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Role of Voltage-Dependent Ionic Currents in Coupling Glucose Stimulation to Insulin Secretion in Canine Pancreatic Islet B-Cells

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Summary. Glucose-induced electrical activity in canine pancreatic islet B cells is distinct from that in rodent islets, though both display Ca^{2+}-dependent insulin secretion. Rodent islet B cells undergo regular bursts of Ca^{2+}-dependent action potentials, while canine islet B cells generate isolated Na^+-dependent action potentials which often give way to a plateau depolarization. Here we present evidence to reconcile the species difference in electrical activity with the similarity of Ca^{2+} dependence of secretion. (i) In canine B cells increasing glucose concentrations produce membrane depolarization and increasing frequency of Na_o-dependent action potentials until a background membrane potential (~ -40 mV) is reached where Na^+ currents are inactivated. (ii) Voltage-dependent Ca^{2+} currents are present which are activated over the voltage excursion of the action potential (-50 to $+20$ mV) and inactivate slowly, (over seconds) in the range of the plateau depolarization (-40 to -25 mV). Hence, they are available to contribute to both phases of depolarization. (iii) Tetrodotoxin (TTX) reduces by half an early transient phase of glucose-stimulated insulin secretion but not a subsequent prolonged plateau phase. The transient phase of secretion often corresponds well in time to the period of initial high frequency action potential activity. These latter results suggest that in canine B cells voltage-dependent Na^+ and Ca^{2+} currents mediate biphasic glucose-induced insulin secretion. The early train of Na^+-dependent action potentials, by transiently activating Ca^{2+} channels and allowing pulsatile Ca^{2+} entry, may promote an early transient phase of insulin secretion. The subsequent sustained plateau depolarization, by allowing sustained Ca^{2+} entry, may permit steady insulin release.

Key Words pancreatic islet B cells · islet cell electrophysiology · stimulus-secretion coupling · Na^+ current · Ca^{2+} current · K^+ current

Introduction

Most studies of the molecular events in nutrient-induced insulin secretion have been performed in rodent pancreatic islet B cells or rodent insulinoma cells. From these studies, a plausible "metabolic stimulation" hypothesis has emerged. That is, nutrient metabolism produces a reduction in resting K^+ permeability which results in cell depolarization. Cell depolarization, in turn, leads to action potential generation and the entry of Ca^{2+} via voltage-dependent Ca^{2+} channels. The resultant rise in cytosolic Ca^{2+} stimulates Ca_i^{2+}-induced exocytosis of insulin granules (*see* Ashcroft & Rorsman, 1991, for review). The generalized applicability of this scheme to B cells in other mammals has not been widely tested.

In our initial investigation of canine islet B cell function (Pressel & Misler, 1990), we were impressed that while glucose-stimulated insulin release is biphasic and dependent on Ca_o^{2+}, much as it is in rodents, the pattern of glucose-induced electrical activity was quite distinct. When exposed to concentrations of glucose which provoke insulin secretion, rodent B cells characteristically display regular "bursts" of Ca_o^{2+}-dependent action potentials riding on periodic plateau depolarizations of -40 to -30 mV. In contrast, canine B cells exposed to similar concentrations of glucose more typically display unitary or brief, irregular clusters of large amplitude Na_o^+-dependent action potentials which arise from membrane potentials between -55 and -40 mV. With prolonged exposure to glucose or a hypoglycemic sulfonylurea, the action potentials often give way to a sustained plateau depolarization averaging -35 to -25 mV; at the latter range of V_m, Na^+ currents show complete steady-state inactivation.

We proposed that the Na^+ action potentials and the sustained plateau depolarization could underlie glucose-induced insulin secretion, *if* several conditions prevailed. (i) Glucose results in concentration-dependent reductions in resting K^+ conductance and baseline membrane potential (V_m). (ii) The frequency of Na_o^+-dependent action potentials is graded with glucose concentration and, therefore, V_m, until a V_m is reached where Na^+ currents are largely inactivated. (iii) Membrane depolarization corresponding to the excursion of the action potential triggers high threshold, voltage-dependent Ca^{2+} currents.

These Ca^{2+} currents inactivate over many seconds at membrane potentials corresponding to the sustained plateau depolarization. Under these conditions intermittent activation of Ca^{2+} currents by large excursion Na^+ action potentials could contribute to linking glucose-dependent depolarization to Ca^{2+} entry during the transient "first phase" of glucose-induced insulin secretion. Likewise, maintained activation of the Ca^{2+} current at the plateau depolarization could maintain steady-state Ca^{2+} entry and contribute to the sustained "second phase" of insulin secretion.

In this paper we present electrophysiological data obtained with the "perforated-patch" variant of whole-cell recording (Horn & Marty, 1988) as well as insulin secretion data obtained with whole islet perifusion. Both lines of evidence strongly suggest that the aforementioned conditions are satisfied. "Perforated-patch" recording proved most advantageous for these studies because it maintains the metabolic responsiveness of B cells and the stability of Ca^{2+} currents in B cells far longer than "conventional" whole-cell recording (Falke et al., 1989). It also permits exchange of intracellular K^+ with Cs^+ (Korn & Horn, 1989), hence reducing the contamination of small amplitude Ca^{2+} currents with K^+ currents. Our results suggest that beneath the obvious differences in electrical activity patterns of rodent and canine B cells, lay common, fundamental mechanisms of Ca^{2+}-mediated stimulus-secretion coupling (in particular, high threshold, voltage-activated Ca^{2+}-channels).

Parts of this work have been reported in abstract form (Pressel & Misler, 1991).

Materials and Methods

Islet Preparation

The islets used in this study were a generous gift from the Islet Transplantation Laboratory, Washington University (David Scharp, Director). Pancreata were harvested from mongrel dogs. Islets were isolated using an automated protocol nearly identical to that previously described for human cadaver pancreases (Ricordi et al., 1988). Aliquots from most islet isolates used for electrophysiological studies responded to secretogogue concentrations of glucose with an increase in insulin release (*see* section below for perifusion details).

Electrophysiological Recording

The methods used to record from islets were nearly identical to those described in a prior study (Pressel & Misler, 1990). Most cells were recorded from using the "perforated-patch" variant of whole-cell recording (Horn & Marty, 1988) as previously applied to islet cells (Falke et al., 1989). Glass micropipettes were filled by immersion of the tip in a "high K^+" or a "high Cs^+" buffer solution and then backfilling with the same solution containing in addition 150–300 μg/ml nystatin (Sigma, St. Louis, MO). The "high K^+" buffer solution contained (in mM): 67.5 KCl, 28.4 K_2SO_4, 11.8 NaCl, 1 $MgCl_2$, 0.5 EGTA, 47.2 sucrose, 20 HEPES titrated to pH 7.3 with KOH. The "high Cs^+" buffer solution contained (in mM): 67.5 CsCl, 28.4 Cs_2SO_4, 11.8 NaCl, 1 $MgCl_2$, 0.5 EGTA, 47.2 sucrose, 20 HEPES titrated to pH 7.3 with tetraethylamine hydroxide.

Immediately after giga-ohm seal formation, cell-attached patch recording permitted physiological identification of a given cell as a B cell (*see below*). Within several minutes of seal formation, the patch of membrane permeabilized due to the insertion, into the membrane, of nystatin molecules present in the pipette solution. The progressive development of electrical continuity between the pipette solution and the cytoplasm was monitored as a drop in access resistance (R_a) from the pipette to the interior of the cell, using the transient compensation circuitry of the EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, FRG).

Current-clamp recording was begun after R_a fell to less than 100 MΩ; voltage-clamp recording was begun after R_a fell to less than 40 MΩ (see *Note Added in Proof*). Steady-state membrane conductance (G_m) was calculated, over a variety of metabolic conditions, from the change in membrane potential (V_m) during the injection of a current (I) (usually −5 pA) using Eq. (1)

$$G_m = I/(\Delta V - IR_a) \quad (1)$$

and the value of R_a read off the the amplifier. By determining G_m from current-clamp recordings, rather than from intermittent voltage-clamp recordings, we were able to avoid the resetting of the cell's spiking pattern often seen on switching between current-clamp and voltage-clamp modes. This permitted stable recording of electrical activity over long time intervals. R_a was assessed intermittently during the course of such an experiment to limit the error in estimating G_m in the face of continuing slow permeabilization of the membrane.

Ca^{2+} currents were recorded using the EPC-9 patch-clamp amplifier which performs on-line leak current subtraction using a P/N pulse protocol. In our experiments the test pulse was followed by four voltage-clamp steps of one-fourth the amplitude of the test step. The four voltage-clamp steps were applied from a hyperpolarized holding potential where there would be no contamination of the leak current with voltage-activated currents. Leak currents measured during the four voltage-clamp steps were summed and subtracted from the test current.

Conventional "whole-cell" recording (Hamill et al., 1981), performed with minimal series resistance compensation, was used in those few experiments where detailed analysis of the current-voltage characteristics of large amplitude (nanoampere) voltage-dependent K^+ currents was attempted. In those experiments the pipette was filled with an "intracellular-like" buffer solution containing (in mM): 144 KCl, 2 $MgCl_2$, 3 Mg_2ATP, 5 EGTA, 20 HEPES titrated to pH 7.3 with KOH.

For all experiments the standard extracellular solution (ES) contained (in mM): 144 NaCl, 5.5 KCl, 2.0 $CaCl_2$, 1 $MgCl_2$, and 20 HEPES titrated to pH 7.3 with NaOH. The composition of the ES was altered by isosmotically substituting a portion of the NaCl content with the test substance or with an isosmotic NaCl solution containing a dilute concentration of the test substance. Unless otherwise indicated, the recordings were made at room temperature (21–23°C) rather than body temperature; this enhanced the mechanical stability of the patches and prolonged the viability of cells subjected to perforated-patch recording.

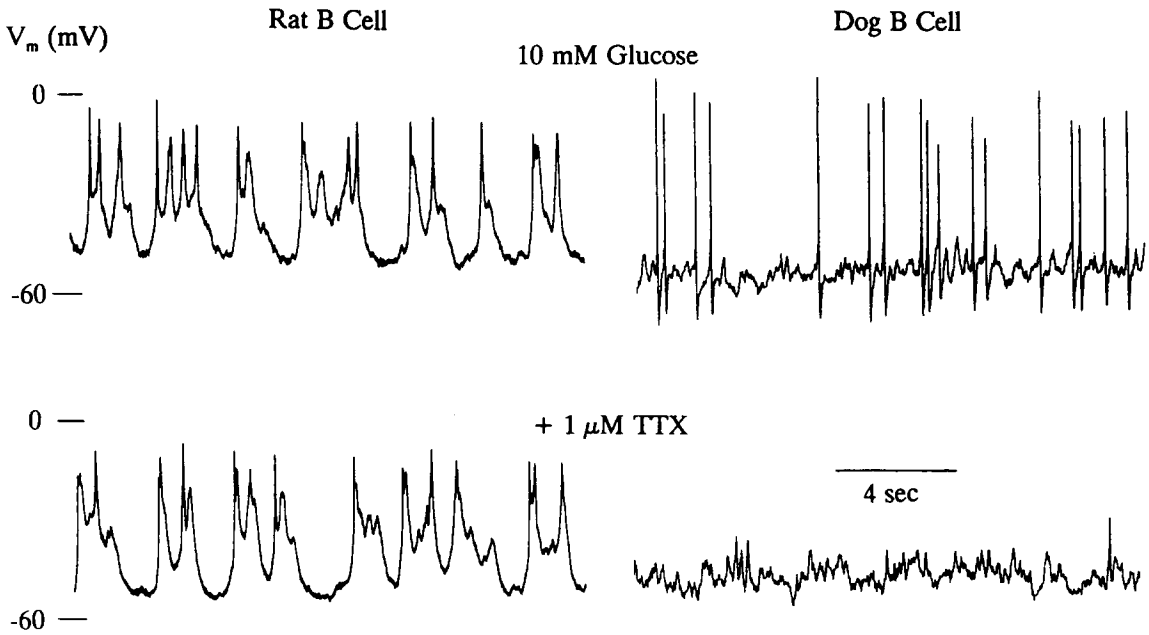

Fig. 1. Glucose-induced electrical activity recorded from rat and canine B cells in the "perforated-patch" mode. *Left panel:* electrical activity in a rat B cell showing lack of effect of tetrodotoxin on action potentials. *Right panel:* Na^+-dependent action potentials seen in canine islets and blocked by tetrodotoxin.

We recorded from isolated islet cells or single islet cells within an islet clump. Cells were "physiologically" identified as B cells during the initial cell-attached patch recording. Two approaches were used. (i) When a "high K^+" buffer solution was used in the pipette, we identified 65-pS metabolically regulated, ATP_i-sensitive K^+ channels which are present in insulin-secreting B cells but not glucagon-secreting A cells of the islet (Rorsman & Hellman, 1988). At 0 mV pipette potential, these channels were identified as 3.5–4.5 pA inward current pulses whose frequency is increased by addition to the bath of 3 mM sodium azide (NaN_3), a rapidly acting and reversible mitochondrial inhibitor. This criterion was developed during our single-channel studies with rat and human islet cells (Misler et al., 1986, 1989). (ii) When a "high Cs^+" buffer solution was used in the pipette, we determined whether the cell under investigation displayed action currents in 5–10 mM glucose and whether the electrical activity is blocked by NaN_3, as might be expected for a B cell. Alternatively, if no spiking activity was apparent, outward currents through the K^+ (ATP) channel were recorded when the patch was depolarized by 110 mV. If single-channel activity increased on addition of NaN_3 to the bath, the cell was identified as a B cell. When a single cell within a small clump of islet cells was voltage clamped to examine membrane currents, care was taken to ensure that the cell was not tightly coupled to its neighbors. That is, we determined that the cell had a C_m of 4.0 to 10.0 pF, which is within the range of values seen in a single B cell and could be well space clamped (Pressel & Misler, 1990).

Measurement of Insulin Secretion

Insulin secretion was measured by in vitro perifusion of isolated islets. After isolation and purification, islets were cultured overnight at 37°C. Equal aliquots of the islet cell suspensions usually containing 75–100 islets were loaded into paired chambers containing a cellulose acetate Millipore filter (8 μm). The chambers were perifused in a 37°C water bath with a solution containing (in mM): 115 NaCl, 24 $NaHCO_3$, 5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 1.0 isobutylmethylxanthine (IBMX) and 0.5% RIA grade albumin. The solution was oxygenated with 95% O_2/5% CO_2. The perifusion flow rate was ~1 ml/min, and effluent from each islet-containing chamber was collected by hand at intervals of from 1–5 min. The samples were frozen and subsequently insulin was measured by the Diabetes Research and Training Center Radioimmunoassay Core Facility at our institution.

Results

Glucose-Induced Depolarization and Action Potential Activity in Canine Islet B Cells

Figure 1 presents the basic observations of glucose-induced electrical activity in rat and canine B cells which prompted this investigation. Perforated-patch recordings were made on islet cells, identified as B cells by the presence of metabolically regulated, ATP_i-sensitive K^+ channels. On exposure to concentrations of glucose which induce insulin secretion, B cells from both species depolarize and generate action potentials even at room temperature. Rat islet B cells display (*see* left panel) short bursts of two to five small amplitude, TTX-resistant action potentials riding on periodic plateau depolarizations of −40 to −30 mV (10/17 cells tested). This electri-

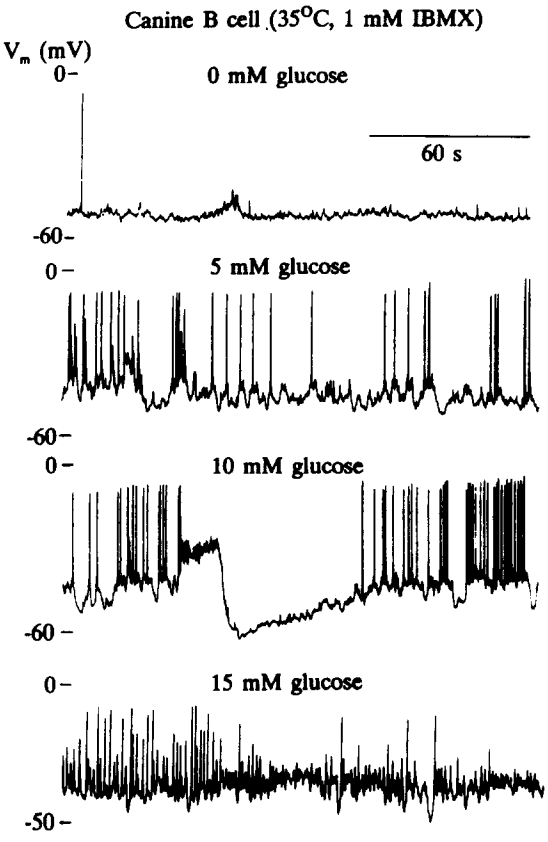

Fig. 2. Glucose-induced electrical activity recorded in the "perforated-patch" mode from a "physiologically identified" B cell in an islet clump maintained at 35°C in the presence of 1 mM IBMX. The pipette contained a "high K^+" buffer with nystatin. Each trace was recorded 5–8 min after bath change to the indicated solution. Note that stepwise increases in extracellular glucose results in increasing cell depolarization and action potential activity. However, "spike" activity is not clustered into regular "bursts" and ultimately degenerates to a depolarized plateau or "wobble" in 15 mM glucose.

cal activity is similar to that seen in mouse islet cells during conventional intracellular recording at subphysiologic temperatures. In contrast, canine islet B cells typically display (*see* right panel) short regular clusters of large amplitude, rapid upstroke Na_o^+-dependent, TTX-blockable action potentials with prominent afterhyperpolarizations (29/38 cells tested). These action potentials arise from a noisy baseline membrane potential of −55 mV. In dog islet cells continuously exposed to a glucose concentration of greater than 10 mM, discrete action potential activity often gives rise to a sustained plateau depolarization from which aborted action potentials occasionally arise (*see* Figs. 2 and 10*b*). In rat, the bursting pattern persists, though individual plateau durations may increase. These interspecies differences were evident even when recording from single cells.

The working hypothesis which arose from these observations is that in canine islet cells Na_o^+-dependent action potentials and the sustained plateau depolarization could contribute to graded and biphasic glucose-stimulated, Ca_o^{2+}-dependent insulin secretion, if at least three conditions were met. (i) Glucose results in concentration-dependent reductions in resting K^+ conductance and, thereby, membrane depolarization. (ii) Action potential activity is graded with glucose concentration and, therefore, the membrane potential, V_m. (iii) Membrane depolarization to voltages positive to −50 mV activated slowly inactivating Ca^{2+} currents. Figures 2 through 4 present evidence that the first two conditions are largely met.

Figure 2 depicts the outstanding features of glucose-induced electrical activity of canine islet cells recorded at near physiological temperature (35°C) and in the presence of 1 mM IBMX, two conditions which optimize glucose-dependent insulin secretion. Note that raising bath glucose concentration over the range of 0–10 mM produces progressive membrane depolarization (from −55 to −45 mV) and greatly enhances action potential activity. The action potentials are isolated or occur in short clusters but no organized or periodic burst of action potentials is seen. Note that at 15 mM glucose, when the underlying V_m reaches values less than −40 mV, action potentials become aborted. This is probably due to the inherent time-dependent inactivation of Na^+ channels at this steady-state voltage. At this time electrical activity takes on the characteristic of a plateau depolarization.

We examined, in more detail, the graded natures of glucose-induced electrical activity and the decrease in background K^+ conductance which permits action potential triggering. Experiments were performed at room temperature where stable perforated-patch recording was routinely possible for many minutes. Figure 3 depicts the results of a set of six experiments performed under current-clamp conditions. Note that raising glucose from 0–3 to 10–20 mM results in a progressive decrease in resting membrane conductance (G_m) measured as the membrane hyperpolarization caused by a current pulse of −5 pA (*see* Fig. 3*a*). Concomitant with this are a progressive membrane depolarization (Fig. 3*b*) and, in most cases, a progressive rise in action potential frequency (Fig. 3*c*). At the higher concentrations of glucose, addition of tolbutamide, a sulfonylurea which specifically targets the K^+(ATP) channel (Trube, Rorsman & Ohno-Shosaku, 1986; Gillis et al., 1989), rapidly results in a further decrease in G_m and further depolarization; this is usually accompanied by the termination of recognizable spike activity and the onset of the plateau depolarization. In the

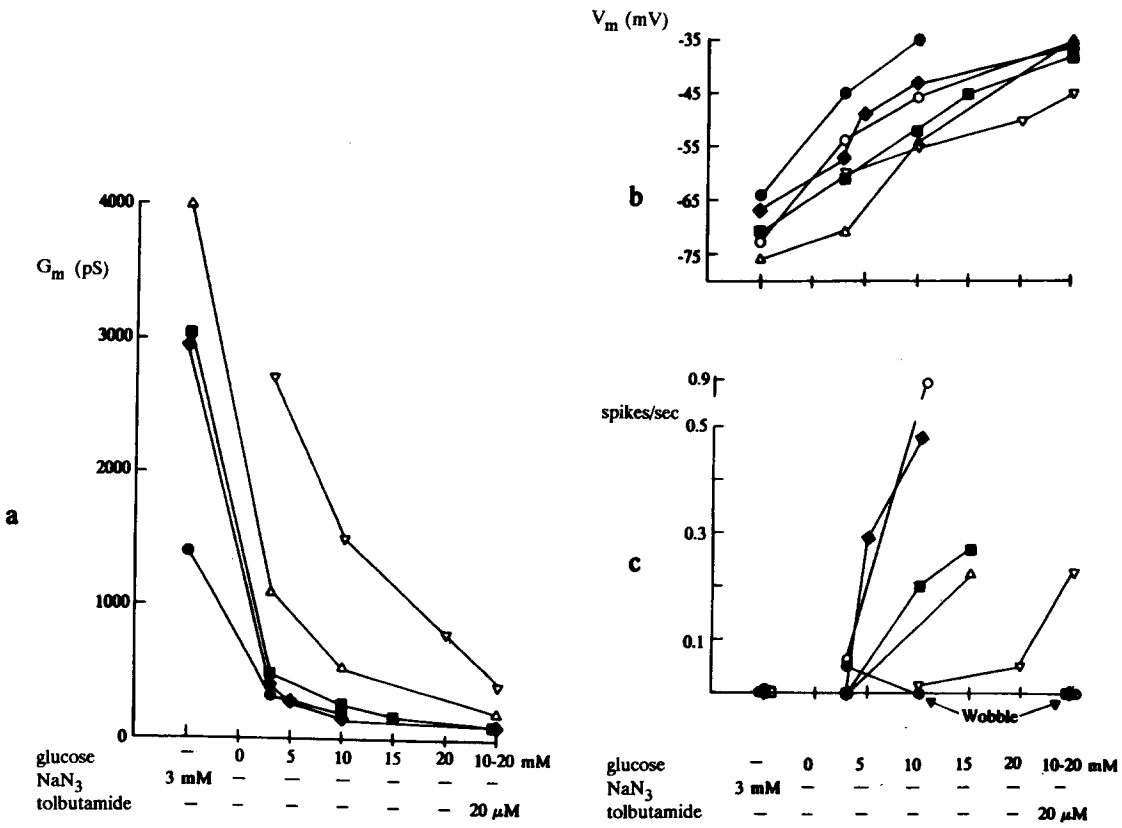

Fig. 3. Compilation of the effects of glucose, tolbutamide and sodium azide on (*a*) average membrane conductance (G_m), (*b*) membrane potential (V_m) and (*c*) action potential frequency (spikes sec^{-1}) determined in the steady state between 3 and 5 min after exposure to the ES's modified as indicated. Data were from a set of "perforated-patch" recordings made on glucose-responsive cells at 20–23°C. G_m was determined by injecting a 1-sec, −5 pA (hyperpolarizing) current pulse. R_a ranged from 30–100 MΩ in these experiments. The same symbol across each panel corresponds to the same B cell, and the 10–20 mM glucose condition is for measurements performed within this concentration range.

presence or absence of glucose, addition of sodium azide (NaN_3), which blocks mitochondrial oxidative metabolism, produces a dramatic increase in G_m and hyperpolarizes the membrane to values of V_m negative to the threshold potential for triggering action potentials.

We noted significant variability among cells in their response to glucose. The data presented in Fig. 3 were obtained from cells which displayed stable decreases in G_m and stable depolarization within 3–4 min after increasing bath glucose to the indicated concentration in the absence of IBMX. Other cells (e.g., Fig. 10*b*) displayed slower, more progressive depolarizations, which ultimately resulted in plateau depolarizations. Yet other cells showed little response to glucose in the absence of IBMX but graded responses to glucose in its presence.

The relative changes in G_m seen with glucose, tolbutamide and NaN_3 roughly parallel the relative changes in K^+(ATP) channel activity produced by these agents in cell-attached patches of B cell membrane held at resting membrane potential (i.e, the pipette held at 0 mV). This reinforces the idea that K^+(ATP) channels are a major contributor to the resting conductance of the B cell. However, in the experiments presented in Fig. 3 significant error in the estimation of G_m might result from (i) difficulty in accurately measuring the small change in V_m in response to a current pulse in the presence of NaN_3 and (ii) the contribution of voltage-dependent channels to G_m at the more depolarized membrane potentials attained in the presence of high glucose or glucose plus tolbutamide. To examine the validity of our basic findings on G_m, in three experiments we intermittently switched from current-clamp recording to voltage-clamp recording. In the voltage-clamp mode we determined the membrane conductance as the slope of the current-voltage curve over the range of V_m (−110 to −60 mV) where voltage-dependent Ca^{2+} and K^+ channels would not be expected to open. Figure 4 shows typical results from one of these experiments which demonstrate that,

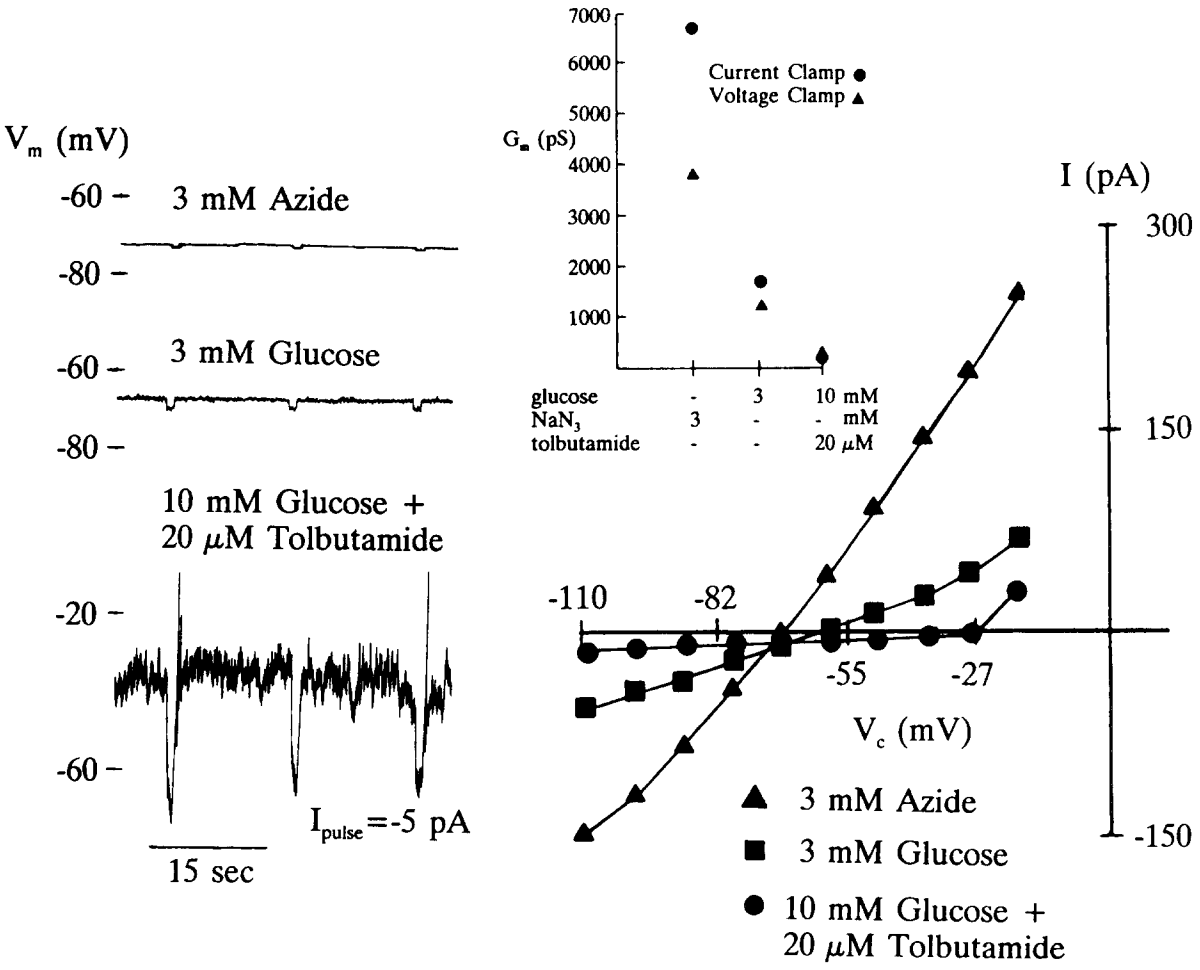

Fig. 4. Comparison of the determination of membrane conductance made on the same cell in the current-clamp and voltage-clamp modes. *Left panel:* Current-clamp recording was initiated when R_a fell below 100 MΩ. *Right panel:* In the voltage-clamp mode the cell underwent a "staircase" depolarization from −110 to −20 mV (10-mV steps at 100-msec intervals). *Inset:* G_m was determined as $\Delta I/\Delta V$ in the voltage-clamp mode and from Eq. (1) in the current-clamp mode.

when V_m is not depolarized beyond −35 to −40 mV, the two methods produce similar estimates of G_m, the largest discrepancy occurring in the presence of NaN_3.

Voltage-Dependent Calcium Currents in Canine Islet B Cells

A third condition expected to be fulfilled if Na_o^+-dependent action potentials were to contribute to Ca_o^{2+}-dependent insulin secretion is that the rapid depolarization, occurring at the start of the action potential, activate a significant voltage-dependent Ca^{2+} current. If this current inactivated over many seconds it could also contribute to Ca^{2+} entry during the plateau depolarization. Two types of experiments performed under current-clamp conditions suggested that Ca^{2+} currents were active in the range of the voltage excursion of the plateau depolarization and the action potential. (i) In two out of five glucose-responsive cells, addition of Bay K 8644, following inhibition of Na^+-dependent action potentials by TTX or their inactivation by sustained depolarization, resulted in the onset of bursts of low amplitude spikes resembling the bursting pattern of Ca^{2+} spikes seen in rodents. (ii) In four out of four cells tested, reduction in $[Ca^{2+}]_o$ from 2 to 0.1 mM during the plateau depolarization resulted in a reversible repolarization from between −35 and −38 mV to between −47 and −50 mV, and the return of spike activity. The latter sets of experiments encouraged us to examine voltage-dependent Ca^{2+} currents in the voltage-clamp mode.

Our most detailed characterization of the voltage-dependent Ca^{2+} current was made in a TEACl-substituted ES containing 2 mM (or near physiological) Ca_o^{2+} and "high Cs^+" nystatin-containing buffer solution in the pipette. Figure 5*a* presents typical voltage-clamp data of the Ca^{2+} current elicited dur-

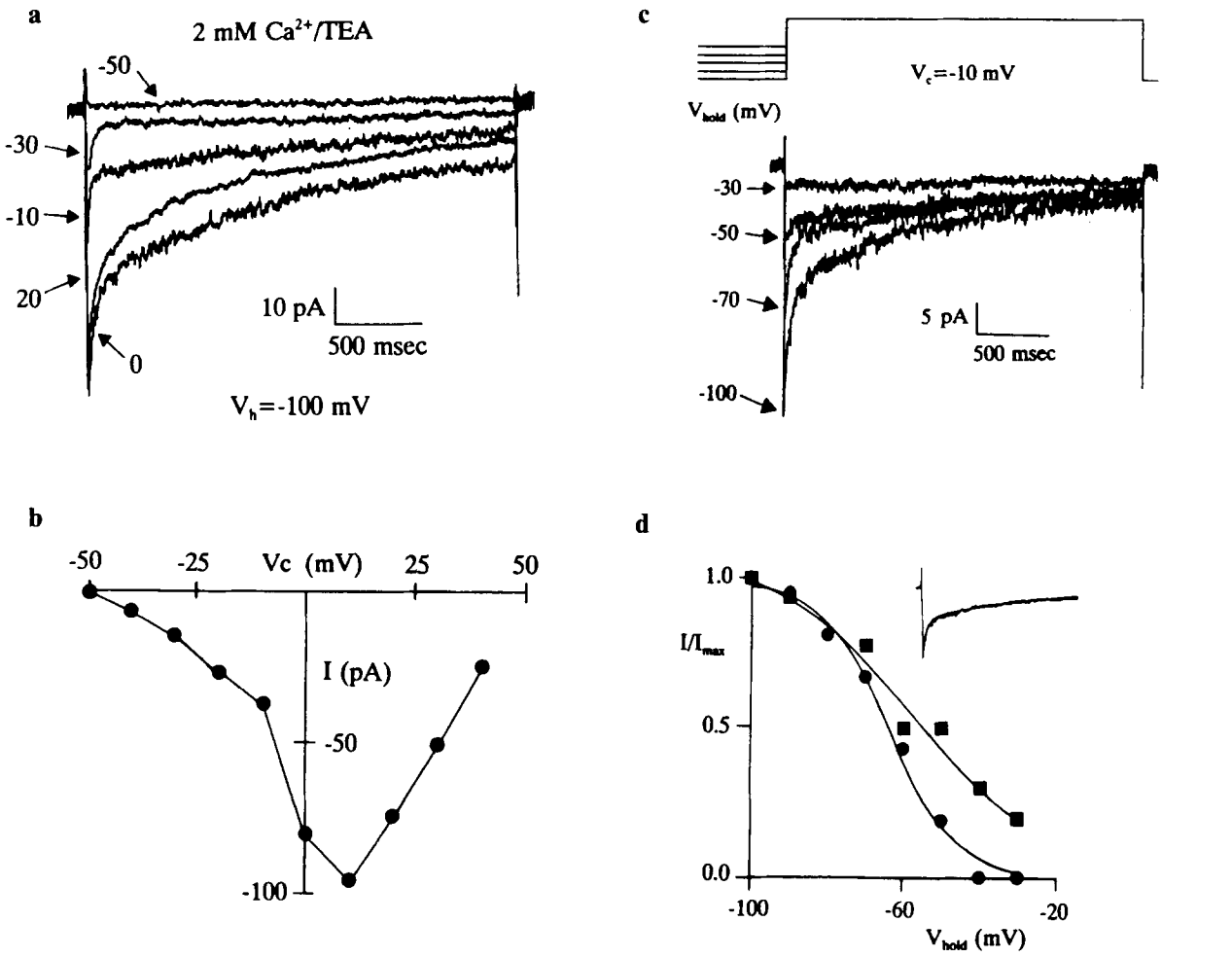

Fig. 5. "Perforated-patch" voltage-clamp recordings of canine B cell Ca^{2+} currents obtained 50–60 min after giga-seal formation using a patch pipette filled with "high Cs^+" buffer solution containing nystatin. Bath solution consisted of a modified ES containing 5 mM glucose, in which NaCl and KCl were replaced with tetraethylamine (TEA) chloride. (*a*) Inward Ca^{2+} currents were activated by stepping from a holding potential of −100 mV to the indicated clamping potential. (*b*) I-V relationship of the peak inward current. (*c*) The cell was stepped from the indicated holding potential after 8 sec to −10 mV. (*d*) The currents elicited at −10 mV were fit by the sum of two exponentials as shown in the inset. The normalized amplitude coefficients of the two exponentials were plotted *versus* holding potential. Round symbols: transient component. Square symbols: sustained component. The lines were drawn to the best fit Boltzmann distribution

$$I/I_{max} = 1/(1 + (\exp[(x - a/b]))$$

where a = 63.5 mV and b = 8.9 for the transient component, and a = −58.0 mV and b = 19.0 for the sustained component.

ing a 2.5-sec duration pulse to various clamping potentials from a holding potential of −100 mV. Inward Ca^{2+} current is activated by steps to membrane potentials positive to −50 mV, increases monotonically to peak at ~+10 mV and then decreases with further depolarization towards a reversal potential of ~+50 mV (Fig. 5*b*). Average peak current density at 0 mV for a group of 16 cells was 11.8 ± 3.7 pA/pF.

The Ca^{2+} current is fully activated within ~5 msec of depolarization and then inactivates along at least a double exponential time course. This suggests that the Ca^{2+} current consists of at least two components: a transient component of approximately 50–100 msec duration and a sustained component present over the entire length of the 2.5-sec pulse. The current elicited at a given clamping potential was fit to the least-squared error (*see* Fig. 5*d*, inset) to Eq. (2) by the simplex routine

$$I_{Ca^{2+}} = a_1 \exp(-t/T_1) + a_2 \exp(-t/T_2). \quad (2)$$

Stepping from holding potentials of −100 to −10 mV the average time constants were T_1 = 52.4 ±

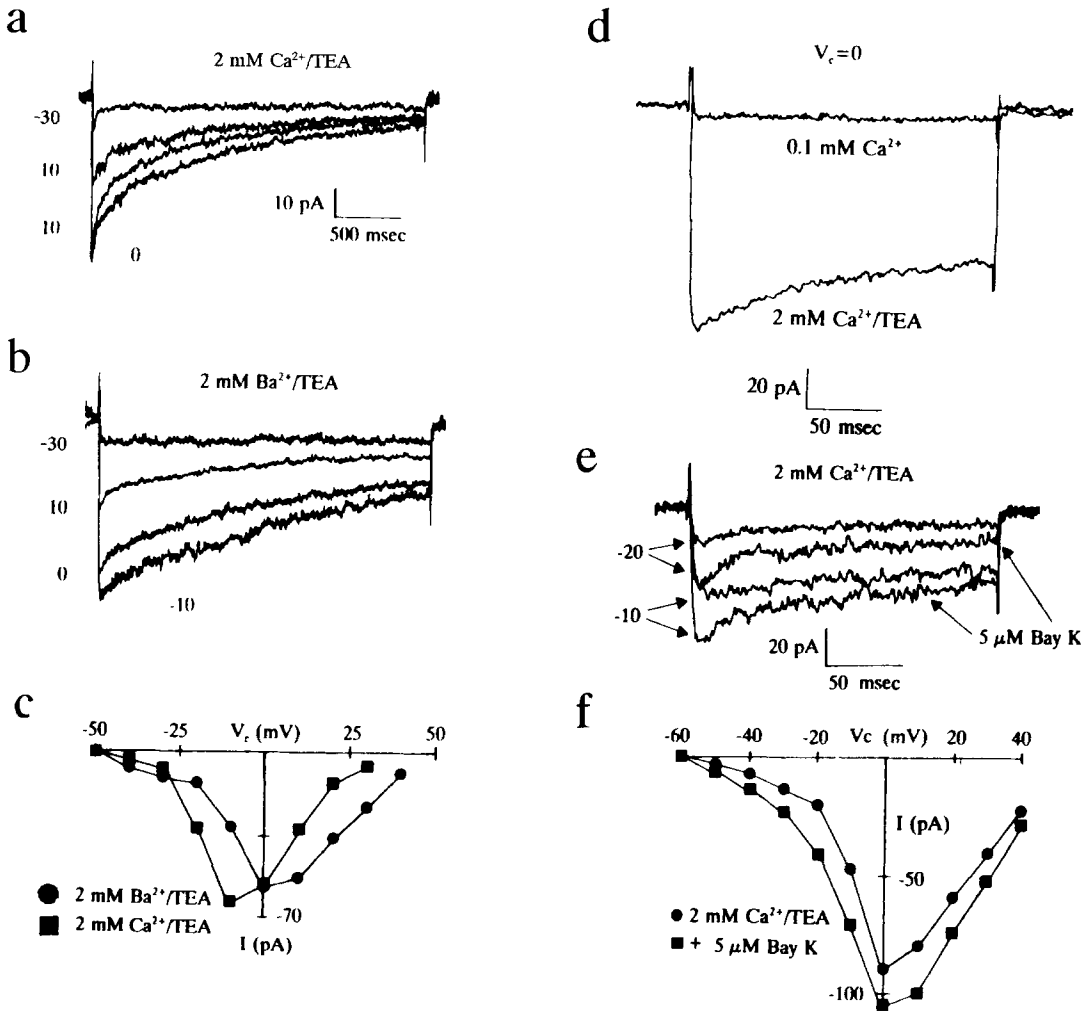

Fig. 6. "Perforated-patch" voltage-clamp recording of inward currents with both Ca^{2+} (*a*) and Ba^{2+} (*b*) as the charge carrier. The cell was stepped from a holding potential of −100 mV to the indicated clamping potential. (*c*) I-V curve of the peak current for both Ca^{2+} and Ba^{2+}. (*d*) The effect of replacement of all but 0.1 mM Ca^{2+} with Mg^{2+} on the inward Ca^{2+} current. (*e* and *f*) Increase in the Ca^{2+} current in another cell by addition of 5 μM Bay K.

16.7 msec and T_2 = 2.33 ± 0.60 sec (n =6). The transient and sustained currents showed different sensitivities to holding potential as shown in Fig. 5*c* and *d*. Here the cell was held at different holding potentials for 8 sec and then stepped to −10 mV. The normalized amplitude coefficients a_1 and a_2 are plotted in Fig. 5*d*. Note that the transient component of this "high voltage-activated" Ca^{2+} current inactivates at a more hyperpolarized potential than the sustained component. Additionally, the sustained component is partially maintained even at potentials closely approximating the plateau depolarization. These data strongly support the hypothesis that high threshold, slowly inactivating Ca^{2+} currents would be rapidly activated during the action potential, yet remain open and contribute to Ca^{2+} entry during plateau level depolarization.

Figure 6 examines the pharmacology of the high voltage-activated Ca^{2+} current. Figure 6*a-c* shows that mole-for-mole replacement of Ba^{2+} for Ca^{2+} caused a 50–100% increase in peak current between −30 and 0 mV, while shifting the peak current 10 mV in the negative direction. Ba^{2+} significantly slowed the inactivation of the sustained current. The inward current is abolished by mole-for-mole replacement of all but 0.1 mM Ca^{2+} with Mg^{2+} (Fig. 6*d*). Figure 6*e* and *f* demonstrates that addition of the Ca^{2+} channel agonist Bay K 8644 increased both the peak transient current and the sustained current. This effect of Bay K on the whole-cell Ca^{2+} current was seen in only one-third of cells tested (12/29). Generally, the current-enhancing action of Bay K was most pronounced at clamping potentials negative to −10 mV.

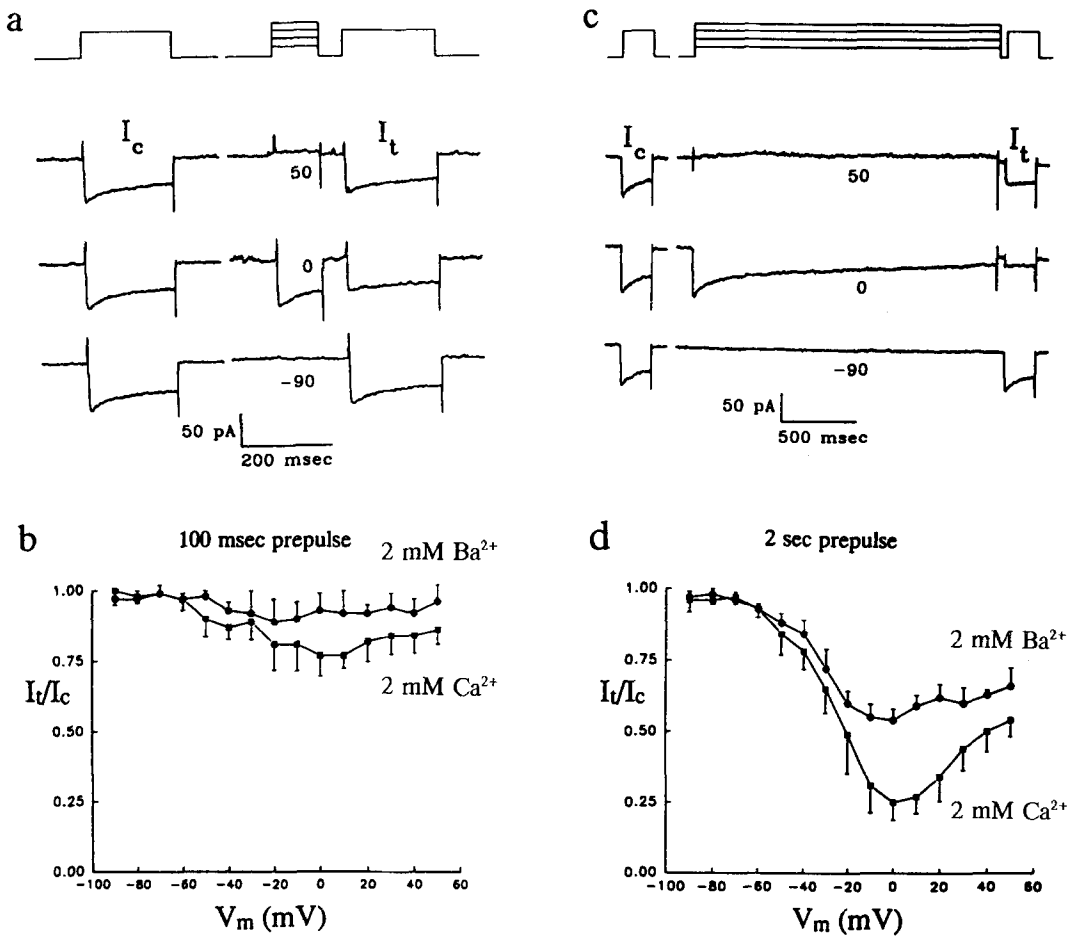

Fig. 7. Calcium-dependent inactivation of the Ca^{2+} current. (*a* and *c*) The pulse protocol and sample current traces for measuring current-dependent inactivation with Ca^{2+} as the charge carrier. The prepulse potentials are indicated under the sample traces. (*b*) The inactivation curve for the 100-msec prepulse duration with Ca^{2+} (n = 7) and Ba^{2+} (n = 5) as the charge carrier. (*d*) The inactivation curve for the 2-sec prepulse duration for Ca^{2+} (n = 9) and Ba^{2+} (n = 6). The error bars are ± the SD.

The more rapid inactivation of Ca^{2+} current with Ca_o^{2+} *vs.* Ba_o^{2+} as a charge carrier (*see* Fig. 6*a*) is often taken as evidence that Ca^{2+} current inactivation is a complex function of both the charge carrier used (i.e., Ca^{2+}-dependent inactivation) as well as the holding potential of the cell (i.e., voltage-dependent inactivation). In mouse B cells, the rapid phase of inactivation of high threshold Ca^{2+} current appears to be largely dependent on the use of Ca^{2+} as the charge carrier and is most prominent at membrane potentials evoking the largest Ca^{2+} current (Plant, 1988; Hopkins, Satin & Cook, 1991). The slower phase of inactivation appears to be chiefly voltage dependent in that it increases steadily with increasing membrane depolarization. As these phenomena might have important functional implications we examined whether they were applicable to dog B cells as well.

Figure 7*a* and *c* illustrates the pulse protocols used to assess the relative contributions of Ca^{2+}-dependent *vs.* voltage-dependent inactivation to the decay of the fast and slowly inactivating components of the canine high voltage-activated Ca^{2+} currents. A 200-msec pulse to 0 mV was used to evoke control current. This was followed, several seconds later, by a prepulse to varying membrane potentials and then 50 msec later by a test pulse to 0 mV. The prepulse-dependent inactivation was determined by computing the ratio of peak current evoked by the test pulse to peak current evoked by the control pulse. Prepulse durations of both 100 msec and 2 sec were used. The effects of Ca^{2+} *vs.* Ba^{2+} as the charge carriers were tested. The sample current traces were recorded with Ca^{2+} as the charge carrier.

Prepulse-dependent inactivation is a "U-shaped" function of the prepulse potential, with maximum inactivation occurring after prepulse potentials (0 and +10 mV) which elicit the greatest

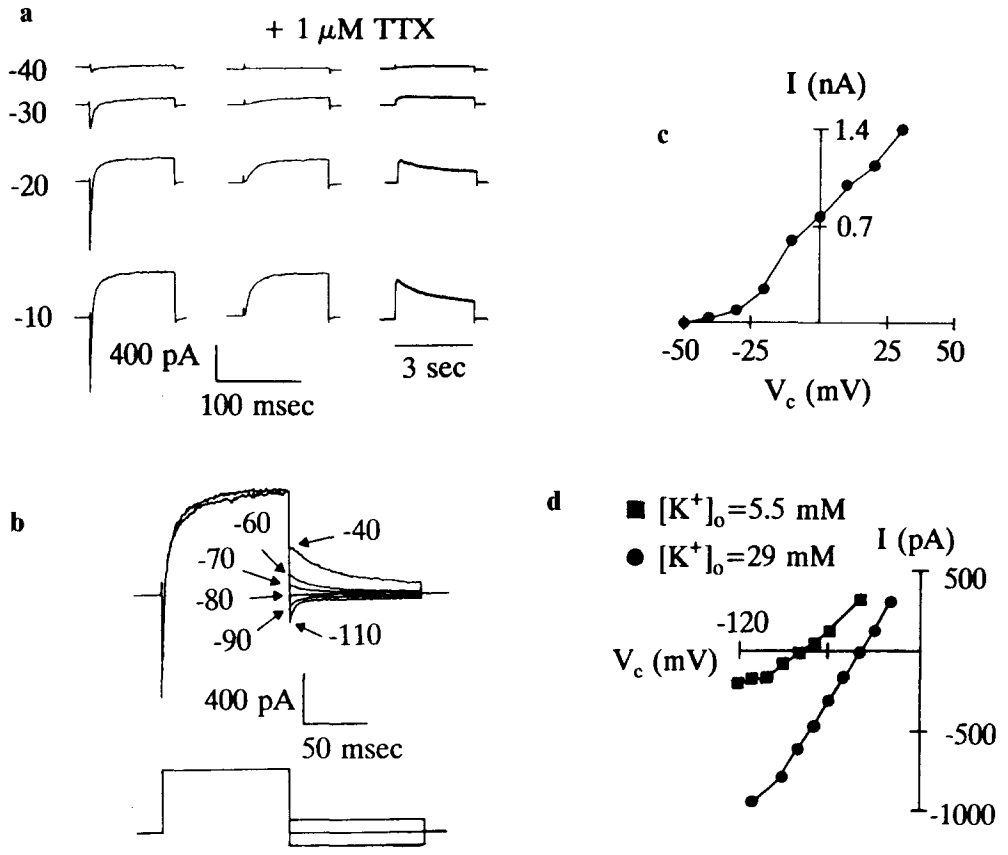

Fig. 8. Macroscopic K^+ currents recorded during conventional "whole cell" with the "intracellular-like" buffer solution in the patch pipette. ES bath contained 5 mM glucose (*a*) Currents evoked by 100-msec steps from −80 mV to the given clamping potential. The transient inward current was blocked by TTX. Sustained depolarizing steps revealed that the outward current underwent slow inactivation. (*b*) The current-voltage curve for the peak outward current shown in *a*. (*c*) Tail currents elicited after a 100-msec depolarizing pulse to 0 mV. (*d*) The reversal potential of the tail current shifted with $[K^+]_o$ in a manner consistent with current carried through a predominantly K^+-selective conductance.

amount of Ca^{2+} current (Fig. 7*b* and *d*). Ca^{2+} currents evoked in response to the test pulse show less inactivation with depolarized prepulses (>30 mV) which elicit little or no Ca^{2+} current. The Ca^{2+} current inactivation and recovery is substantially greater for the 2-sec prepulse than for the 100-msec prepulse protocol. The Ba^{2+} current curve shows less inactivation than the Ca^{2+} current for both prepulse durations. The U-shaped inactivation curve and the diminished inactivation seen with Ba^{2+} as the charge carrier have been taken to indicate that a fraction of the current inactivation is due to the Ca^{2+} current (Eckert & Chad, 1984).

Voltage-Dependent K^+ Currents

Figure 8 presents the voltage-dependent ionic currents recorded in the "whole-cell" configuration using a pipette filled with an "intracellular-like" buffer solution (*see* Materials and Methods). The bath contained sufficient tolbutamide to block nearly all of the current through the K^+(ATP) channels. Voltage-clamp pulses from −80 mV to potentials positive to −40 mV evoke an initial inward current which is followed by a slowly developing and sustained outward current (Fig. 8*a*). The time course of development of the outward current is more clearly seen after blockade of the inward current with TTX. During sustained depolarization the outward currents display slow inactivation. The current-voltage curve for the peak, whole-cell K^+ current is shown in Fig. 8*b*. The reversal potential (E_{rev}) of the tail currents seen on repolarization of the membrane from a fixed clamping potential to a range of test potentials varies with $[K^+]_o$ (Fig. 8*c* and *d*). Increasing $[K^+]_o$ from 5.5 to 29 mM resulted in a +40-mV shift in E_{rev} from −80 to −40 mV; a +43-mV shift is predicted by the Nernst equation. In careful experiments taking into account the voltage drop across the pipette access

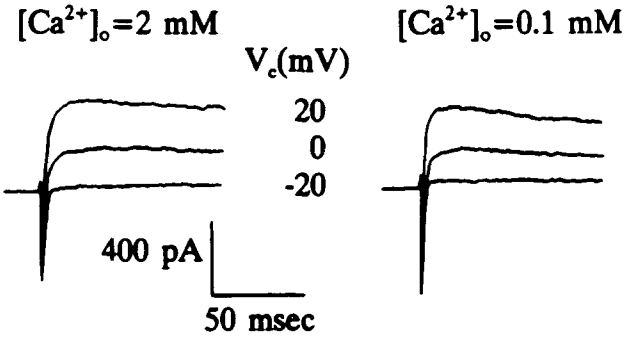

Fig. 9. Lack of effect of Ca_o^{2+} on macroscopic K^+ currents recorded with a "high K^+" buffer solution with nystatin in the pipette and an ES bath. Note that reducing Ca_o^{2+} to 0.1 mM and increasing Mg_o^{2+} to 3 mM had no significant effect on outward K^+ current at any potential.

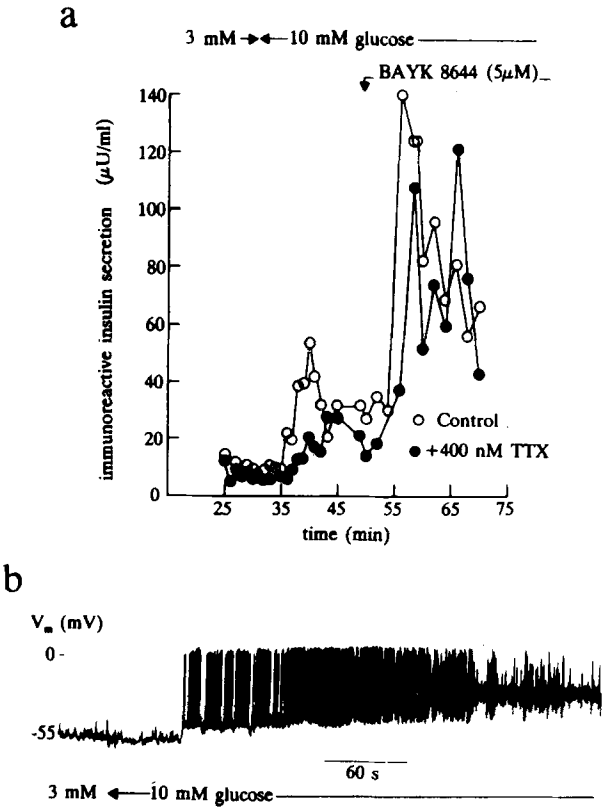

Fig. 10. (*a*) Effects of Na^+ channel blockade by TTX and Ca^{2+} channel enhancement by BAY K 8644 on glucose-induced insulin secretion (1 mM IBMX present throughout). In this paired experiment basal insulin release and peak insulin release in BAY K are nearly comparable in the presence and absence of TTX. However, in the presence of TTX (400 nM) the initial or "first phase" of insulin secretion occurring between 35 and 45 min is reduced nearly threefold. Dramatic increases in insulin secretion are evident in either case after the addition of BAY K 8644. (*b*) Time course of onset and degeneration of high frequency activity of Na^+ action potentials after exposure of a B cell to 10 mM glucose during "perforated-patch" current-clamp recording (*see* text for discussion).

resistance it was found that the K^+ conductance initially increases by *e*-fold per 5 mV. Altogether these features suggest that the outward current is a K^+ current of the "delayed-rectifier" variety.

Recently, there has been renewed interest in the possible contribution of Ca^{2+}-activated K^+ currents to voltage-dependent outward current recorded "whole cell" (Satin & Cook, 1989). Using the canine islet B cells, we re-examined this issue in the "perforated-patch" recording mode where buffering of cytosolic Ca^{2+} should remain more intact. Figure 9 demonstrates that reducing $[Ca^{2+}]_o$ concentration (by equimolar substitution with Mg_o^{2+}) to levels which eliminate the Ca^{2+} current, has virtually no effect on K^+ current at a given clamping potential. The slight increase in Na^+ current caused by this maneuver might be attributable to a small increase in negative surface potential which would occur if Ca^{2+} tended to bind as well as screen surface charge density thought to be associated with the external surface of the Na^+ channels of excitable cells (Hille, 1984).

Role of Ca^{2+} and Na^+ Currents in Insulin Secretion

Figure 10*a* presents evidence showing the contribution of Na^+ and Ca^{2+} currents to glucose-induced insulin secretion. In this experiment, addition of 10 mM glucose to the bath resulted in a clear transient (or "first") phase of insulin secretion followed by a more sustained plateau. In the paired experiment, where the perifusion medium contained 400 mM TTX throughout, the transient phase was not evident. This experiment is typical of four out of five such paired experiments from two different batches of islets as shown in the Table. In these experiments, addition of TTX reduced initial peak insulin secretion by nearly twofold in comparison with control, while exerting no consistent effect on sustained plateau secretion or basal secretion. No effect of TTX was seen in the one experiment where paired control islets lacked initial peak secretion. When the time delay of the perifusion system (~3 min from stock solution to islet-containing chamber) is taken into account, the onset and the duration of the initial phase of insulin secretion corresponds to the period of most intense glucose-induced spike activity (*see* Fig. 10*b*, for example). These results suggest that Na^+ currents make a significant contribution to "first" phase secretion.

Figure 10*a* also shows that addition of the Ca^{2+} channel agonist BAY K 8644 during the plateau pro-

Table. Effect of tetrodotoxin on first and second phase insulin secretion

	Control			Tetrodotoxin (200–400 nM)		
Trial	Basal	Peak	Plateau	Basal	Peak	Plateau
1	9.9 ± 5.1	44.1 (4.5)	24.4 ± 4.6 (2.4)	12.4 ± 3.2	26.8 (2.2)	18.4 ± 4.7 (1.5)
2	10.7 ± 1.4	53.6 (5.0)	30.1 ± 4.3 (2.8)	7.9 ± 2.0	20.9 (2.6)	23.3 ± 10.1 (2.9)
3	18.3 ± 5.0	43.6 (2.4)	25.3 ± 5.2 (1.4)	15.9 ± 2.3	26.6 (1.7)	25.0 ± 7.1 (1.6)
4	6.0 ± 0.9	36.2 ± (6.0)	22.0 ± 7.9 (3.7)	5.7 ± 1.7	20.6 (3.6)	21.2 ± 4.3 (3.7)
5	4.6 ± 0.8		19.3 ± 1.6 (4.2)	7.1 ± 0.7	—	23.2 ± 3.3 (3.3)
	Mean stimulation index 4.48*		2.90		2.53*	2.60
	$s^2 = 2.30$		1.21		0.65	1.00

The insulin data is given in μU/ml. The numbers in parentheses are the stimulation index, i.e., peak or plateau secretion divided by basal secretion. Test of difference between two means by one-tailed Student's *t* test, *P < 0.05.

duces a dramatic increase in insulin secretion both in the presence and absence of TTX. Results very similar to those obtained with BAY K were also obtained on addition of 20 mM KCl, though the effects of high K^+ were less persistent. These results suggest that the plateau or "second" phase of insulin secretion may correspond to the sustained period of plateau "wobble" deoplarization. Taken together with the slow time course of inactivation of voltage-dependent Ca^{2+} currents, these data suggest that Ca^{2+} currents may contribute significantly to sustained secretion.

Discussion

We have investigated the relationship between electrical activity and insulin secretion in canine islet B cells stimulated by glucose. Canine B cells display biphasic Ca_o^{2+}-dependent insulin secretion on exposure to glucose but do not display the regular "bursts" of Ca^{2+} action potentials, which have become the *sine qua non* of electrical activity in rodent islet B cells. Rather, on exposure to concentrations of glucose which stimulate insulin secretion, canine B cells display an initial nonpatterned salvo of Na^+ action potentials, which is often followed by a prolonged plateau depolarization. Here we present three new lines of evidence which appear to reconcile the observed electrical behavior to Ca_o^{2+}-dependent secretion. (i) Over the range of membrane potentials where canine B cells can trigger Na^+ action potentials, the frequency of these spikes increases with the magnitude of glucose-induced depolarization. (ii) Long-lasting, voltage-activated Ca^{2+} currents are recruitable over the voltage excursion of the action potential. In the voltage range of the plateau depolarization inactivation is sufficiently slow that Ca^{2+} currents are still measurable many seconds after the onset of depolarization. (iii) When clearly evident, "first phase" insulin secretion is significantly reduced by the addition to the bath of a concentration of TTX (>200 nM) which inhibits the Na^+ current by greater than 95%. Second phase insulin secretion is enhanced by the elevation of K_o^+, which should further depolarize the cells, or by the addition of BAY K 8644, which is known to enhance the probability of opening of some types of Ca^{2+} channels. Both maneuvers should enhance the Ca^{2+} entry into the B cells. Together this evidence suggests that on exposure to glucose the early barrage of Na^+ action potentials, arising from a background of modest depolarization, facilitates pulsatile entry of Ca^{2+} through voltage-dependent Ca^{2+} channels, and thereby, contributes to the first phase of insulin secretion. Later, the plateau depolarization sustains Ca^{2+} entry through the slowly inactivating Ca^{2+} channels, and thereby, contributes to the second phase of insulin secretion. It has been reported in RINm5F cells that secretogogue-induced electrical activity and increases in cytosolic Ca^{2+} are dependent on extracellular Na^+ and are blocked by TTX (Dunne et al., 1990).

The whole-cell, voltage-dependent Ca^{2+} currents recorded in these experiments have the following characteristics. (i) They are of the high threshold variety, being activated at clamping potentials equal or positive to −40 mV. (ii) They peak at 0 to +20 mV in 2 mM Ca^{2+}. (iii) During sustained depolarization they slowly inactivate with two discernable time constants, one rapid (~50 msec) and the other slow (~2.5 sec). Both components of inactivation display a combination of voltage- and current-dependent features. (iv) The Ca^{2+} currents are enhanced by equimolar substitution of Ba^{2+} for Ca^{2+}. (v) Last, they show variable enhancement by the dihydropyridine BAY K 8644, with the rapidly inactivating component being at least as well enhanced as the slowly

inactivating one. In preliminary single-channel recording experiments, performed in the cell-attached patch or the outside-out patch mode, with 90 mM $BaCl_2$ as the charge carrier, we recorded a voltage-dependent, slowly inactivating, ~22-pS conductance inwardly directed single-channel currents between −40 and 0 mV. In four out of eight experiments, addition of 5 μM BAY K at least doubled mean single-channel activity (Pressel & Misler, 1989; and *unpublished observations*). Taken together all of these features suggest that a significant fraction of canine B cell Ca^{2+} current shares many characteristics with high threshold, voltage-activated, Ca^{2+} current seen in a host of secretory cells (McCleskey et al., 1986). Similar currents have been recorded in mouse B cells (Rorsman & Trube, 1986; Plant, 1988; Rorsman, Ashcroft & Trube, 1988), rat B cells (Ashcroft, Kelly & Smith, 1990), neonatal rat B cells and HIT cells (Satin & Cook, 1988).

In whole cell patch-clamp experiments we also identified voltage-dependent K^+ currents which are activated by voltage excursions into the range of V_m's traversed by the action potential. These currents are only fully activated after tens of milliseconds of depolarization and then inactivate over hundreds of milliseconds. Often they reach peak amplitudes of almost 1 nA at 0 mV. Due to the intensity of activity of small amplitude channel currents seen at most depolarized V_m's, we have not been able to characterize, in patches of membrane, the single-channel current underlying the macroscopic current.

A previous study using conventional whole-cell recording showed that expression of a $K^+(Ca^{2+})$ current was dependent on the extent of intracellular Ca^{2+} buffering by the patch pipette solution (Satin et al., 1989). The role of maxi-$K^+(Ca^{2+})$ channels has been uncertain even in rodent B cells (Henquin, 1990; Smith et al., 1990) where channel density may be as high as 5–6/μm^2 (Tabcharani & Misler, 1989). In perforated-patch experiments on dog B cells, where cellular Ca^{2+} buffering is intact and Ca^{2+} channels show minimal rundown, we found that reducing extracellular Ca^{2+} had little effect on whole-cell K^+ currents. This data is in concert with our very infrequent sightings of maxi-$K^+(Ca^{2+})$ channels in cell-attached patches, even when the patch is depolarized by >100 mV, or in the inside-out excised patch, even when the cytoplasmic side of the membrane is exposed to Ca^{2+} concentrations >100 μM (D.M. Pressel & S. Misler, *unpublished observations*).

Our study raises a variety of issues which remain unsettled. First and most broadly, while intensive interest has focused on the "bursting pacemaker" paradigm of rodent islet electrical activity, evidence from our studies with canine islets as well as human islet B cells (*see* Falke et al., 1989; Pressel & Misler, 1990; Misler, Pressel & Gillis, 1991) suggests that a wide variety of electrical activity patterns, including nonpatterned spiking behavior and even nonspiking depolarization, can occur in other species. This study suggests that at least one alternate form of electrical activity satisfactorily underlies secretion. This raises the question of whether the bursting regime is optimal or even critical for triggering secretion or merely a consequence of a fortuitous balance of Ca^{2+}, K^+, and other channels in the plasma membrane in some species. Undoubtedly the study of the efficacy of a pattern or changing patterns of electrical activity in stimulating secretion will require simultaneous measurement of electrical activity and exocytotic secretion on single cells. These studies are now in progress using combined "perforated-patch recording" and membrane capacitance measurement by phase detection (Gillis & Misler, 1991).

The plateau potential represents a "quasi stable" level of membrane potential at which both the delayed rectifier K^+ and sustained Ca^{2+} currents are active. Since the magnitude of the delayed rectifier K^+ current is several times larger than that of the Ca^{2+} current, other ionic currents might contribute to the sustained plateau potential. There are several possibilities. One is a nonselective cation channel resembling those seen in insulinoma cells (Sturgess et al., 1987) and human islet cells (Misler et al., 1989). A second is a Ca^{2+} (or voltage) activated anion (or Cl^-) current resembling that seen in AtT-20 pituitary cells (Korn & Horn, 1988). In the latter cells, such currents underlie long duration tail currents seen after activating Ca^{2+} channels and may contribute to the plateau phase of the action potential in these cells (Korn, Bolden & Horn, 1991). Flux studies in B cells have found a Cl^- conductance which is enhanced by glucose and depolarization (Sehlin, 1987). A third possibility is a Na^+-Ca^{2+} exchanger. A Na^+-Ca^{2+} exchanger is present in B cells and can provide net inward Na^+ current in exchange for Ca^{2+} (Plasman Lebrun & Herchuelz, 1990).

Second, the high voltage-activated Ca^{2+} currents recorded here were strikingly similar in their threshold voltages, amplitudes and inactivation kinetics to those studied intensively in mouse islet cells (Hopkins et al., 1991). However, the role of Ca^{2+} currents in the generation of electrical activity in dog is very different than their role in mouse. In the mouse, voltage-activated Ca^{2+} currents underlie "bursting pacemaker" type electrical activity; in the dog, they do not. A recent model for mouse B cell excitability has proposed that several features of Ca^{2+} current activation and inactivation contribute to B cell electrogenesis (Cook, Satin & Hopkins,

1991). There are three salient points. (i) Activation of high threshold Ca^{2+} currents at ~ -40 mV initiates both the plateau and the superimposed action potentials. (ii) Ca^{2+}-dependent inactivation contributes to individual spike repolarization during the plateau. (iii) Slow, voltage-dependent inactivation contributes to the repolarization of the plateau. In dog, no Ca_o^{2+}-dependent spike activity is seen until BAY K 8644 is added. In principle, subtle differences in Ca^{2+} current characteristics might contribute to the different patterns of excitability. For instance, the transient component of the Ca^{2+} current in dog appears to undergo more rapid voltage-dependent inactivation than in mouse (Hopkins et al., 1991). Whether such small differences in the degree of Ca^{2+} channel inactivation underlie the lack of Ca^{2+} action potentials in dog, or whether differences in the densities of other channels account for the different patterns of electrical activity remains to be resolved.

Third, we noted that up to one-half of cultured canine islet B cells did not generate an electrical response to glucose unless IBMX, a potent phosphodiesterase inhibitor, was also present. This is in sharp contrast to rat and human islet cells where upwards of 90% of cells with K^+(ATP) channels depolarize in response to 10–15 mM glucose (S. Misler, J. Tabcharani, K. Gillis & L. Falke, *unpublished data*). Interestingly, IBMX is also needed to ensure glucose-stimulated insulin secretion in isolated canine islets (Scharp et al., 1989). We are now studying the effect of IBMX on glucose-induced electrical activity and insulin secretion in canine islet B cells.

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We dedicate this work to the memory of our cherished colleague and friend, Lee C. Falke, Jr. His insights and achievements shall be a timeless source of inspiration to our lab.

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Note Added in Proof

Based on a recent detailed analysis of membrane currents recorded with the perforated patch technique (Sala et al., 1991), we estimate that the Ca^{2+} currents we recorded were attenuated by at most 10–15%, as compared with those achievable with conventional whole cell recording.