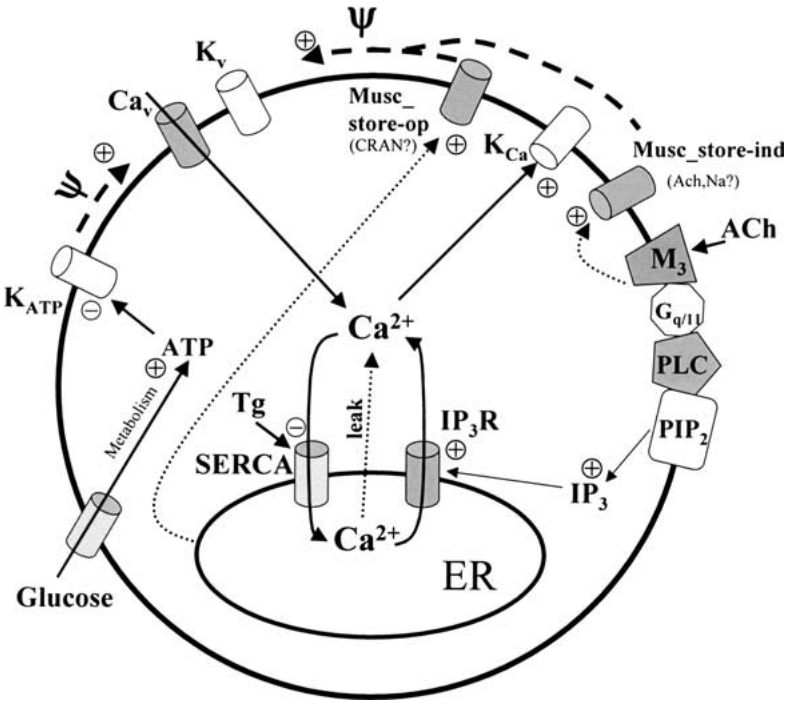

Fig. 9. Role of I_{musc} in β-cell electrical activity. The scheme shows proposed roles for the two components of I_{musc} in modulation of glucose-induced electrical activity by cholinergic agonists and SERCA inhibitors. Potassium channels are shown as white cylinders, calcium and nonselective ion channels are shown as gray cylinders. *Abbreviations*: K_{ATP}, adenosine triphosphate sensitive K^+ channel; ATP, adenosine triphosphate; ψ, membrane potential; Ca_v, voltage-dependent Ca^{2+} channel; K_v, voltage-dependent K^+ channel; Tg, thapsigargin; SERCA, sarco-/endoplasmic reticulum Ca^{2+}-ATPase; ER, endoplasmic reticulum; IP_3; inositol trisphosphate; IP_3R, inositol trisphosphate receptor; leak, passive Ca^{2+} leak pathway; Musc_store-op, store-operated component of I_{musc}; CRAN, Ca^{2+} release-activated nonselective ion channel; K_{Ca}, Ca^{2+}-activated K^+ channel; Musc_store-ind, store-independent component of I_{musc}; $I_{Ach,Na}$, acetylcholine-activated Na^+ channel; Ach, acetylcholine; M_3, muscarinic acetylcholine receptor (M_3 subtype); PLC, phospholipase C; $G_{q/11}$, PLC-activating G-protein; PIP_2, phosphatidylinositol 4,5-bisphosphate.

highly buffered in these experiments and the currents activated by Tg and CCh were sustained rather than transient, the activating signal for the Ca^{2+} store-dependent current is most likely store depletion per se, rather than a transient increase in $[Ca^{2+}]_i$ caused by Ca^{2+} release. Our results provide the first direct electrophysiological evidence that both cholinergic agonists and SERCA inhibitors activate a store-operated current in insulin-secreting cells.

Thapsigargin reportedly empties intracellular Ca^{2+} stores more effectively than cholinergic agonists (Miura, Gilon & Henquin, 1996). Nevertheless, we noted that the store-dependent component of the current activated by 30 μM CCh had essentially the same whole-cell conductance as that activated by 1 μM Tg (Fig. 5). This observation suggests that Tg-sensitive Ca^{2+} stores do not need to be completely empty to maximally activate the store-operated current. The fact that CCh activated the store-operated current in whole-cell experiments indicates that agonist-generated IP_3 is effective in releasing Ca^{2+} in this configuration, which is consistent with previous observations in other cell types (Tse, Tse & Hille, 1994).

The store-operated component of I_{musc} is similar to the current that Worley and colleagues (1994a,b) identified by removing extracellular Ca^{2+} to passively deplete intracellular Ca^{2+} stores (I_{CRAN}). Both I_{CRAN} and the store-operated component of I_{musc} are nonselective currents, and both are blocked by SKF 96365. The presence of a non-selective, store-operated current (SOC) in the β-cell accounts for the membrane depolarization produced by exposure to Tg, and by other manipulations that empty β-cell Ca^{2+} stores (Worley et al., 1994b; Bertram et al., 1995; Mears et al., 1997). The small magnitude of the current is consistent with the observation that SERCA inhibitors do not depolarize the β-cell at basal glucose levels (Worley et al., 1994b), when open K_{ATP} channels dominate the membrane conductance. Nonselective SOCs carry some Ca^{2+} under physiological conditions (Leech & Habener, 1998), so the store-operated component of I_{musc} could account for the small, voltage-independent Ca^{2+} influx observed in β-cells following Ca^{2+} store depletion (Liu & Gylfe, 1997; Miura et al., 1997). However, the influence of such Ca^{2+} influx on intracellular Ca^{2+} levels in the β-cell is expected to be small compared to influx through voltage-gated Ca^{2+} channels. Therefore, the principal role of the nonselective SOC in the β-cell is most likely to increase Ca^{2+} in the presence of glucose indirectly, by depolarizing the membrane to stimulate additional voltage-dependent Ca^{2+} entry.

I_{musc} Has a Store-independent Component

The observation that I_{musc} also has a component that is insensitive to blockade by heparin is consistent with the report by Rolland and co-workers (2002b) showing that acetylcholine activates a Na^+-selective current ($I_{Ach,Na}$) in mouse β-cells that is unaffected by pre-treatment with Tg. Although our measurements of the store-independent component of I_{musc} in HIT cells suggest that it is a nonselective current, we cannot rule out the possibility that $I_{Ach,Na}$ contributes to this current or that they are, in fact, the same current. Like $I_{Ach,Na}$, the store-independent compo-

nent of I_{musc} is G-protein independent. Furthermore, the *I-V* curve of the store-independent component of I_{musc} (Fig. 4) hints at inward rectification, suggesting that the reversal potential may be more positive than 0 mV. We have found that both the store-independent and store-operated components of I_{musc} are reduced in amplitude in the absence of Na^+ (*data not shown*). However, we cannot as yet conclude that Na^+ removal completely inhibits the store-independent current, which would be consistent with its identification as $I_{Ach,Na}$, or simply reduces its magnitude to below the resolution of our recording system. The sensitivity of $I_{Ach,Na}$ to SKF 96365 was not previously reported.

Roles for I_{musc} in β-Cell Electrical Activity

Identifying the functional roles of I_{musc} in the β-cell is impeded by a lack of selective agonists and antagonists for either component of the current. Nevertheless, a number of observations are consistent with a role for I_{musc} in cholinergic stimulation of β-cell electrical activity. Nonselective currents carry predominantly Na^+ under physiological conditions, so a role for I_{musc} is accordant with the observation that cholinergic agonists stimulate Na^+ uptake and increase intracellular Na^+ in β-cells (Gagerman et al., 1980; Gilon & Henquin, 1993). However, cholinergic electrical activity is completely inhibited by Na^+ removal (Henquin et al., 1988), whereas our results show that I_{musc} is only partially blocked by this manipulation. This observation does not rule out a role for I_{musc} in cholinergic stimulation of β-cell electrical activity, which likely reflects a balance between depolarizing and hyperpolarizing electrical effects of the agonists (Sanchez-Andres & Soria, 1991; Gilon et al., 1997; Rolland et al., 2002a). In fact, in Na^+-free solutions, acetylcholine causes a slight inhibition of glucose-induced β-cell electrical activity (Henquin et al., 1988). It is therefore possible that in the absence of Na^+, the reduced depolarizing influence of I_{musc} is no longer able to overcome the hyperpolarizing effects of cholinergic agonists. Finally, a role for the two components of I_{musc} in cholinergic modulation of β-cell electrical activity is consistent with the observation that these agents produce a greater rise in intracellular Na^+ than SERCA inhibitors (Miura et al., 1997), which activate only the store-operated component.

What is the relative contribution of each component of I_{musc} to cholinergic agonist-induced depolarization? At the agonist concentration that we used (30 μM) the store-independent and store-operated components have similar whole-cell conductance, accounting for 57% and 43% of the total whole-cell conductance of I_{musc}, respectively. However, because of its more positive reversal potential, the store-independent component of I_{musc} accounts for 67–85% of the total inward current at potentials ranging from −70 mV to −40 mV (Fig. 4). This component is therefore expected to have a much greater depolarizing influence on the β-cell.

Often, particularly at high agonist concentrations, the action of acetylcholine on glucose-induced bursting is triphasic, consisting of an initial period of rapid spiking followed by a transient hyperpolarization, with the membrane finally depolarizing again to enter a phase of fast bursting (Gilon & Henquin, 2001). Bordin and colleagues (1995) have provided evidence that the transient hyperpolarization is due to activation of Ca^{2+}-activated K^+ channels (K_{Ca}) by release of stored Ca^{2+}. If the store-operated component of I_{musc} activates with a similar time course to the K_{Ca} channels, one would expect that this current participates in the steady-state, depolarized phase of cholinergic electrical activity rather than the initial phase. Bertram and co-workers (1995) proposed this mechanism in a theoretical study. Unfortunately, limitations in our perfusion system prevent us from determining the exact time course of activation of the components of I_{musc}, so the relative contribution of each component to the different phases of cholinergic electrical activity remains to be determined. The fact that the phase of Ca^{2+}-release-induced hyperpolarization is only observed at high agonist concentrations does not preclude that the store-operated component of I_{musc} is activated by lower agonist concentrations. In fact, the recent observation that capacitative Ca^{2+} influx can be induced by as low as 3.6 μM CCh (Dyachok & Gylfe, 2001) suggests that the store-operated component is likely to affect membrane potential at these concentrations as well.

In summary, we have identified a multi-component, nonselective ionic current activated by muscarinic agonists in insulin-secreting cells. As shown in the scheme in Fig. 9, the presence of this current suggests that both Ca^{2+} store-dependent and -independent mechanisms contribute to cholinergic potentiation of glucose-induced β-cell electrical activity. The regulation of β-cell membrane potential by these mechanisms likely plays an important role in cholinergic stimulation of insulin secretion, by enhancing Ca^{2+} influx through voltage-gated Ca^{2+} channels.

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