A Role for Calcium Release-Activated Current (CRAC) in Cholinergic Modulation of Electrical Activity in Pancreatic β-Cells

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ABSTRACT S. Bordin and colleagues have proposed that the depolarizing effects of acetylcholine and other muscarinic agonists on pancreatic β-cells are mediated by a calcium release-activated current (CRAC). We support this hypothesis with additional data, and present a theoretical model which accounts for most known data on muscarinic effects. Additional phenomena, such as the biphasic responses of β-cells to changes in glucose concentration and the depolarizing effects of the sarco-endoplasmic reticulum calcium ATPase pump poison thapsigargin, are also accounted for by our model. The ability of this single hypothesis, that CRAC is present in β-cells, to explain so many phenomena motivates a more complete characterization of this current.

INTRODUCTION

The endocrine pancreas is controlled by blood glucose concentration and both sympathetic and parasympathetic input (Woods and Porte, 1974). Insulin secretion is elicited by an increase in blood glucose or parasympathetic stimulation. Secretion is inhibited by sympathetic input. In agreement with these in vivo observations, in vitro experiments on pancreatic islets of Langerhans show that cholinergic drugs potentiate glucose-induced insulin secretion (Henquin et al., 1988), whereas catecholamines inhibit insulin secretion (Ashcroft and Rorsman, 1989). We address the cholinergic effects on membrane potential and cytosolic Ca^{2+} in this paper.

Increases in insulin release from islet β-cells typically parallel increases in average free cytosolic Ca^{2+} concentration (Ca_i), although there are exceptions (Gilon et al., 1993). Free cytosolic Ca^{2+} levels are determined largely by the electrical activity of the β-cell (Santos et al., 1991). In low glucose, islet β-cells are hyperpolarized and Ca_i is low. Application of acetylcholine (ACh) leads to a small depolarization and a small release of insulin (Henquin et al., 1988). In higher glucose concentrations, β-cells burst in synchrony, a periodic behavior consisting of a phase of hyperpolarization during which Ca_i is low followed by an active phase of spiking accompanied by elevated Ca_i (Santos et al., 1991). There is great islet-to-islet variability when a cholinergic drug is applied under these conditions. The bursting is often superseded by a transient period of high-frequency spiking, followed by a transient membrane hyperpolarization. This is followed by a slow depolarization culminating in "muscarinic bursting", with a shortened period and a depolarized silent phase (Cook et al., 1981; Sánchez-Andrés et al., 1988; Henquin et al., 1988; Santos and Rojas, 1989; S. Bordin, A. C. Boschero, E. M. Carneiro, and I. Atwater, submitted for publication). In single human β-cells, cytosolic Ca^{2+} was observed to increase dramatically upon application of ACh, muscarine, or oxotremorine-m, a muscarinic agonist (Rojas et al., 1994). We present data here showing that in mouse islets Ca_i is also dramatically elevated following application of agonist (see Fig. 4 *B*).

At low glucose (3 mM), ACh (20 μM) increases the influx of $^{22}Na^+$, but not $^{45}Ca^{2+}$ (Henquin et al., 1988). When Na^+ is removed from the bath, ACh fails to depolarize the cells, suggesting that the depolarizing action of ACh is through a current carried by Na^+. Unpublished observations reported by F. Ashcroft and P. Rorsman (1989) have also suggested that activation of muscarinic receptors opens a channel permeable to Na^+.

ACh activates muscarinic receptors in the β-cell (Santos and Rojas, 1989), activating phospholipase C and producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular stores, while DAG activates protein kinase C, which sensitizes the secretory machinery to Ca^{2+} (Berridge and Irvine, 1989; Jones et al., 1985; Bozem et al., 1987). The mechanism by which muscarinic agonists open membrane channels and depolarize the β-cell is unknown. We argue that it is a calcium release-activated current (CRAC), activated by depletion of Ca^{2+} stores in the endoplasmic reticulum (ER), and carried by Na^+ and K^+.

A linkage mechanism between Ca_{er} and membrane channels was first proposed by Putney and coworkers (Takemura et al., 1989; Putney, 1990). This "depletion hypothesis" states that when the ER calcium concentration becomes too low, a second messenger is released that diffuses outward to the cell membrane, where it opens Ca^{2+} channels. This increases Ca_i and refills ER calcium stores. Evidence for the diffusible "calcium influx factor" (CIF) has been reported in

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a lymphocyte cell line (Randriamampita and Tsien, 1993). Evidence for CRAC current has been reported in mast cells (Hoth and Penner, 1992), lymphocytes (Zweifach and Lewis, 1993), *Xenopus* oocytes (Parekh et al., 1993), parotid acinar cells (Mertz et al., 1990; Randriamampita and Tsien, 1993), and pancreatic acinar cells (Bahnson et al., 1993).

Although there is no evidence for CIF in β-cells, there have been reports of nonselective cation currents with properties consistent with I_{CRAC}. Silva et al. (1994) showed that raising the concentration of extracellular calcium in the presence of the L-type Ca^{2+} channel blocker nifedipine elicited a pronounced rise in Ca_i, attributed to increased influx of Ca^{2+} through nonselective voltage-independent channels. The rise in Ca_i was enhanced by depletion of ER Ca^{2+}. Worley et al. (1994) measured an increased voltage-independent inward current in β-cells upon bath application of EGTA and removal of external Ca^{2+}, which presumably deplete the ER and activate CRAC channels. Because of the persistence of this current in the absence of extracellular Ca^{2+}, they suggested that it is carried by Na^+ and K^+. They also showed that the sarco-endoplasmic reticulum calcium ATPase (SERCA) pump blocker thapsigargin (Tg) depolarizes islets. In HIT-T15 cells, Leech et al. (1994) showed that Tg induces Mn^{2+} influx. We present additional supporting data on Tg, including its slow transient effects (see Fig. 2).

Using a theoretical model, we investigate the implications of the hypothesis that CRAC current is present in β-cells. We show that this model accounts for the effects of Tg and ACh, as well as the biphasic responses to changes in glucose concentration (Meissner and Atwater, 1976; Henquin, 1992; Roe et al., 1993). Thus, this single hypothesis suffices to explain many previously unexplained phenomena.

Some of this work has been presented previously in abstract form (Bertram et al., 1994).

MATERIALS AND METHODS

Electrophysiology

The details of the intracellular recording technique for microdissected mouse islets of Langerhans have been reported elsewhere (Atwater et al., 1978). Briefly, single microdissected mouse islets were continuously perifused with a modified Krebs solution containing, in mM, 120 NaCl, 25 $NaCO_3$, 5 KCl, 2.5 $CaCl_2$, and 1.1 $MgCl_2$, which was equilibrated with 95% O_2/5% CO_2 to yield a pH of 7.4 at 37°C. Glucose was added to the medium without adjusting for osmotic changes. Cells were impaled with high resistance microelectrodes (150–250 MΩ), and β-cells were identified based on bursting electrical activity in 11.1 mM glucose. Thapsigargin was added to the medium from a 10 mM stock solution in DMSO; acetylcholine was added from a 10 mM stock solution in water, kept on ice. A 10 mM diazoxide stock solution was obtained by first dissolving in a few drops of 1N NaOH, then adding deionized water to the desired concentration (<2% 1N NaOH). Carbamylcholine was added to the medium from 10 mM stock solution in H_2O. Membrane potential recordings in Fig. 2 were made with an EPC-7 amplifier (List Electronics, Darmstadt-Eberstadt, Germany), and those in Fig. 4 were made with an Axoclamp IIB amplifier (Axon Instruments, Foster City, CA). Membrane potential records were printed on a chart recorder and recorded on magnetic tape for future analysis.

Ca_i measurements

Islets of Langerhans were isolated by collagenase digestion as previously reported (Lernmark, 1974). Once isolated, islets were incubated for 60 min at 37°C in standard medium with the following composition (mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 1.1 $MgCl_2$. Glucose (5 mM) and 3% bovine serum albumin were added. The medium was continuously equilibrated with a mixture of O_2 (95%) and CO_2 (5%) for a final pH of 7.4. After incubation, islets were loaded at room temperature with 2 μM of Indo-1/AM (Molecular Probes, Eugene, OR) in the same medium for 60 min. Loaded islets were placed in the superfusion chamber and left to settle down. Most of the islets adhered spontaneously to the bottom of the chamber within 3–6 min. The experimental superfusion chamber (volume 300 μl) was mounted on the stage of a Nikon-Diaphot inverted microscope and superfused at a rate of 1 ml/min with standard incubation medium. Glucose (11 mM) was added to the medium. Carbamylcholine was added to the medium from 10 mM stock solution in H_2O. Bath temperature was maintained at 36 ± 1°C by heating a stainless steel ring controlled by a thermostat. The temperature of the chamber was continuously monitored with a microthermistor. Cytosolic free calcium, expressed as the ratio of the two wavelengths (F_{410}/F_{480}), was monitored by Indo-1 (Grynkiewicz et al., 1985), as reported (Valdeomillos et al., 1989).

The mathematical model

The mathematical model consists of three components: one capable of bursting independently, one describing calcium handling, and one providing direct feedback from the ER to the cell membrane (CRAC current). The first component includes two fast calcium currents (I_{Caf} and I_{Cas}), one of which (I_{Cas}) inactivates slowly; a delayed rectifying potassium current (I_K); and an ATP-inactivated potassium current ($I_{K(ATP)}$):

$$C_m \frac{dV}{dt} = -[I_{Caf} + I_{Cas} + I_K + I_{K(ATP)}] \quad (1)$$

$$\frac{dn}{dt} = (n_\infty(V) - n)/\tau_n(V) \quad (2)$$

$$\frac{dj}{dt} = (j_\infty(V) - j)/\tau_j(V). \quad (3)$$

V represents membrane potential, n is activation of I_K, and j is inactivation of I_{Cas}. Ionic currents are given by:

$$I_{Caf} = \bar{g}_{Caf} m_{f,\infty}(V)(V - V_{Ca}) \quad (4)$$

$$I_{Cas} = \bar{g}_{Cas} j m_{s,\infty}(V)(V - V_{Ca}) \quad (5)$$

$$I_K = \bar{g}_K n(V - V_K) \quad (6)$$

$$I_{K(ATP)} = \bar{g}_{K(ATP)}(V - V_K). \quad (7)$$

This is based on the model of Chay and Cook (1988), with calcium-dependent inactivation of I_{Cas} replaced by voltage-dependent inactivation and with the addition of $I_{K(ATP)}$. Other parameter changes have been made to accommodate these modifications. In particular, I_{Cas} now activates at a more depolarized voltage than I_{Caf}, consistent with data from Satin and Cook (1988). Expressions for the infinity and time constant functions are given in the Appendix, as are values of all parameters.

Action potentials are generated by the interaction of the instantaneously activated Ca^{2+} currents and the less rapidly activated delayed rectifier, while $I_{K(ATP)}$ is a background current, whose conductance is a decreasing function of glucose concentration. Bursting is driven by the V-dependent modulation of the inactivation variable j, which is much slower than V and n.

The second component of our model includes equations for calcium handling, based on Li and Rinzel (1994), and a calcium-activated potassium current ($I_{K(CA)}$). Equations for the concentrations of free cytosolic (Ca_i) and free ER calcium (Ca_{er}) are:

$$\frac{V_i}{f_i}\frac{dCa_i}{dt} = J_{er} + J_{mem} \tag{8}$$

$$\frac{V_{er}}{f_{er}}\frac{dCa_{er}}{dt} = -J_{er}. \tag{9}$$

J_{mem} and J_{er} represent Ca^{2+} flux through the plasma and ER membranes, respectively. V_i and V_{er} represent cytosolic and ER volumes, while f_i and f_{er} are the ratios of free to total calcium in each compartment. Expressions for the Ca^{2+} fluxes are:

$$J_{mem} = -\alpha(I_{Caf} + I_{Cas}) - k_{Ca}Ca_i \tag{10}$$

$$J_{er} = (P_{leak} + P_{ip3}O_\infty)(Ca_{er} - Ca_i) - J_{er,p}. \tag{11}$$

α is the conversion factor from current to flux, $k_{Ca}Ca_i$ and $J_{er,p}$ represent fluxes through the plasma membrane pumps and SERCA pumps, P_{leak} is the calcium leakage permeability through the ER membrane, and P_{ip3} is calcium permeability through IP_3-activated Ca^{2+} channels in the ER membrane. The fraction of open IP_3-activated channels is $O_\infty = a_\infty b_\infty h_\infty$, where a_∞ represents activation by Ca_i; b_∞, IP_3 activation; and h_∞, inactivation by Ca_i (unlike Li and Rinzel (1994), we model this inactivation as instantaneous, a simplification that has no significant effect on our simulations). Detailed expressions for the pumps and O_∞ are contained in the Appendix.

There is experimental evidence for both voltage-dependent (Cook et al., 1984) and voltage-independent (Ämmälä et al., 1991, 1993) K(Ca) current in the β-cell. For simplicity, we include only a voltage-independent current:

$$I_{K(Ca)} = \bar{g}_{K(Ca)}\frac{Ca_i^5}{Ca_i^5 + (0.55)^5}(V - V_K). \tag{12}$$

This expression is identical to that used in the model of Keizer and De Young (1993) to explain the periodic interruptions of tonic spiking due to repetitive release of Ca^{2+} observed in high concentrations of IP_3 and glucose (Ämmälä et al., 1991, 1993). Our model exhibits this behavior if it is extended to provide dynamics for IP_3 channel inactivation ($h \neq h_\infty(Ca_i)$).

The first two components of our model provide a glucose-induced bursting mechanism and give a reasonable glucose dose-response curve. However, it is only with the addition of the third component, a CRAC current (I_{CRAC}), that the model is capable of producing the effects of Tg and ACh. We model this current by:

$$I_{CRAC} = \bar{g}_{CRAC}r_\infty(Ca_{er})(V - V_{CRAC}) \tag{13}$$

where r represents Ca_{er}-dependent activation. Lacking data on the kinetics of this current in β-cells, we model it as instantaneous. Because the emptying and filling of the ER is slow, any activation delay of I_{CRAC} would have only a minor effect, as we have verified with the model (not shown). There is also a lack of data on the levels of Ca_{er} at which I_{CRAC} is activated, or even typical values of Ca_{er}. We assume that I_{CRAC} is half activated when Ca_{er} = 3 μM ($r_\infty(3) = 0.5$), corresponding to 30–40% depletion of the ER. The half-activation level could be adjusted up or down to accommodate future measurements of ER Ca^{2+} concentration. Finally, we assume that I_{CRAC} carries Na^+ and K^+ and reverses at 0 mV.

With the addition of the K(Ca) and CRAC currents, the voltage equation becomes:

$$C_m\frac{dV}{dt} = -[I_{Caf} + I_{Cas} + I_K + I_{K(ATP)} + I_{K(Ca)} + I_{CRAC}]. \tag{14}$$

Equations were integrated numerically using a Gear method implemented in the software package LSODE. This method works well on systems such as ours with multiple time scales. All computations were carried out on an IBM RS/6000 computer.

RESULTS

Simulated glucose-induced bursting

In our model, membrane potential reaches action potential threshold through the depolarizing effects of I_{Cas}. At the start of the active phase of bursting the inactivation variable j is high, so I_{Cas} is strong. During the active phase, j decreases until there is not enough depolarizing current to sustain the electrical activity and the cell enters a hyperpolarized quiescent phase, during which j increases and the cell is brought back to threshold (Fig. 1). In contrast, Ca_i rises almost immediately at the onset of the active phase and falls quickly at the onset of the quiescent phase. This fast variation of Ca_i, which is too fast to drive glucose-induced bursting, is consistent with experiments (Santos et al., 1991). Also consistent with experiments is the property that an increase in glucose concentration, through reduction in $\bar{g}_{K(ATP)}$, increases the ratio of active phase duration to burst period (plateau fraction),

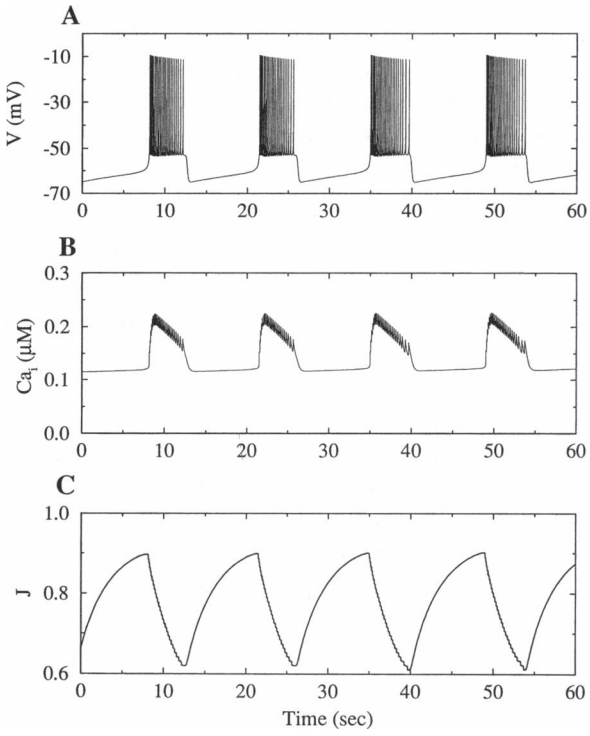

FIGURE 1 Simulated glucose-induced bursting ($\bar{g}_{K(ATP)}$ = 150 pS, IP_3 = 0 μM). (*A*) Membrane potential. (*B*) Cytosolic Ca^{2+}. (*C*) *j*, the voltage-dependent inactivation of I_{Cas}. Following the Hodgkin-Huxley convention, *j* represents channel availability. Its slow decline terminates the active phase of bursting; its slow recovery terminates the silent phase.

while having little effect on the amplitude of the action potentials (not shown). Thus, average Ca_i is increased, while peak Ca_i is not.

$I_{K(Ca)}$ and I_{CRAC} contribute little to the simulated glucose-induced bursting. Ca_i is never large enough to significantly activate $I_{K(Ca)}$, and Ca_{er} is too high to activate I_{CRAC}. Blocking either or both of these currents causes only a minor change in burst frequency. This is consistent with Kukuljan et al. (1991), who showed that the charybdotoxin-sensitive K(Ca) channel does not participate in glucose-induced electrical activity.

Application of thapsigargin

Fig. 2 shows the effect of Tg on β-cell electrical activity. The leftmost portion of the top trace illustrates normal bursting induced by 11.1 mM glucose. Addition of 1 μM Tg to the perifusion medium (as indicated by the arrow in the top trace) had little immediate effect on the burst pattern. During the 24 minutes between traces A and B in Fig. 2, the Tg concentration was progressively increased to 3 μM. This was accompanied by a gradual increase in the plateau fraction and burst frequency. Shortly after the Tg concentration was changed to 5 μM (Fig. 2 *B*, *arrow*), the silent phase potential began to depolarize and both the plateau fraction and burst frequency increased further. At the time indicated by the arrow in Fig. 2 *C*, Tg was removed completely from the perifusion medium and the electrical activity shifted to a pattern of almost continuous spiking, with no hyperpolarization during the brief silent phases. Addition of 100 μM ACh (Fig. 2 *D*) rapidly brought the cell to continuous spiking. The combined effect of Tg and ACh on membrane potential was irreversible, as indicated by the maintenance of continuous activity following removal of ACh (Fig. 2 *D*, *right*).

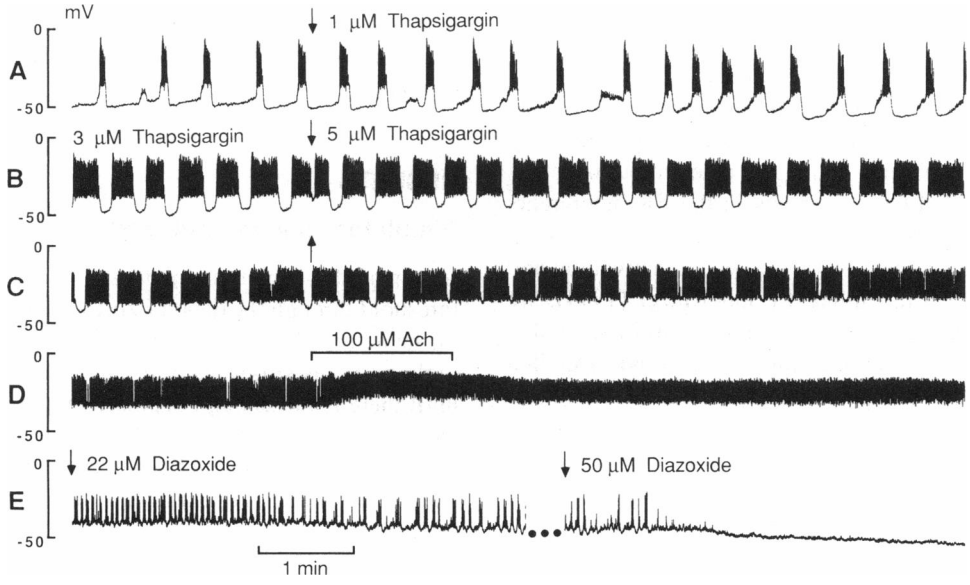

FIGURE 2 Effect of Tg on β-cell membrane potential. Glucose concentration was 11.1 mM throughout the recording. (*A*) 1 μM Tg was added as indicated by the arrow. During the time between the top two traces (24 min), Tg concentration was progressively increased to 3 μM. (*B*) Tg concentration was increased to 5 μM at the arrow. 30 s elapsed between the end of *B* and the start of *C*. (*C*) Tg was removed at the arrow. (*D*) (Continuation of trace *C*) The islet was exposed to a 90-s pulse of 100 μM ACh as indicated. Time between *D* and *E* was 14 min. (*E*) Burst pattern restored by 22 μM Dz (*left*). Increasing Dz to 50 μM (6 min between left and right sides of E) abolished spiking.

Five cells were subjected to a similar experimental protocol, with Tg concentration varying from 1 to 10 μM. Two other cells were exposed to brief pulses of Tg (5 μM for 90 s and 1 μM for 180 s). In all cases the membrane potential records during Tg treatment or following Tg pulses were qualitatively similar to Fig. 2, with the silent phase progressively (over 30–60 min) depolarizing, eventually leading to continuous spiking. These results indicate that thapsigargin is irreversible and the stimulation of elevated electrical activity by Tg is critically dependent on the time required for Tg to take effect rather than the Tg concentration.

We will argue (see below) that Tg and ACh both depolarize the β-cell by emptying the ER of Ca^{2+}, thus activating I_{CRAC}. Their effects on the electrical activity of the cell differ in the extent to which they deplete the ER, Tg being more effective at the concentrations used. Since the effects of Tg are irreversible, its depolarization of the islet can only be attenuated by adding an outward current to oppose the depletion-activated inward I_{CRAC}. If our hypothesis is correct, it should be possible to bring the islet from the Tg-induced continuous spiking state (Fig. 2 *D*) to a state of muscarinic bursting by titrating diazoxide (Dz) into the Krebs solution, enhancing the outward $I_{K(ATP)}$.

When Dz was progressively titrated into the Krebs solution, a muscarinic-like burst pattern (cf. Fig. 4 *A*) was indeed generated, having properties quite different from the control bursts in Fig. 2 *A*. With Dz concentration of 22 μM (Fig. 2 *E*, *left*), the bursts were extremely rapid (10–16/min), and there was no hyperpolarization in the silent phase. In 50 μM Dz, the spiking ceased and the cell hyperpolarized. When Dz was removed, continuous spiking was restored (not shown).

The detailed mechanisms through which Tg inactivates the SERCA pumps are unknown. Nor is it known why Tg takes so long to act. For these reasons, we simulated the steady state behavior of the β-cell after Tg application (after all SERCA pumps are inactivated), by setting $v_{er,p} = 0$ (so that $J_{er,p} = 0$; see Appendix). We omit the long transient period during which pumps are presumably being inactivated. A control bursting pattern is shown in Fig. 3 *A*, while Fig. 3 *B* shows the electrical activity with $J_{er,p} = 0$. The depolarizing current responsible for taking the cell to continuous spiking is I_{CRAC}, which was activated by the depletion of ER Ca^{2+} accompanying the inactivation of the SERCA pumps.

Titration of Dz in the presence of Tg was also simulated, by stepping $\bar{g}_{K(ATP)}$ from control to progressively higher values. As in Fig. 2 *E*, this induced muscarinic bursting, with a short burst period and a depolarized silent phase (Fig. 3 *C*). When $\bar{g}_{K(ATP)}$ was made sufficiently large, all spiking ceased and the membrane hyperpolarized (Fig. 3 *D*). Tonic spiking resumed when Dz was removed ($\bar{g}_{K(ATP)}$ returned to control).

We also simulated the effect of partial blockage of the SERCA pump by reducing $v_{er,p}$ to a non-zero value. When $v_{er,p}$ is one-half its standard value, muscarinic bursting is produced (not shown), rather than continuous spiking (Fig. 3 *B*). When $v_{er,p}$ is 25% of its standard value, the model cell spikes continuously (not shown).

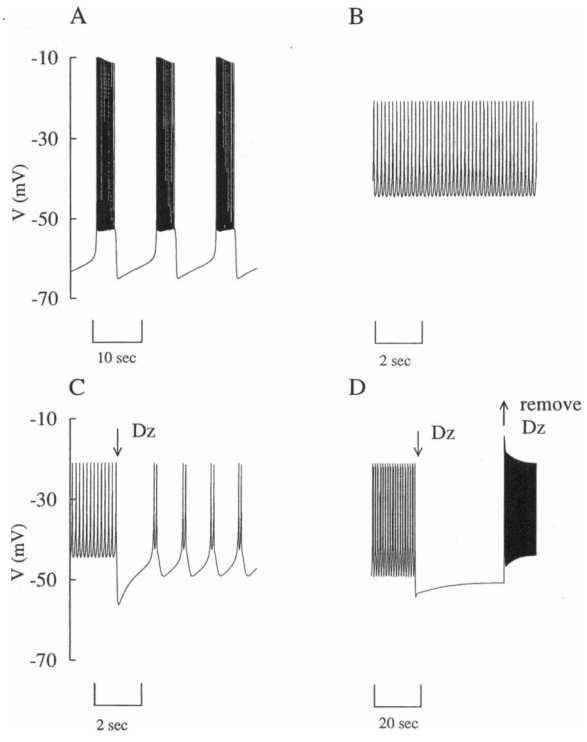

FIGURE 3 Simulated application of Tg to a bursting cell ($\bar{g}_{K(ATP)}$ = 150 pS, $IP_3 = 0$, $k_{Ca} = 0.1$). (*A*) Control. (*B*) Following inactivation of SERCA pumps, modeled by setting $J_{er,p} = 0$. Transients are omitted. (*C*) Addition of Dz, modeled by increasing $\bar{g}_{K(ATP)}$ to 250 pS, at the arrow. (*D*) Dz concentration increased ($\bar{g}_{K(ATP)}$ = 300 pS), at the first arrow. At the second arrow Dz is removed ($\bar{g}_{K(ATP)}$ returned to 150 pS).

We have observed experimentally (data not shown) that after preincubating an islet in Tg for 20 min or longer in the absence of glucose, adding stimulatory glucose (11.1 mM) to the medium leads to an immediate depolarization and continuous spiking. This was also shown by Worley et al. (1994) and is similar to what is observed when Dz is removed from an islet preincubated with both Tg and stimulatory glucose, as described above. Both procedures reduce $I_{K(ATP)}$ and, since I_{CRAC} is maximally activated due to previous Tg-induced blockade of the SERCA pumps, rapidly depolarize the cell to a tonic-spiking state.

Application of muscarinic agonists

Islet response to muscarinic agonists is glucose-dependent. In low glucose, agonists induce small depolarizations (Henquin et al., 1988; S. Bordin, A. C. Boschero, E. M. Carneiro, and I. Atwater, submitted for publication). In stimulatory glucose, low agonist concentrations lead to an increased burst frequency. High agonist concentrations induce a multiphasic response (Fig. 4 *A* and Sánchez-Andrés et al., 1988; S. Bordin, A. C. Boschero, E. M. Carneiro, and I. Atwater, submitted for publication). First, the cell often spikes with high frequency during one last burst. This is followed by membrane hyperpolarization, which can last for tens of seconds. This hyperpolarization is accompanied by a decrease

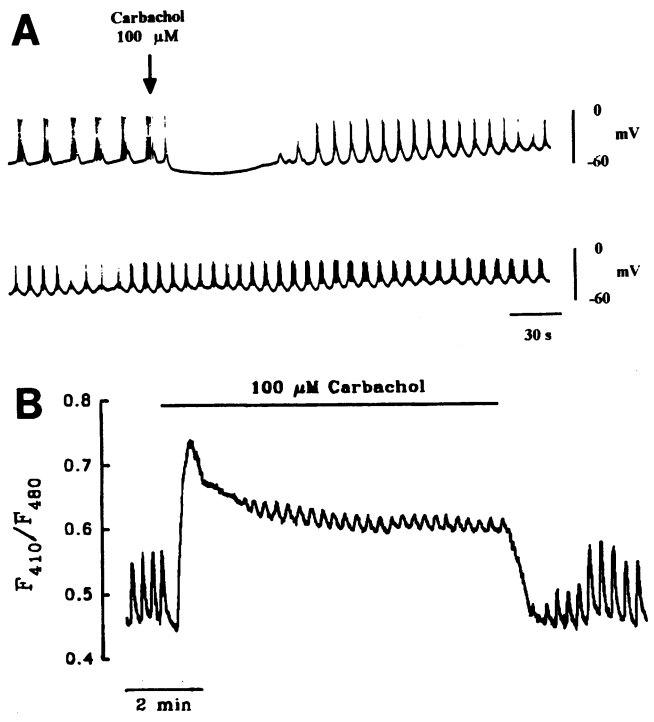

FIGURE 4 Effects of muscarinic agonist on membrane potential and Ca_i. (*A*) Continuous recording of the effects of 100 μM carbamylcholine (carbachol) on glucose-induced electrical activity. Carbamylcholine was added as indicated by the arrow and removed before the lapse. Lapse: 15 min. Glucose concentration was 11 mM throughout the recording. (*B*) Effect of 100 μM carbamylcholine on glucose-induced Ca_i oscillations in a single islet of Langerhans. Glucose concentration was 11 mM throughout the recording. Ca_i is expressed as the ratio between the fluorescence emission at two wavelengths, 410 nm and 480 nm. According to calibration estimates, peak Ca_i during glucose-induced bursting is 0.23 μM and during muscarinic bursting is 0.3 μM. The overall peak is 0.45 μM.

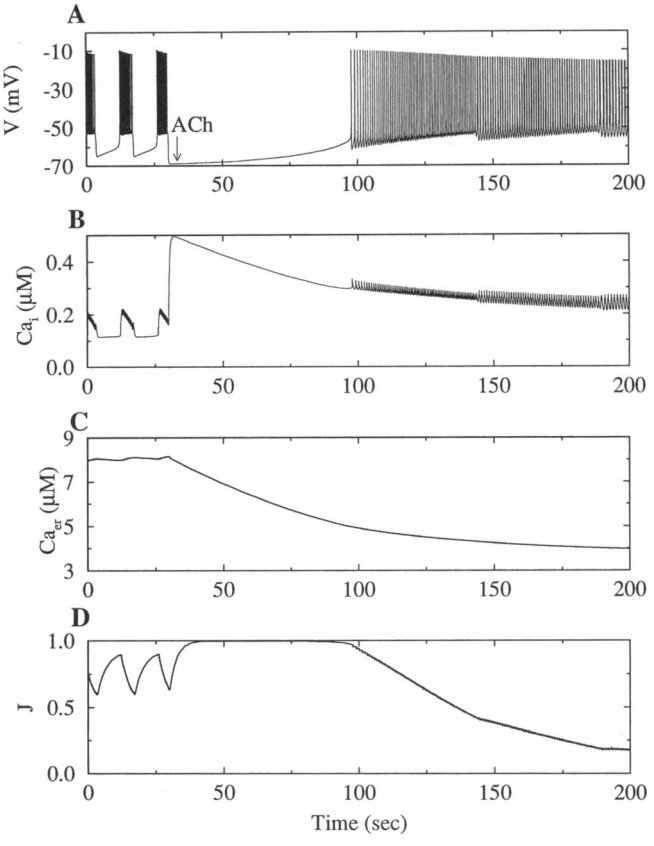

FIGURE 5 Simulated application of a muscarinic agonist. $\bar{g}_{K(ATP)}$ = 150 pS throughout. At the arrow, IP_3 concentration was increased from 0 to 0.6 μM. I_{CRAC} (not shown) follows Ca_{er}. During the silent phase of glucose-induced bursting, I_{CRAC} = −0.1 pA. During the silent phase of muscarinic bursting, I_{CRAC} = −1.2 pA. Ca_i, rather than *j*, drives muscarinic bursting.

in the input resistance (unpublished observations by J. Sánchez-Andrés and B. Soria). Then there is a slow depolarization and the cell enters a muscarinic bursting state, consisting of short high frequency bursts with slower rising and falling phases than glucose-induced bursts and depolarized silent phases. Muscarinic bursting persists as long as agonist is present.

Accompanying this response in membrane potential is a multiphasic Ca_i response (Fig. 4 *B*). This consists of an initial large increase in Ca_i, followed by a partial decay to a plateau concentration well above the average in stimulatory glucose alone. Superimposed on this plateau are small oscillations corresponding to muscarinic bursts. We believe the initial rise in Ca_i is due mainly to release from intracellular stores. The decrease in Ca_i from this peak value presumably corresponds both to the decreased flux of Ca^{2+} from intracellular stores as they empty and the cessation of Ca^{2+} influx across the cell membrane when hyperpolarized. The final, sustained phase is, we believe, due to influx accompanying muscarinic bursting.

Application of a muscarinic agonist was simulated in our mathematical model by increasing IP_3 concentration, which produced multiphasic responses in membrane potential and Ca_i (Fig. 5). Ca^{2+} channels in the ER membrane are activated by the IP_3, causing a large initial release of Ca^{2+} from the ER, which activates $I_{K(Ca)}$. The resulting hyperpolarization shuts off Ca^{2+} influx through voltage-dependent plasma membrane channels, causing Ca_i to decrease. As Ca_i decreases, deactivating $I_{K(Ca)}$, so too does Ca_{er} (Fig. 5 *C*), activating I_{CRAC}, with the combined effect of depolarizing the membrane above spike threshold. This leads to a transient period of tonic spiking, followed by muscarinic bursting.

Whereas glucose-induced bursting in our model is driven by slow oscillations in the inactivation variable *j*, muscarinic bursting is driven by significantly faster oscillations in Ca_i. The range of values over which Ca_i oscillated before agonist application was too low to significantly activate $I_{K(Ca)}$. With the addition of agonist, average Ca_i is elevated and $I_{K(Ca)}$ is sufficiently activated to influence the membrane and drive the burst. I_{CRAC} provides a depolarizing background current, which opposes $I_{K(Ca)}$ and prevents the membrane from hyperpolarizing. The inactivation variable *j*, which varies on a longer time scale, changes little over a muscarinic burst period and plays no role in driving the muscarinic burst (Fig. 5 *D*).

When this simulation is repeated using a smaller value of $\bar{g}_{K(Ca)}$ (not shown), two qualitative changes in the *V* record are evident: the duration of the initial hyperpolarization is re-

duced, and the cell evolves to a state of continuous spiking, rather than muscarinic bursting. Under these conditions, muscarinic bursting occurs only transiently, between the hyperpolarization and the tonic spiking.

Biphasic response to glucose

There is a characteristic biphasic response of β-cell electrical activity to application of stimulatory glucose. The islet depolarizes from rest to a state of continuous spiking lasting for a minute or more before synchronized bursting begins (Meissner and Atwater, 1976). The initial tonic electrical activity is accompanied by a sustained elevation of Ca_i (Roe et al., 1993). The first phase of spiking and elevated Ca_i has not been adequately explained with earlier models, which produce at best short initial phases. Simulations with the present model, however, produce a significant first phase (Fig. 6).

We propose that the first phase is due to an initial excess of I_{CRAC}. In low glucose the ER is largely depleted of Ca^{2+}, so that when glucose is added ($\bar{g}_{K(ATP)}$ reduced) I_{CRAC} is large, which, along with the reduction of $I_{K(ATP)}$, depolarizes the cell into continuous spiking. The resulting influx of Ca^{2+} raises both Ca_i and Ca_{er}, deactivating I_{CRAC} until it is insufficient to maintain tonic electrical activity, and the cell settles into a bursting state.

Our simulation does not show the decrease in Ca_i before the onset of electrical activity that is observed experimentally. This decrease is attributed to a metabolic increase in Ca^{2+} pump activity, which precedes the block of K(ATP) current (Roe et al., 1993; Nadal et al., 1994). We have simulated this with the model by increasing Ca^{2+} pump flux parameters before decreasing $\bar{g}_{K(ATP)}$, but do not show it here so as to emphasize that the first transient phase of spiking can be accounted for solely by I_{CRAC}. Our simulations show that the increase in Ca_{er} resulting from the increased SERCA pump rate is small, and its effect through I_{CRAC} on the duration of the first phase of spiking is marginal.

The dramatic rise in Ca_i after application of stimulatory glucose is similar in appearance to the initial rise induced by ACh. Why, then, is the electrical activity so different? First, there is a difference in the magnitude of the rise in Ca_i. The higher Ca_i induced by ACh activates more $I_{K(Ca)}$ than is activated by glucose. Next, because the cell was bursting at the time when ACh was applied (Fig. 5), Ca_{er} was high and I_{CRAC} was almost completely deactivated, leaving the hyperpolarizing current $I_{K(Ca)}$ largely unopposed. In contrast, Ca_{er} was low before the application of glucose, so that I_{CRAC} was present to counterbalance, and overwhelm, $I_{K(Ca)}$. Thus, the duration of the initial ACh hyperpolarization is determined primarily by the decay rate of Ca_i from its high peak value, whereas the duration of the initial glucose-induced depolarization is determined primarily by the ER filling rate.

A reverse biphasic response is sometimes observed upon stepping glucose from one stimulatory concentration to a lower, but still stimulatory, concentration. After this reduction, the bursting cells hyperpolarize for up to several minutes before bursting is resumed (Beigelman et al., 1977; Cook, 1984; Henquin, 1992). Insulin secretion is also transiently reduced after reduction in glucose (Grodsky et al., 1967). According to our model, this biphasic response in electrical activity is again related to I_{CRAC}. In high glucose, Ca_{er} is elevated and I_{CRAC} almost completely deactivated. If glucose concentration is subsequently reduced to an almost substimulatory level, where I_{CRAC} becomes necessary to bring the membrane potential to spike threshold, the membrane will hyperpolarize until Ca_{er} falls far enough to activate I_{CRAC}.

In summary, we argue that the initial tonic spiking following glucose application and the initial hyperpolarization following reduction of glucose are caused by an excess or a deficit, respectively, of I_{CRAC}. If glucose concentration is ramped slowly enough for Ca_{er} to adjust to the new conditions, our model indicates that the transients will be eliminated. This was in fact observed experimentally when glucose concentration was ramped from 0 to a stimulatory concentration (Beigelman et al., 1977). The first phase of elevated insulin secretion was also eliminated when glucose concentration was slowly ramped, rather than abruptly increased, to a stimulatory concentration (Grodsky, 1972).

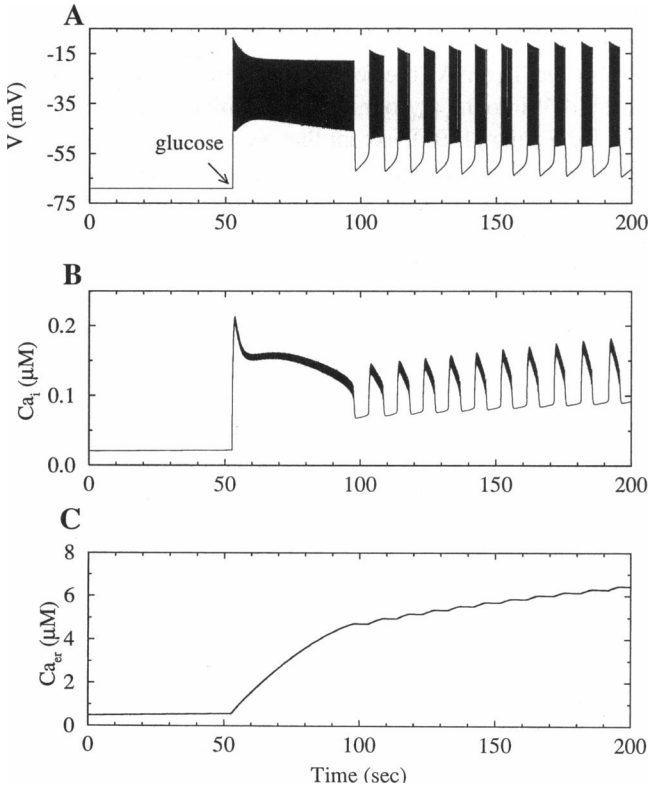

FIGURE 6 Biphasic response to glucose, simulated by decreasing $\bar{g}_{K(ATP)}$ from 5000 to 150 pS, at the arrow. I_{CRAC} = −4.5 pA before glucose application.

DISCUSSION

We have shown that the SERCA pump blocker Tg depolarizes pancreatic islets in the presence of stimulatory glucose (Fig. 2), consistent with the hypothesis that a depolar-

izing current, which is activated by depletion of ER calcium, is present. The heightened electrical activity induced by Tg was maintained even after the drug was removed (Fig. 2 *D*, *right*). Cells that were given only brief exposures to Tg also exhibited elevated electrical activity after 30–60 minutes, indicating that considerable time is required for Tg to empty the intracellular Ca^{2+} stores. In Fig. 2 *C* there remains a well defined silent phase in 5 μM Tg. However, almost immediately after Tg was removed, the silent phase ceased to hyperpolarize, and the electrical activity became nearly continuous. The same effect was seen in four other cells, indicating that in addition to emptying the intracellular Ca^{2+} stores, Tg may have some hyperpolarizing effect on the cell membrane, which is reversible upon removal of the drug.

We next showed that carbamylcholine, a muscarinic agonist, produces a multiphasic response in β-cell membrane potential and cytosolic Ca^{2+} concentration in the presence of stimulatory concentrations of glucose (Fig. 4). The first phase is characterized by a dramatic rise in Ca_i, which we believe is due mainly to release of Ca^{2+} from intracellular stores. This is followed by membrane hyperpolarization, during which Ca_i falls toward a plateau level. We attribute this hyperpolarization to Ca^{2+} activation of a K(Ca) current. Finally, the cells enter a muscarinic bursting state with short period and depolarized silent phase. This bursting is accompanied by oscillations in Ca_i due solely to influx through plasma membrane Ca^{2+} channels, which range over much higher concentrations than in glucose-induced bursting.

The transient hyperpolarization after application of a muscarinic agonist has been observed before (Sánchez-Andrés et al., 1988; Santos and Rojas, 1989), but is not always present (Cook et al., 1981). There appears to be a relationship between the duration of the hyperpolarization and the agonist concentration, with longer hyperpolarizations accompanying greater agonist concentrations (the authors, unpublished observation). Another feature often observed following application of agonist is a period of high-frequency spiking preceding the transient hyperpolarization. The ionic mechanism behind this is still unclear, and at present cannot be accounted for by the model. Differences in experimental conditions, such as different agonist perfusion rates, may be partly responsible for the response variability.

The effects of Tg and muscarinic agonists can be accounted for by the hypothesis that CRAC current is present in the β-cell. To show this, we constructed a theoretical model and simulated the application of the drugs (Figs. 3 and 5). This hypothesis also accounts for the differing effects of glucose and ACh on membrane potential: 1) Electrical activity can be initiated by increasing glucose concentration, whereas ACh can only depolarize the cell slightly in the absence of stimulatory glucose. This is consistent with the hypothesis that ACh activates a CRAC current that is much smaller than the K(ATP) current inhibited by glucose. 2) A transient hyperpolarization precedes muscarinic bursting, while a transient depolarization precedes glucose-induced bursting. The former is due to activation of $I_{K(Ca)}$ following release of ER Ca^{2+}, while the latter is due to excess CRAC current activated in low glucose when the ER is depleted of Ca^{2+}. 3) Increasing glucose concentration raises the plateau fraction of bursting, whereas adding ACh has little effect. The burst frequency is higher and the silent phase depolarized in muscarinic bursting. These are by-products of the different mechanisms driving glucose-induced bursting and muscarinic bursting. 4) Finally, our model predicts that addition of ACh lowers ER calcium concentration, while addition of glucose raises it.

Our belief that the Tg and ACh effects share a common mechanism, CRAC current, is supported by the observation that muscarinic-like bursting is obtained when Dz is added to an islet with prior exposure to Tg (Fig. 2 *E*). In our simulations (Fig. 3 *C*), bursting produced in this way is indeed "muscarinic", in that it is driven by variations in Ca_i acting through $I_{K(Ca)}$.

We showed that the biphasic response to glucose can be attributed to CRAC current, which is strongly activated in low glucose. The initial phase is terminated by the rise in Ca_{er}, and consequent deactivation of I_{CRAC}, which accompanies spiking. If glucose is slowly ramped up to a stimulatory level, then there should be no first phase. If the rise in Ca_{er} is inhibited by prior application of Tg, the first phase persists indefinitely.

Our explanation contrasts with the hypothesis of Roe et al. (1993) that the initial phase is the result of depolarization-triggered Ca^{2+} release from intracellular stores. In our simulations, the ER actually takes up Ca^{2+} after glucose application, and the initial increase in Ca_i is due solely to influx of Ca^{2+} through the plasma membrane. Indeed, one strength of our model is that it explains the simultaneous elevation of both average Ca_i and electrical activity during the first phase of the glucose response; without CRAC current the rise in Ca_i would tend to hyperpolarize the islet.

Can the Tg or ACh effects be explained without CRAC current? Without I_{CRAC} our model is similar to that of Keizer and De Young (1993), except that we assume instantaneous relaxation of the IP_3 inactivation variable to its steady state ($h = h_\infty(Ca_i)$). Keizer and De Young (1993) showed that their model is capable of displaying a wide range of behaviors due to IP_3-induced oscillations in the ER and cytosolic Ca^{2+} concentrations, but they did not address muscarinic bursting or the effects of Tg. We simulated the application of Tg and ACh with our model, setting $g_{CRAC} = 0$ to see if the effects of these drugs could be reproduced in the absence of I_{CRAC}. When the application of Tg was simulated, we found that the burst frequency increased dramatically, but there was no overall depolarization. The increased burst frequency is due to $I_{K(Ca)}$, which is activated by the higher levels of Ca_i attained in the absence of ER Ca^{2+} buffering. The absence of depolarization is to be expected, since the only plasma membrane current linked to Ca^{2+} is the inhibitory $I_{K(Ca)}$. Indeed, it is hard to envision any mechanism for the Tg-induced depolarization that does not include an excitatory current linked to ER or cytosolic calcium. When application of ACh was simulated, only a transient period of high-frequency bursting was seen, followed by normal bursting.

The claim that muscarinic agonists act through I_{CRAC} is further supported by data (Henquin et al, 1988, Fig. 3) showing that β-cells depolarized by as much as 5 mV when ACh was added

to a low glucose solution, this depolarization lasting as long as the agonist was present (~6 min). We argue that, unless this depolarization is transient, it is due either to a direct effect of ACh on a plasma membrane current (perhaps through G-protein coupling), or to activation of CRAC current. An alternative, that the depolarization represents a change in equilibrium membrane potential brought about by a Ca_i-dependent plasma membrane current, can be dismissed through a calcium-balance argument. Membrane potential and cytosolic Ca^{2+} are coupled through plasma membrane ion channels and pumps. Cytosolic Ca^{2+} and ER Ca^{2+} are coupled through an IP_3-activated Ca^{2+} current, SERCA pumps, and Ca^{2+} leakage. However, ER Ca^{2+} and membrane potential are directly coupled only if CRAC current is present. Hence, in the absence of CRAC current, equilibrium membrane potential and cytosolic calcium concentration are determined independently of Ca_{er} and any ER parameters (such as IP_3). This argument is independent of any mathematical model.

In our simulations we addressed the possibility that the Tg and ACh effects are mediated through G-protein coupling to plasma membrane channels, by adding a voltage-independent leakage current to the model, whose conductance is zero unless Tg or ACh is present. When application of either Tg or ACh was simulated, the model cell behaved as it did when I_{CRAC} was present. However, the leakage current is not activated in glucose alone, and thus does not account for the biphasic response to glucose. Experimentally, one could test whether CRAC current or an agonist-activated leakage current is involved in the Tg or ACh responses by applying the agents to an excised membrane patch. If this results in an opening of ion channels, then this is evidence for an agonist-activated leakage mechanism. (This experiment is inconclusive if no channels are opened, since this could be explained either by the absence of an ER or the absence of G proteins.)

In this paper we have investigated the implications of a hypothetical CRAC current in β-cells with a minimal mathematical model. We assumed that slow inactivation of a Ca^{2+} current is responsible for driving glucose-induced bursting, but the results do not depend upon the specific burst mechanism. Other burst mechanisms, such as slow modulation of a K^+ current, could be incorporated into the model with little impact on the agonist-induced behaviors. We have done this, for example, with a model driven by slow oscillations in K(ATP) current (Smolen and Keizer, 1992). This is an important point, since the mechanism behind glucose-induced bursting is still a matter of debate (Satin and Smolen, 1994). We have also assumed that a voltage-independent K(Ca) current is present in the β-cell and is important in muscarinic bursting, but the role of this current in muscarinic bursting could be played by a voltage-dependent K(Ca) current. Similarly, the results do not depend upon our choice of the ER Ca^{2+} handling model. Other models, such as that due to Keizer and De Young (1993) or even a linear ER model, would be equally effective. Although there is now evidence supporting the presence of CRAC current in the β-cell (Silva et al., 1994; Worley et al., 1994; Leech et al., 1994), it has not been well characterized. Therefore, we have made several assumptions regarding the conductance and ER calcium sensitivity of this channel, as well as typical levels of free Ca^{2+} concentration in the ER. If future measurements of one quantity, such as ER Ca^{2+} concentration, show one of our assumptions to be invalid, this does not necessarily invalidate our model since other parameters, such as the sensitivity of the CRAC channel, can be adjusted to account for the new data. However, future data can invalidate the model if it shows, for instance, that ER Ca^{2+} concentration is much greater than the activation level for I_{CRAC}. It is our hope that the explanatory potential of the CRAC current demonstrated in this paper will motivate further experimentation to test some of the predictions made with the model and to identify other behaviors that can be attributed to this current.

APPENDIX

In our computations we use an alternate form of the calcium handling equations (Eqs. 8 and 9):

$$\frac{dCa_i}{dt} = \frac{1}{\lambda}\left[\left(\frac{P_{leak}}{P_{ip3}} + O_\infty\right)(Ca_{er} - Ca_i) - \frac{J_{er,p}}{P_{ip3}}\right] + \frac{J_{mem}}{V_{i,eff}} \quad (15)$$

$$\frac{dCa_{er}}{dt} = -\frac{1}{\sigma\lambda}\left[\left(\frac{P_{leak}}{P_{ip3}} + O_\infty\right)(Ca_{er} - Ca_i) - \frac{J_{er,p}}{P_{ip3}}\right]. \quad (16)$$

Effective volumes are defined as $V_{i,eff} = V_i/f_i$ and $V_{er,eff} = V_{er}/f_{er}$. Several parameters have been combined into the time scale parameter, $\lambda = V_{i,eff}/P_{ip3}$, and the effective volume ratio, $\sigma = V_{er,eff}/V_{i,eff}$.

$O_\infty = a_\infty b_\infty h_\infty$ is the fraction of open IP_3-activated Ca^{2+} channels, where $a_\infty = Ca_i/(Ca_i + 0.1)$, $b_\infty = IP_3/(IP_3 + 0.2)$, and $h_\infty = 0.4/(Ca_i + 0.4)$. SERCA pump flux is expressed as a Hill function, $J_{er,p}/P_{ip3} = v_{er,p}\, Ca_i^2/(Ca_i^2 + 0.09^2)$, where $v_{er,p} = 0.24$ μM.

Other parameter values used in the calcium handling equations are: $\lambda = 250$ ms, $P_{leak}/P_{ip3} = 0.02$, $f_i = 0.01$, $\alpha/V_i = 3.6 \times 10^{-6}$ fA^{-1} μM ms^{-1}, $k_{Ca}/V_i = 0.07$ ms^{-1}, $\sigma = 5$, and $V_{i,eff} = 7.19 \times 10^6$ μm^3. For more details on the ER calcium handling model, see Li and Rinzel (1994).

Ionic current infinity functions have the form $z_\infty = 1/(1 + \exp[(vz - V)/sz])$: $vmf = -20$, $smf = 7.5$, $vms = -16$, $sms = 10$, $vj = -53$, $sj = -2$, $vn = -15$, $sn = 6$ (mV). The CRAC current activation function is $r_\infty = 1/(1 + \exp[Ca_{er} - 3])$. Time constants are $\tau_n = 4.86/(1 + \exp[(V + 15)/6])$ and $\tau_j = (5 \times 10^4)/(\exp[(V + 53)/4] + \exp[-(V + 53)/4]) + 1.5 \times 10^3$ (ms).

We assume a cell radius of 7 μm, so $C_m = 6158$ fF. Maximum ionic current conductances are $\bar{g}_{Caf} = 810$, $\bar{g}_{Cas} = 510$, $\bar{g}_K = 3900$, $\bar{g}_{K(Ca)} = 1200$, $\bar{g}_{CRAC} = 75$ (pS). Reversal potentials are $V_{Ca} = 100$, $V_K = -70$, $V_{CRAC} = 0$ (mV). Values for the parameters $\bar{g}_{K(ATP)}$, k_{Ca}/V_i, and IP_3 are given in figure legends.

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