A Role for Ca^{2+}-Sensitive Nonselective Cation Channels in Regulating the Membrane Potential of Pancreatic β-Cells

Colin A. Leech and Joel F. Habener

The incretin hormones, glucagon-like peptide 1 and pituitary adenylyl cyclase–activating polypeptide, are proposed to activate a maitotoxin (MTX)-sensitive, Ca^{2+}-dependent nonselective cation current in pancreatic β-cells and insulinoma cells. This MTX-sensitive current is present in human β-cells as well as in mouse and rat β-cells, and is accompanied by a rise in cytosolic Ca^{2+} in voltage-clamped cells in which the activation of voltage-dependent Ca^{2+} channels is prevented. Activation of the nonselective cation current is inhibited by reduction of disulfide bonds with intracellular, but not extracellular, dithiothreitol, and is also abolished by intracellular dialysis with trypsin. The nonselective cation channels that carry this current have a conductance of about 30 pS, with Na^+ as the major extracellular cation. We estimate that these cation channels are expressed on β-cells at a density similar to that of ATP-sensitive potassium channels (K_{ATP} channels) and exhibit spontaneous activity at basal glucose concentrations. We propose that this spontaneous cation channel activity constitutes at least part of the depolarizing background conductance that permits changes in the activity of K_{ATP} channels to regulate the resting potential of β-cells. *Diabetes* 47:1066–1073, 1998

Elevation of blood glucose stimulates insulin secretion through closure of ATP-sensitive potassium channels (K_{ATP} channels), leading to membrane depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs), and the influx of Ca^{2+} triggers secretion (1). The inhibition of K_{ATP} is augmented by hormones that stimulate insulin secretion, such as glucagon-like peptide 1 (GLP-1) (2,3), pituitary adenylyl cyclase activating polypeptide (PACAP) (4), and glucagon (5). Conversely, somatostatin and leptin, hormones that inhibit insulin secretion, hyperpolarize β-cells by increasing the membrane K conductance through activation of a low-conductance, G-protein–regulated K channel (1,6) or of K_{ATP} (7–9), respectively. However, the nature of the depolarizing background conductance that permits these changes in the activity of K_{ATP} (or G-protein–regulated K channels) to regulate the membrane potential of β-cells has not been clearly defined.

The insulinotropic hormones GLP-1 (10) and PACAP (11) elevate intracellular cAMP levels and are potent insulin secretagogues in the presence of slightly elevated glucose levels. These hormones activate voltage-independent, nonselective cation currents (4,12,13) that are proposed to be identical to maitotoxin (MTX)-sensitive currents (14). These currents may, therefore, play an important role in depolarizing β-cells to regulate insulin secretion. We investigated the single-channel properties of this current to determine whether these channels are similar to the Ca^{2+}-activated, nonselective cation channels (NSCCs) previously reported in insulinoma cells (15–17), whose physiological role is uncertain but which could potentially underlie the depolarizing background conductance and the GLP-1/PACAP/MTX-sensitive currents.

This study reports on the expression of a MTX-sensitive, nonselective cation current in human pancreatic β-cells (also present in mouse β-cells and insulinoma cells) and the single-channel properties of the channels underlying this current. MTX appears to bind to an extracellular receptor, and activation of MTX-sensitive currents is inhibited by prolonged dialysis of cells with ATP-free solutions. We further demonstrate that NSCCs, with the same conductance and reversal potential as MTX-sensitive channels, are spontaneously active under basal conditions, and that these channels may thus play an important role in determining the resting potential of pancreatic β-cells. We also report that cytosolic dithiothreitol (DTT; a sulfhydryl-reducing agent), but not extracellular DTT, inhibits activation of the nonselective cation current in HIT-T15 and βTC3 insulinoma cells and also in mouse β-cells. Activation of the current by MTX is also inhibited by intracellular dialysis with trypsin, suggesting that an intracellular site(s) plays an essential role in regulating channel activity.

RESEARCH DESIGN AND METHODS

Preparation of cell cultures. HIT-T15 cells were obtained from the American Type Culture Collection, and human islets were obtained from Dr. C. Riccordi (Diabetes Research Institute, University of Miami, FL). Mouse (c57/blk) islets were prepared by collagenase digestion of isolated pancreases and handpicked. Mouse and human islets were then dissociated into single cells by tryptic digestion and trituration, and maintained in RPMI 1640 plus 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin. HIT-T15 cells (passages 64–75) were maintained in HAM's F-12 medium containing 10 mmol/l glucose, 10% heat-inac-

From the Laboratory of Molecular Endocrinology, Howard Hughes Medical Institute, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts.

Address correspondence and reprint requests to Dr. Colin A. Leech, Laboratory of Molecular Endocrinology, Massachusetts General Hospital, 55 Fruit St., WEL 320, Boston, MA 02114-2696. E-mail: leech@helix.mgh.harvard.edu.

Received for publication 12 November 1997 and accepted in revised form 3 April 1998.

$[ATP]_i$, intracellular ATP concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DTT, dithiothreitol; GLP-1, glucagon-like peptide 1; K_{ATP} channel, ATP-sensitive potassium channel; MTX, maitotoxin; NMG, *N*-methyl-D-glucamine; NSCC, nonselective cation channel; PACAP, pituitary adenylyl cyclase activating polypeptide; PTX, palytoxin; SES, standard extracellular buffered saline; TTX, tetrodotoxin; VDCC, voltage-dependent Ca^{2+} channel.

tivated horse serum, 2.5% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Cells were plated onto glass coverslips coated with 1 mg/ml of type V concanavalin A (Sigma, St. Louis, MO), which facilitates their adherence to glass. Cultures were maintained at 37°C in a 5% CO_2 atmosphere incubator, and experiments were conducted 1–5 days after plating.

Test solutions. Cells were bathed in a standard extracellular buffered saline (SES) containing 138 mmol/l NaCl, 5.6 mmol/l KCl, 2.6 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 10 mmol/l HEPES (295 mOsm; pH adjusted to 7.4 with NaOH; ~4 mmol/l), and 0.8 mmol/l D-glucose, unless otherwise indicated. Na^+-free solutions were prepared using 138 mmol/l *N*-methyl-D-glucamine (NMG) substituted for NaCl and adjusted to pH 7.4 with HCl. Ca^{2+}-free solutions were prepared by substituting $MgCl_2$ for $CaCl_2$; other extracellular solutions containing KCl, CsCl, LiCl, or CholineCl were prepared by substitution of NaCl.

Test solutions containing MTX were applied to individual cells by focal application from micropipettes using a PicoSpritzer II pressure ejection system (General Valve, Fairfield, NJ). A gravity-fed bath superfusion system was used to exchange and refresh bath solutions. MTX, glyburide, ATP, and trypsin were obtained from Sigma. Tetrodotoxin (TTX) was obtained from Calbiochem (La Jolla, CA). DTT was obtained from Boehringer Mannheim (Indianapolis, IN), and tetraethylammonium chloride (TEA) and 4-aminopyridine were obtained from Aldrich (Milwaukee, WI).

Patch clamp recording techniques. Membrane currents were measured under voltage clamp using tight-seal, patch-clamp techniques (18). Patch pipettes were pulled from borosilicate glass (Kimax-51) and fire polished. Pipettes were filled with Cs- or K-pipette solutions containing 95 mmol/l Cs_2SO_4 (or K_2SO_4), 7 mmol/l $MgCl_2$, 5 mmol/l HEPES (pH adjusted to 7.4 with CsOH [or KOH]; ~2 mmol/l). Nystatin (240 µg/ml) was added to this solution for perforated patch recording (19,20). Pipettes for whole-cell recordings had resistances of 1.4–3.6 MΩ.

The patch pipette was connected to a Heka Electronik EPC-9 patch clamp amplifier (Instrutech, Mineola, NY) interfaced with a personal computer running Pulse v8.0 software (Instrutech). Continuous records of single-channel currents were stored on videotape using an Instrutech VR-1 OA digital data recorder for subsequent analysis using pClamp v6.0 software (Axon, Foster City, CA).

RESULTS

Stimulation of β-cells with GLP-1 at constant glucose concentration results in membrane depolarization and a rise of the intracellular Ca^{2+} concentration. Figure 1*A* shows records of membrane potential (*top*) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*bottom*) measured simultaneously from the same mouse β-cell. Stimulation with 10 nmol/l GLP-1 (indicated by bar) at a constant glucose concentration (7.5 mmol/l) produced membrane depolarization and activation of VDCCs, and Ca^{2+}-dependent action potentials generated a rise of $[Ca^{2+}]_i$. The initial depolarization to elevate the membrane potential to the threshold for activation of VDCCs can occur through enhanced inhibition of K_{ATP} (2,3) and also by activation of a nonselective cation current independent of K_{ATP} (Fig. 1*B*) (4,13,14). The mouse β-cell shown in Fig. 1*B* was held at –70 mV in perforated patch voltage clamp, with membrane current (*top*) and $[Ca^{2+}]_i$ (*bottom*) recorded simultaneously. Note that $[Ca^{2+}]_i$ increased with a time course, similar to the amplitude of the inward current under voltage clamp at –70 mV and below the threshold for activation of VDCCs (1). Comparison of the pharmacological properties and time course of these GLP-1–activated currents with MTX-sensitive currents led to the proposal that these currents are carried through the same channels (14). MTX-sensitive, nonselective cation currents are also expressed in human β-cells (Fig. 1*C*, *top*). Activation of the inward current in human β-cells is accompanied by a rise of $[Ca^{2+}]_i$ (Fig. 1*C*, *bottom*) under voltage clamp at –70 mV, independent of the activation of VDCCs. The cation current in human β-cells has a linear current-voltage relationship (Fig. 1*D*), and its reversal potential, measured using voltage ramps, was –1.7 ± 1.6 mV (n = 4), essentially identical to currents in rodent β-cells and insulinoma cells (14,21).

Nonselective cation channels in β-cells have a conductance of 25–30 pS. To investigate the properties of the channels underlying the nonselective cation current, we recorded single-channel activity in cell-attached patches from HIT-T15 cells (Fig. 2*A*). Cells were bathed in SES, with the same solution plus 10 nmol/l MTX in the patch pipette. MTX was used in these single-channel studies because it reliably activates whole-cell currents in all cells tested. Single-channel current amplitudes were measured at different pipette potentials, and a linear current-voltage relation was obtained, from which the mean channel conductance was estimated to be 25 pS (Fig. 2*B*). In these cell-attached recordings, the currents reversed at about +60 mV relative to

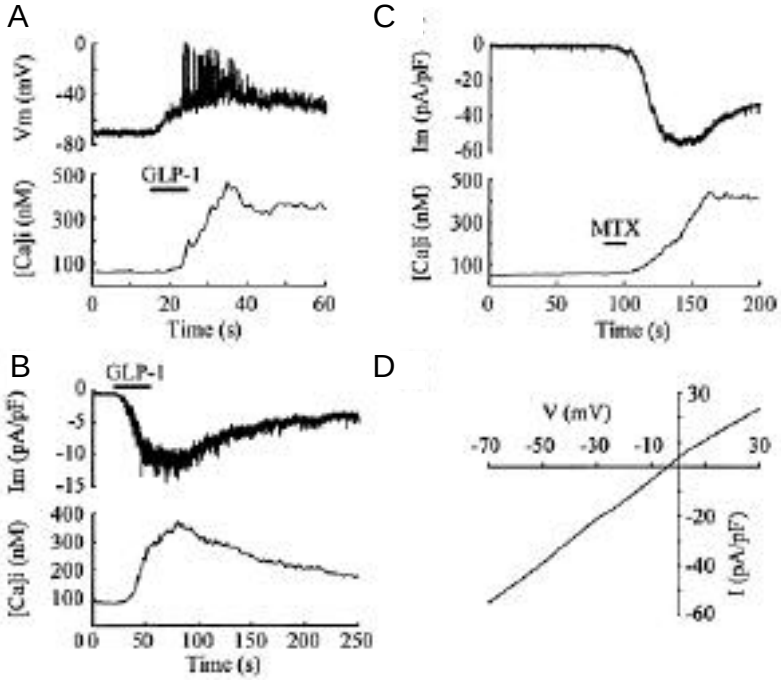


FIG. 1. GLP-1 depolarizes β-cells and activates a MTX-sensitive nonselective cation current. *A*: Records of membrane potential (*top*) and $[Ca^{2+}]_i$ (*bottom*) in a mouse β-cell bathed in 7.5 mmol/l glucose under perforated patch current clamp. Application of 10 nmol/l GLP-1(7-37) at constant glucose caused membrane depolarization, initiation of Ca^{2+}-dependent action potentials, and a rise of $[Ca^{2+}]_i$. *B*: Records from a mouse β-cell under voltage clamp at –70 mV under the same conditions as in *A*. A pulse of 10 nmol/l GLP-1 activates an inward, nonselective cation current (*top*) (C_M 10.9 pF) and a rise of $[Ca^{2+}]_i$ (*bottom*). *C*: Records of membrane current (*top*) (C_M 11.2 pF) and $[Ca^{2+}]_i$ (*bottom*) from a human β-cell bathed in 5.5 mmol/l glucose and held at –70 mV under perforated patch voltage clamp. The cell was stimulated with 10 nmol/l MTX (indicated by bar) and an inward current and a rise of $[Ca^{2+}]_i$ resulted. *D*: Reversal potential of the current was obtained by subtracting averaged membrane currents (elicited by a series of four voltage ramps from –70 to +30 mV over 100 ms) before stimulation with MTX from ramp currents recorded after stimulation with MTX.

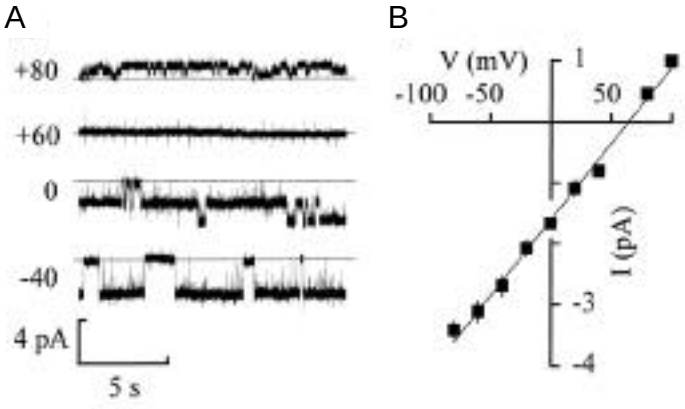


FIG. 2. Nonselective cation channels in on-cell patches. *A*: Series of current traces from an on-cell patch of a HIT-T15 cell at different potentials relative to the resting potential of the cell. Cells were bathed in SES plus 5 mmol/l TEA and 1 µmol/l TTX; the pipette contained this same solution plus 10 nmol/l MTX. Hyperpolarization increased the amplitude of the currents, which reversed at about +60 mV. Dotted lines mark the closed state of the channels. Gaussian fits to all-points histograms were used to obtain single-channel current amplitudes for estimation of the channel conductance from current-voltage relations. *B*: Mean (± SE where larger than symbols) current-voltage relation from five on-cell patches from HIT cells. The line was fitted by linear regression to obtain the mean conductance of the nonselective cation channel. A conductance of 29 pS was obtained for the patch illustrated in *A* and the mean value in five on-cell patches was 25 pS (*B*).

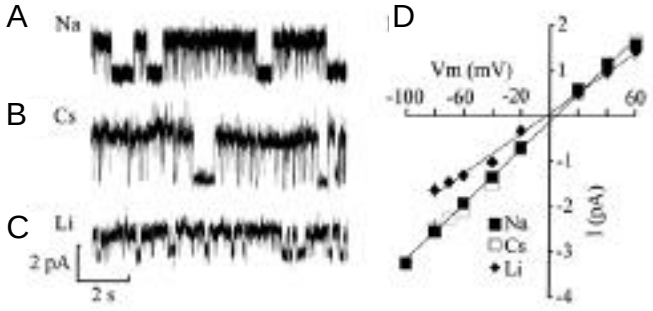
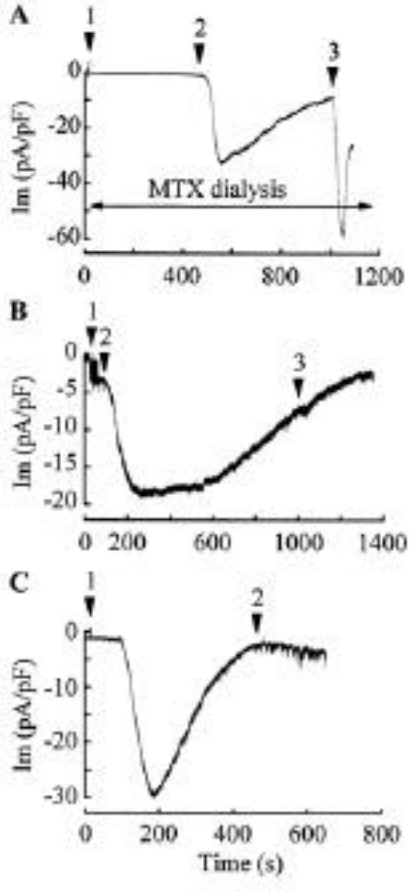


FIG. 3. Nonselective cation currents in outside-out patches. Outside-out patch recordings from insulinoma cells were performed using Cs^+ pipette solution and bath solutions with different major cations. *A–C*: Records from patches held at –60 mV where the major extracellular cations are Na^+ (*A*), Cs^+ (*B*), or Li^+ (*C*) with CsCl or LiCl substituted for NaCl, respectively, in SES. *D*: Mean current amplitudes (± SE where larger than symbols) for Na^+ (■), Cs^+ (□), and Li^+ (◆). The lines drawn were fitted by linear regression to obtain the mean conductances with different extracellular cations and gave values of 30.6 pS (n = 8) with Na^+, 30.8 pS (n = 4) with Cs^+, 22.4 pS (n = 5) with Li^+, and 31.7 pS (n = 5, data not shown) for K^+.



the resting potential of the cell, predicted to be about –60 mV from current clamp recordings under these recording conditions (4,13). This indicated that the reversal potential of these single-channel currents was ~0 mV, similar to the reversal potential of whole-cell currents. The linear current-voltage relation and reversal potential of the single-channel currents suggest that they arise from NSCCs that carry the MTX-sensitive whole-cell current.

To confirm the ion selectivity of the MTX-sensitive single-channels, outside-out patches were obtained from cells after activation of whole-cell inward currents by MTX (Fig. 3). Single channels with a conductance of 30 pS were observed

FIG. 4. Nonselective cation currents are regulated by extracellular, but not intracellular, MTX, and are also regulated by intracellular ATP. Whole-cell records from HIT cells bathed in 0.8 mmol/l glucose SES. The records in *A–C* start in the on-cell configuration, and the patch membrane was ruptured to enter the whole-cell recording mode at *arrowhead 1*. *A*: Dialysis of the cell (C_M 28.3 pF) with Cs-pipette solution + 50 µmol/l EGTA + 4 mmol/l Na_2ATP + 100 pmol/l MTX for about 480 s failed to activate a significant current. A 20-s pulse of extracellular 100 pmol/l MTX (*arrowhead 2*) then generated a current and a second MTX pulse (*arrowhead 3*) again reactivated the current. The sudden decrease in current at the end of the trace was accompanied by a decrease in the apparent cell capacitance, suggesting that a vesicle formed at the pipette tip. *B*: HIT cell (C_M 17.9 pF) dialyzed with Cs-pipette solution + 10 µmol/l EGTA (ATP-free). A 5-s pulse of 100 pmol/l MTX (*arrowhead 2*) shortly after break-in activated an inward current, whereas a second 10-s pulse (*arrowhead 3*) about 1,000 s after break-in had little or no effect. The activation of a current by MTX after about 1,000 s of whole-cell dialysis with 4 mmol/l ATP, but not after a similar period if dialysis with ATP-free solution, suggests that washout of intracellular ATP inhibits activation of the current by MTX, rather than this inhibition being a nonspecific washout phenomenon. *C*: HIT cell (C_M 10.8 pF) under the same recording conditions as in *B*. Dialysis with the ATP-free solution alone activated an inward current and subsequently desensitized the cell to a 20-s pulse of MTX (*arrowhead 2*). In batches of cells that exhibited current activation by dialysis with ATP-free solution, this activation was completely inhibited by the addition of 4 mmol/l ATP to the pipette solution, and partially inhibited by 2 mmol/l ATP (data not shown).

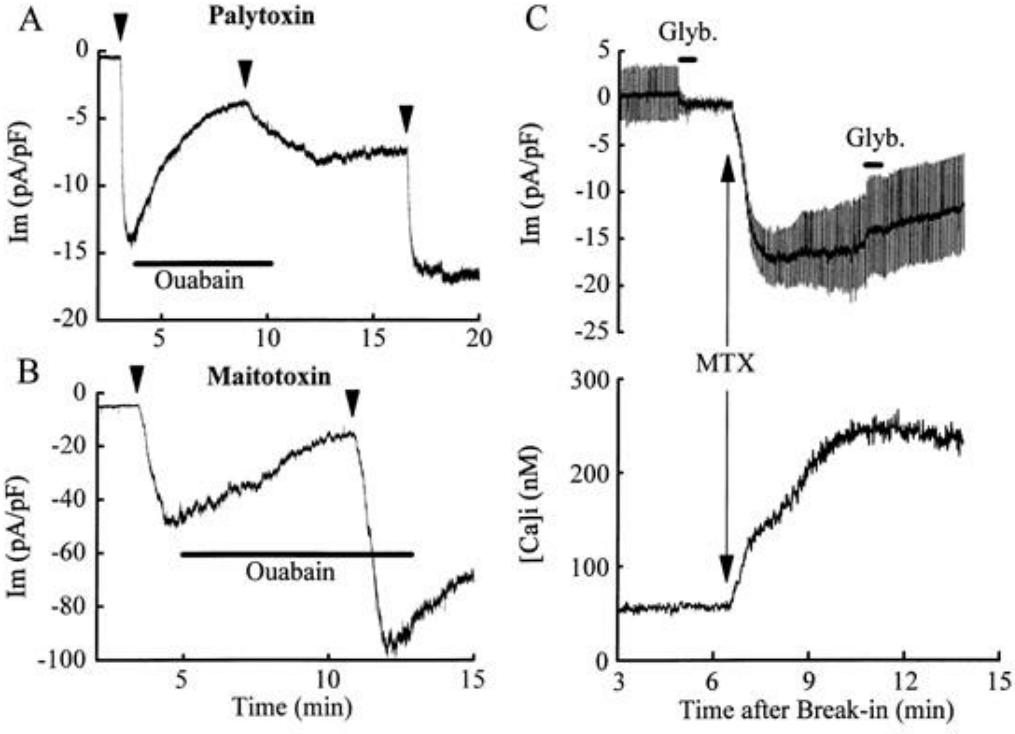


FIG. 5. MTX-activated currents are not inhibited by ouabain or by glyburide. *A*: Whole-cell record from a HIT cell (C_M 21.7 pF) bathed in 0.8 mmol/l glucose SES and dialyzed with Cs-pipette solution + 50 µmol/l EGTA + 4 mmol/l Na_2ATP. The records in *A* and *B* start 2 min after break-in to the whole-cell configuration, and cells were clamped at –70 mV. Three 5-s pulses of 100 pmol/l PTX were applied (*arrows*). The first pulse of PTX elicited a rapidly activating nonselective inward current. At the peak of this current response, the bath solution was changed to one containing 1 mmol/l ouabain (indicated by bar). In the presence of ouabain, the PTX-induced current rapidly reversed; a second pulse of PTX elicited a smaller and much slower activating current. Following washout of ouabain from the bath solution, the PTX-induced current persisted and a subsequent pulse of PTX again elicited a rapidly activating current that shows little inactivation. *B*: Whole-cell record from another HIT cell (C_M 12.0 pF) from the same plating using the same bath and pipette solutions as in *A*. Two pulses of 50 pmol/l MTX (*arrows*) were applied in the absence (first pulse) or presence (second pulse) of 1 mmol/l ouabain in the bath solution. No difference in the rate of activation of the MTX-induced currents was observed in the presence or absence of ouabain, indicating that PTX and MTX induce currents by different mechanisms. *C*: Whole-cell record from a HIT cell (C_M 14.3 pF) bathed in 0.8 mmol/l glucose SES and dialyzed with K-pipette solution + 2 mmol/l Na_2ATP + 25 µmol/l K_5Fura2. This record starts 3 min after break-in to the whole-cell configuration. The membrane conductance was monitored by applying 750-ms steps of ± 10 mV from the holding potential of –70 mV. Application of 100 nmol/l glyburide (Glyb; indicated by bars) inhibited the membrane conductance through K^+_{ATP} channels, indicated by the decreased amplitude of the current deflections elicited by the voltage steps. A 5-s pulse of 50 pmol/l MTX induced an inward current, increase in membrane conductance (*top*), and rise in $[Ca^{2+}]_i$ (*bottom*). A second pulse of glyburide did not affect the current or membrane conductance, indicating that sulfonylurea receptors are unlikely to play a role in generating the MTX-sensitive current.

in outside-out patches with SES plus 1 µmol/l TTX (to block voltage-gated Na^+ channels) and 10 µmol/l nimodipine (to block voltage-dependent, L-type Ca^{2+} channels) in the bath and Cs-pipette solution (to block K channels). Similar recordings were then made using bath solutions in which the NaCl of SES was substituted by CsCl, LiCl, or KCl; the channel conductance was unchanged (30 pS) by extracellular Cs^+ or K but was reduced to about 22 pS with extracellular Li^+ (Fig. 3). These data are consistent with previous data indicating that the reversal potential of MTX-activated whole-cell currents is not significantly changed with Na^+, K, Cs^+, or Li^+ bath solutions (14). MTX-activated single-channel currents were also blocked by extracellular application of Na^+-free, NMG-substituted saline; further, prolonged incubation (~2 h) in Ca^{2+}-free solution reversibly inhibited activation of the channels by MTX (data not shown). Whole-cell nonselective cation currents activated by MTX, GLP-1, PACAP, and analogs of cAMP were Ca^{2+}-dependent and impermeant to NMG (4,13,14), supporting the suggestion that the 30 pS nonselective channels carry these whole-cell currents.

Evidence that MTX is unlikely to act as an ionophore. It is important to consider the possibility that MTX generates ion currents by acting as an ionophore (22). To test this possibility, we dialyzed cells with Cs-pipette solution plus 50 µmol/l EGTA, 4 mmol/l ATP, and 100 pmol/l MTX (Fig. 4*A*). Intracellular dialysis with this MTX-containing solution did not stimulate the activation of a current and did not block activation of the current by 100 pmol/l extracellular MTX, suggesting that MTX binds to an extracellular site to activate the current.

It is possible that MTX interacts with an extracellular receptor that permits insertion of the toxin into the membrane, or that the toxin associates with such a membrane protein to form a channel. Two pulses of extracellular MTX at ~480 and ~1,000 s after break-in to form the whole-cell configuration both activated currents (Fig. 4*A*). This activation of currents by consecutive pulses of MTX might be predicted if MTX forms channels by being inserted into the membrane. However, cells dialyzed with ATP-free solution that responded to extracellular MTX when applied shortly after break-in failed to respond to

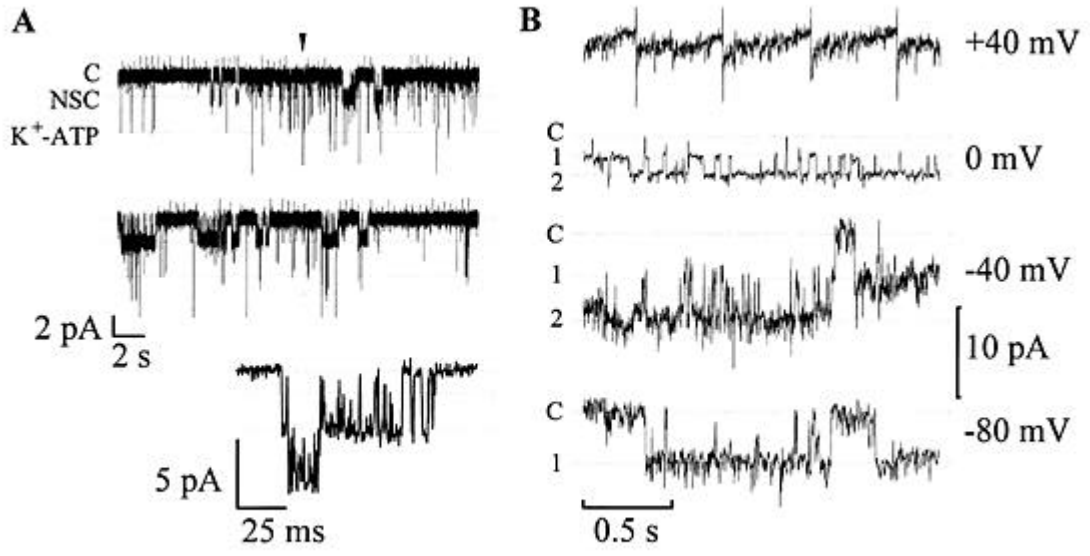


FIG. 6. Spontaneous activity of nonselective cation channels. *A*: An on-cell record from a mouse β-cell bathed in 5.5 mmol/l glucose SES. The patch pipette contained 140 KCl solution. The pipette was held at 0 mV and spontaneous openings of both nonselective cation channels (NSC) and K_{ATP} channels (K^+-ATP) from the closed state (*C*) were observed. The top two traces are continuous records and the lower trace shows K^+_{ATP} channel activity on an expanded time scale from the point indicated by the arrow head in the top trace. *B*: Shows continuous on-cell records from a human β-cell bathed in 2 mmol/l glucose SES. The patch pipette contained 140 KCl solution, and the patch was held at the potentials indicated, relative to the resting potential of the cell. This patch contained at least two channels (C, closed; 1, one channel open; 2, two channels open). This cell generated action currents at positive potentials. The conductance of these channels was estimated to be 24 pS, and the mean conductance in human β-cells estimated from four similar records was 25.6 ± 1.1 pS.

a second pulse of MTX at about 1,000 s after break-in (Fig. 4*B*). Therefore, if MTX is inserted into the membrane to form a channel by association with an integral membrane protein, this must be an ATP-dependent process. Similarly, dialysis with ATP-free solution sometimes resulted in the activation of an inward current without stimulation by MTX and, after such activation, cells subsequently failed to respond to MTX (Fig. 4*C*). These data suggest that intracellular ATP may normally hold these nonselective channels in the closed state, similar to the case for K_{ATP} channels, and that ATP may also participate in the activation of the channel by MTX, perhaps through a tyrosine-phosphorylation mechanism (14).

Palytoxin (PTX) is another marine toxin that has been suggested to interact with the Na:K-ATPase to form a nonselective cation channel (23,24) with a conductance of ~10 pS (25), though this mechanism has been disputed (26). PTX-stimulated HIT cells show an inward current (Fig. 5*A*) that is accompanied by a rise of $[Ca^{2+}]_i$ (not shown) in voltage-clamped cells. As previously reported (24,26), the PTX-induced current in HIT cells is inhibited by ouabain (Fig. 5*A*). In contrast, ouabain has no effect on MTX-induced currents (Fig. 5*B*), indicating the MTX does not act by forming a pore in association with the Na:K-ATPase. Further evidence to support this observation is shown in Fig. 5*C*. The sulfonylurea compound glyburide inhibits the Na:K-ATPase in HIT cells, in addition to its effects on K_{ATP} channels, by binding to a low-affinity site with an IC_{50} for the Na:K-ATPase of 20–40 nmol/l (27). Figure 5*C* shows that 100 nmol/l glyburide caused inhibition of the membrane conductance due to K_{ATP} channel activity in a whole-cell recording, but did not inhibit activation of the nonselective cation current by MTX.

A role for NSCCs in controlling the membrane potential of β-cells. The physiological role of Ca^{2+}-dependent NSCCs has remained elusive because, when studied in inside-out patches, they require cytosolic Ca^{2+} concentrations >0.1 mmol/l before they exhibit significant activation (16). Pancreatic acinar cells also express a Ca^{2+}-dependent cation current that shows a marked decrease in Ca^{2+}-sensitivity over a period of a few minutes (28). Activation of nonselective cation currents at physiological $[Ca^{2+}]_i$ levels by MTX, GLP-1, or PACAP (4,13,14) suggests that, in vivo, the Ca^{2+}-dependence of these channels is much lower than in inside-out patches.

In view of the ability of NSCCs to activate at physiological $[Ca^{2+}]_i$ levels, they could potentially play a role in controlling the membrane potential of β-cells under both stimulatory and nonstimulatory conditions for insulin secretion. The maximum current amplitudes in response to 10 nmol/l MTX that we have observed are about –11 nA (C_M 24.0 pF) at –70 mV, giving a maximum whole-cell conductance of about 160 nS. Under identical recording conditions, the single-channel conductance is about 30 pS and, assuming a channel open probability of 1, this gives an estimated number of channels within the cell membrane as >5,000, similar to estimates of the number of K_{ATP} channels in β-cells (1). Given this large number of NSCCs, a small level of spontaneous activity of these channels could account for the depolarized level of β-cell–resting potentials relative to the estimated K equilibrium potential. Spontaneous activity of NSCCs in a cell-attached patch from an unstimulated mouse β-cell at a pipette potential of 0 mV is shown in Fig. 6*A*. When the cell was bathed in 5.5 mmol/l glucose SES plus 10

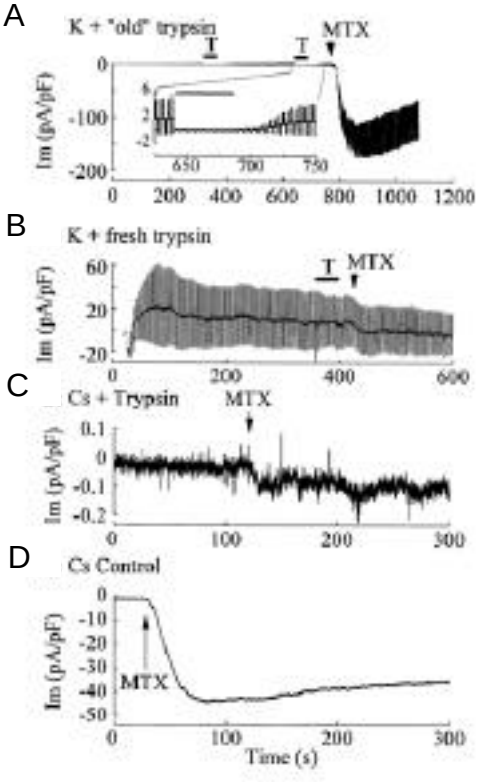


FIG. 7. Inhibition of the MTX-induced current by intracellular trypsin. *A*: Record of membrane current from a HIT cell (C_M 15.9 pF) dialyzed in the whole-cell configuration with K-pipette solution containing 20 µg/ml trypsin that had been at room temperature for about 5 h. The membrane conductance was monitored by applying ± 10 mV steps of 750-ms duration, increased during dialysis due to activation of K^+_{ATP} channels, indicated by the reversible inhibition of the membrane current steps by 100 µmol/l tolbutamide (T; shown on expanded scales in inset). A 1-s pulse of 10 nmol/l MTX (*arrow*) generated an inward membrane current. *B*: Membrane current from another HIT cell (C_M 12.0 pF) dialyzed with a fresh 20 µg/ml trypsin solution. The same tolbutamide test solution as used in *A* failed to inhibit the membrane conductance, consistent with the reported effects of trypsin to reduce the sulfonylurea sensitivity of K^+_{ATP} channels (22), suggesting that this trypsin solution was effective. A subsequent 1-s pulse of MTX (the same 10 nmol/l test solution as used in *A*) had only a very weak effect, suggesting that activation of the current is inhibited by intracellular trypsin. *C* and *D*: Traces showing similar effects in cells dialyzed with Cs-pipette solution plus 4 mmol/l ATP and 20 µg/ml trypsin (*C*; C_M 10.5 pF) or a control cell (*D*; no trypsin, C_M 38.0 pF). These results again demonstrate inhibition of the MTX-induced current by intracellular trypsin under conditions in which K^+ currents are inhibited by intracellular Cs^+. The cells in *C* and *D* were tested using the same 10 nmol/l MTX solution.

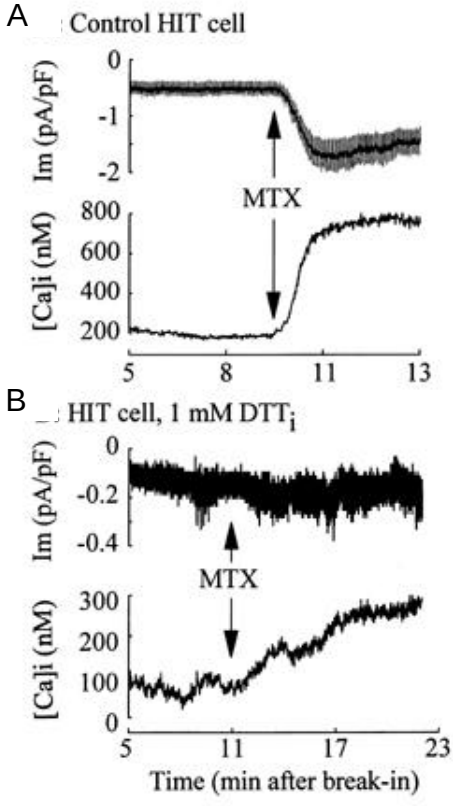


FIG. 8. Intracellular DTT inhibits MTX-sensitive currents in β-cells. *A* and *B*: Records obtained in the whole-cell configuration. Cells were dialyzed with Cs-pipette solution (to block K^+ currents) plus 25 µmol/l pentapotassium fura2 and 2 mmol/l ATP. *A*: Control records of membrane current (C_M 27.5 pF; *top*) and $[Ca^{2+}]_i$ (*lower*) starting 5 min after break-in. A 1-s pulse of MTX (*arrow*) generated an inward current and rise of $[Ca^{2+}]_i$. *B*: Similar records from a cell dialyzed with 1 mmol/l DTT added to the pipette solution. The cell (C_M 12.7 pF) showed very little inward current and a slow rise of $[Ca^{2+}]_i$.

µmol/l nimodipine and the pipette was filled with 140 KCl solution, spontaneous openings of both nonselective cation channels and K_{ATP} channels were observed. This observation is consistent with the suggestion that NSCCs induce depolarization of β-cells and thereby stimulate Ca^{2+}-dependent action potentials. Spontaneous activity of NSCCs was also observed in on-cell patches from human β-cells (Fig. 6*B*) with 140 KCl pipette solution. Cells were bathed in 2 mmol/l glucose SES; depolarizing the patch by 40 mV induced action currents (Fig. 6*B*, *top*). From the current amplitudes at hyperpolarized potentials, the mean conductance of NSCCs in human cells under these conditions was 25.6 ± 1.1 pS (n = 4).

Nonselective cation currents are trypsin sensitive. It has been shown previously that genistein, a tyrosine kinase inhibitor, inhibits the MTX-induced current (14), raising the possibility that tyrosine phosphorylation at an intracellular site may play a role in the activation of the current. Intracellular trypsin has been shown to modulate the activity of K_{ATP} channels in β-cells (29). We investigated the possible role of trypsin-sensitive sites in the activation of the nonselective cation current by dialysis of HIT cells with K-pipette solution plus 20 µg/ml trypsin. The K-pipette solution was used initially so that the decreased inhibitory effect of the sulfonylurea tolbutamide (100 µmol/l) could be used as a control for the efficacy of dialysis with the trypsin pipette solution in whole-cell recording. Control experiments in the absence of intracellular trypsin or with "old" trypsin solutions in which the proteolytic efficacy of the solution had diminished over 5–6 h were used to test for the inhibition of K_{ATP} by tolbutamide and activation of cation currents by the MTX test solution (Fig. 7*A*). Figure 7*B* shows that dialysis with fresh trypsin solution removes both inhibition of K_{ATP} by tolbutamide and activation of the inward current by MTX. Figure 7*C* shows inhibition of the inward current by intracellular trypsin when dialyzed into the cell in Cs-pipette solution to inhibit K currents; currents from a control cell using the same MTX test solution as in Fig. 7*C* are illustrated in Fig. 7*D*. These results suggest that activation of cation channels by MTX involves an intracellular, trypsin-sensitive site(s).

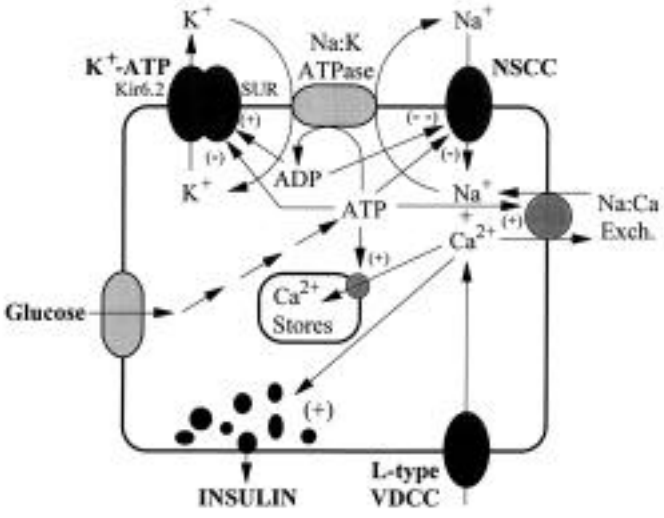


FIG. 9. A role for NSCCs in regulating the membrane potential of β-cells. The β-cell K^+_{ATP} channel is a hetero-octamer of the type 1 sulfonylurea receptor and the Kir 6.2 pore-forming subunit (38,39). Activity of K^+_{ATP} channels alone would set the cell membrane potential at their reversal potential ($V_{K\text{-}ATP}$). Spontaneous activity of NSCCs will depolarize the membrane relative to $V_{K\text{-}ATP}$, as the current through these channels has a reversal potential of –5 to –10 mV (14). A decrease in the activity of K^+_{ATP} channels will then result in cell depolarization as the membrane potential moves toward the reversal potential of NSCCs. This depolarization will activate VDCCs and stimulate insulin secretion. The increase of intracellular [Na^+] resulting from the influx of Na^+ through NSCCs and efflux of K^+ through K^+_{ATP} channels will then be corrected by activity of the Na:K-ATPase; activity of this pump may play a role in setting the submembrane ATP:ADP ratio that controls the activity of K^+_{ATP} channels, and may also influence the activity of NSCCs and the Na:Ca exchanger.

Nonselective cation currents are inhibited by cytosolic DTT, a reducing agent that disrupts disulfide bonds. Thiol reagents have long been known to influence insulin secretion from β-cells (30) and have been shown to modulate K_{ATP} and voltage-dependent Ca^{2+} channels in these cells (31–33). Sulflhydryl group–oxidizing (mercury, thimerosal, and oxidized glutathione) or –reducing (DTT and reduced glutathione) agents have also been shown to influence the activity of NSCCs (34,35); therefore, we investigated the potential role of sulfhydryl groups in the regulation of nonselective cation currents in β-cells (Fig. 8).

Dialysis of HIT-T15 cells with pipette solutions containing 100 nmol/l (n = 2) or 500 nmol/l (n = 4) $HgCl_2$ did not inhibit activation of the cation current by MTX and did not cause activation of the current alone (data not shown). However, dialysis with DTT inhibited the MTX-sensitive current in β-cells (Fig. 8). HIT cells dialyzed with Cs-pipette solution in the absence of DTT generated an inward current in response to 10 nmol/l MTX (Fig. 8*A*), whereas cells dialyzed with DTT failed to generate a significant inward current (Fig. 8*B*). When the same experiment was performed on mouse β-cells (Fig. 8*C* and *D*), dialysis with 1 or 2 mmol/l DTT partially inhibited currents activated by 10 nmol/l MTX. In primary mouse β-cells, currents were decreased from a control value of –273 ± 24 pA/pF (n = 2) to –112 ± 30 pA/pF (n = 6; P < 0.03), and in mouse-derived βTC3 cells, currents decreased from –443 ± 130 pA/pF (n = 5) to –88 ± 15 pA/pF (n = 5; P < 0.005) in the presence of 2 mmol/l intracellular DTT. Application of 1 mmol/l DTT to the extracellular face of the cell membrane did not inhibit activation of inward cation currents by MTX (data not shown).

DISCUSSION

We have shown that the Ca^{2+}-dependent nonselective cation current in β-cells is carried through 25–30 pS channels whose activation is inhibited by intracellular dialysis with trypsin and intracellular DTT, but not by extracellular DTT. It is well established that membrane-permeant thiol oxidizers inhibit insulin secretion (30), an effect that likely results from the inhibition of both K_{ATP} and voltage-dependent Ca^{2+} channels (33). Inhibition of K_{ATP} channels in β-cells by sulfhydryl-oxidizing agents is reversed by DTT (31–33); the opposite effect of the thiol redox state on the activity of K_{ATP} and NSCCs would have synergistic effects on the cell membrane potential. For example, reduction of intracellular disulfide bonds would inhibit NSCCs and activate K_{ATP}, effects that would lead to membrane hyperpolarization. However, the physiological basis for any such changes in channel activity as a result of the redox state of the cell remains speculative.

The MTX-sensitive NSCCs are also regulated by intracellular ATP levels ($[ATP]_i$). Dialysis with ATP-free solutions spontaneously activated currents in some batches of cells, suggesting that $[ATP]_i$ may inhibit channel activity. However, prolonged incubation with ATP-free solutions inhibited activation of NSCCs by MTX. These observations would be consistent with a mechanism through which ATP binds to an intracellular site to hold the channels in the closed state, with channels having a very low open probability at 4 mmol/l $[ATP]_i$, similar to the effects of the resting physiological $[ATP]_i$ on K_{ATP} channels. Activation of the channels could then occur through unbinding of ATP or through ATP utilization by a phosphorylation mechanism, consistent with the inhibition of MTX-induced current activation by the tyrosine kinase inhibitor, genistein (14). A phosphorylation mechanism might explain the inhibitory effect of prolonged dialysis with ATP-free solutions. ADP is a more potent inhibitor of Ca-NS channels in insulinoma cells than ATP (15), washout of ATP and ADP could then activate channels by unbinding these inhibitors, and channel desensitization could occur by dephosphorylation.

We also demonstrated that channels with the same conductance as MTX-sensitive channels show spontaneous activity under physiological conditions, and suggest that they play an important role in setting the membrane potential of pancreatic β-cells. Spontaneous activity of NSCCs, and activity of Na^+-coupled transporters, will generate an influx of Na^+ that will be compensated by the ouabain-sensitive Na:K-ATPase (Na-pump, Fig. 9). It has been suggested that there is a functional coupling between K_{ATP} channel activity and Na-pump activity in β-cells (27,36,37) in which ATP utilization by the Na-pump decreases the submembrane ATP:ADP ratio, thereby increasing the activity of K_{ATP} channels. Such a change in the ATP/ADP ratio might also cause increased inhibition of NSCCs, as ADP is a more effective inhibitor of these channels than ATP (15), providing a mechanism for feedback inhibition of NSCCs. Such sensitivity of NSCCs to the ATP/ADP ratio would provide a complementary regulatory mechanism to that of K_{ATP} channels. An increase in the ATP/ADP ratio in response to elevation of blood glucose would not only increase inhibition of K_{ATP}

channels but also decrease inhibition of NSCCs, with both effects combining to produce depolarization.

In conclusion, we propose that MTX-sensitive, Ca^{2+}-dependent NSCCs may play a critical role in setting the membrane potential of β-cells. Spontaneous activity of these channels will move the membrane potential positive to the reversal potential of K_{ATP} channels ($V_{K\text{-}ATP}$); this depolarizing conductance would allow the cell to depolarize when K_{ATP} channel activity is reduced by a rise in blood glucose or by hypoglycemic sulfonylureas. Similarly, an increase in the activity of K_{ATP} channels against a constant background activity of these NSCCs will cause hyperpolarization, as the membrane potential is able to move toward $V_{K\text{-}ATP}$.

ACKNOWLEDGMENTS

J.F.H. is an investigator with the Howard Hughes Medical Institute. We thank Maurice Castonguay for maintenance of cell cultures.

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