Maitotoxin (MTX) activates a Ca\(^{2+}\)-dependent non-selective cation current (I\(_{\text{Ca-NS}}\)) in insulinoma cells whose time course is identical to non-selective cation currents activated by incretin hormones such as glucagon-like peptide-1 (GLP-1), which stimulate glucose-dependent insulin secretion by activating cAMP signaling pathways. We investigated the mechanism of activation of I\(_{\text{Ca-NS}}\) in insulinoma cells using specific pharmacological reagents, and these studies further support an identity between MTX- and GLP-1-activated currents. I\(_{\text{Ca-NS}}\) is inhibited by extracellular application of genistein, econazole, and SKF 96365. This inhibition by genistein suggests that tyrosine phosphorylation may play a role in the activation of I\(_{\text{Ca-NS}}\). I\(_{\text{Ca-NS}}\) is not inhibited by incubation of cells in glucose-free solution, by extracellular tetrodotoxin, nimodipine, or tetraethylammonium, or by intracellular dialysis with 4-aminopyridine, ATP, ryanoctidine, or heparin. I\(_{\text{Ca-NS}}\) is also not significantly inhibited by staurosporine, which does, however, partially inhibit the MTX-induced rise of intracellular Ca\(^{2+}\) concentration. These effects of staurosporine suggest that protein kinase C may not be involved in the activation of I\(_{\text{Ca-NS}}\) but that it may regulate intracellular Ca\(^{2+}\) release. Alternatively, I\(_{\text{Ca-NS}}\) may have a small component that is carried through separate divalent cation-selective channels that are inhibited by staurosporine. I\(_{\text{Ca-NS}}\) is neither activated nor inhibited by dialysis with KF, KP + ATP, or GTP\(\gamma\)S (guanosine 5\(^\prime\)-O-(3-thiotriphosphate)), suggesting that GTP-binding proteins do not play a major role in the activation of this current.

The consensus model of glucose-stimulated insulin secretion is that closure of ATP-sensitive K\(^{-}\) channels (K\(^{-}\text{ATP}\)) permits membrane depolarization, activation of voltage-dependent Ca\(^{2+}\) channels (VDCCs) and the influx of Ca\(^{2+}\). Individual β-cells, however, are often unresponsive to glucose alone, but can become responsive by combined stimulation with glucose and hormones, such as insulinotropic hormone glucagon-like peptide-1 (GLP-1; Ref. 2), that elevate intracellular cAMP levels (3–5). One mechanism underlying this increased responsiveness is the enhanced closure of K\(^{-}\text{ATP}\) channels (2). A second mechanism by which GLP-1, pituitary adenylate cyclase-activating polypeptide (PACAP), and cAMP can induce β-cell depolarization is through the activation of voltage-independent, non-selective cation currents (6, 7). Similar cation currents are also activated by MTX, a polyether toxin isolated from dinoflagellates that in β-cells has been shown to stimulate insulin secretion and inositol trisphosphate (Ins\(1,4,5\)P\(_3\)) production (8) and to enhance the influx of monovalent cations (9).

MTX-sensitive currents are activated by depletion of intracellular Ca\(^{2+}\) stores (10), and GLP-1 enhances intracellular Ca\(^{2+}\) mobilization through the potentiation of ryanodine-sensitive Ca\(^{2+}\)-induced Ca\(^{2+}\) release at βTC3 cells (11, 12). Increased cAMP levels stimulate Ca\(^{2+}\) release from secretory granules and reduce mitochondrial Ca\(^{2+}\) uptake in β-cells (13, 14). These observations raise the possibility that the activation of non-selective cation currents by PACAP and GLP-1 may be a secondary consequence of glucose- and cAMP-dependent intracellular Ca\(^{2+}\) release. The physiological role of Ca\(^{2+}\)-release-activated currents in β-cells remains controversial, but such currents have been suggested to play a role in the cholinergic modulation of electrical bursting activity (15), and may control the membrane potential and intracellular Ca\(^{2+}\) oscillations in response to nutrient stimulation (10).

Both PACAP (16) and GLP-1 (17) are potent insulin secretagogues in the presence of slightly elevated glucose levels. The activation of a voltage-independent, non-selective cation current by these hormones under conditions that stimulate insulin secretion suggests that this current may play an important role in depolarizing β-cells to initiate insulin secretion. The aim of this study is to examine the mechanism of activation of the MTX-sensitive current and to compare the properties of I\(_{\text{Ca-NS}}\) with the current activated by GLP-1, PACAP, and cAMP to determine whether these currents are likely to be carried through the same channels.

**MATERIALS AND METHODS**

**Preparation of Cell Cultures**—HIT-T15 cells were obtained from the American Type Culture Collection. βTC6 cells were obtained from Dr. Shimon Efrat (Albert Einstein College of Medicine, New York, NY). HIT-T15 cells (passages 67–75) were maintained in Ham’s F-12 medium containing 10 mM glucose, 10% heat-inactivated horse serum, and 2.5% fetal bovine serum. βTC6 cells (passages 24–59) were maintained in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 15% horse serum, and 2.5% fetal bovine serum. Culture media also contained 100 units/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), which facilitates their adherence to glass. Cultures were
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MTX activates a non-selective cation current mainly carried by Na⁺. Panel A shows the whole cell current from a perforated patch (Cs-pipette solution) voltage-clamped βTC6 cell (C m 11.3 pF) initially bathed in SES (142 mM Na⁺). All bath solutions contained 5 mM TEA, 2 μM TTX, and 100 μM Cd²⁺ (to inhibit Ca²⁺-activated K⁺ channels, voltage-dependent Na⁺ channels, and VDCCs respectively). FIG. 1 shows that a hyperpolarizing voltage clamp step from 0 mV to −70 mV caused a rapid and reversible increase of [Ca²⁺]i that has a reversal potential of −7.8 mV (Fig. 1) that has a reversal potential of −7.8 mV. This current was observed in all cells tested, and its reversal potential becomes more negative as [Na⁺] increases (Fig. 1, Table I), although the shift is less than predicted from the Nernst potential for Na⁺. ICa-NS is also observed in solutions where extracellular Na⁺ is replaced by Cs⁺, K⁺, Li⁺, choline⁺, or NMG⁺ and in cells dialyzed with K⁺.

MTX activates a non-selective cation current in βTC6 cells (Fig. 1) that has a reversal potential of −7.8 ± 1.0 mV (n = 72) in SES (142 mM Na⁺). This current was observed in all cells tested, and its reversal potential becomes more negative as [Na⁺] increases (Fig. 1, Table I), although the shift is less than predicted from the Nernst potential for Na⁺. ICa-NS is also observed in solutions where extracellular Na⁺ is replaced by Cs⁺, K⁺, Li⁺, choline⁺, or NMG⁺ and in cells dialyzed with K⁺.

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Table I
Sodium dependence of $I_{Ca-NS}$ reversal potential (mV)

Table shows the effect of changing [Na\(^+\)], on the reversal potential (mV) of the MTX-activated current ($I_{Ca-NS}$) in βTC6 cells. The reversal potential shifted by less than would be predicted from the Nernst potential for Na\(^+\) suggesting that the channels are non-selective. Other values given in this table are control values obtained from cells subsequently transferred to different [Na\(^+\)] solutions. The number of cells recorded is indicated in parentheses.

| 142 Na | 70 Na/70 NMG | 70 Na/50 Ca | 14 Na/NMG | Na-free/NMG | Na-free/100 Ca |
|--------|--------------|------------|-----------|-------------|---------------|
| −7.8 ± 1.0 (72) | −21.6 ± 2.1 | −30.6 ± 2.9 | −38.4 ± 2.5 | −39.7 ± 1.8 | −25.0 ± 1.4 |

Table II
Ionic dependence of reversal potentials (mV) for MTX-induced currents

Table shows the effect of extracellular (bath) and intracellular (pipette) ion substitutions on the reversal potential of $I_{Ca-NS}$. The reversal potential with SES (Na\(^+\)) bath solution and Cs\(^+\)-pipette solution is taken to be the reference value and ion substitutions that produced a significant change in the reversal potential are indicated. These data confirm the non-selective nature of the MTX-sensitive channels. *p < 0.001; **, p = 0.005; ***p = 0.014.

| Cytosol | Bath | Na | K | Cs | Li | Choline |
|---------|------|----|---|----|----|---------|
| Ca      | −7.8 ± 1.0 (72) | −2.6 ± 0.7 (5) | +2.7 ± 1.7 (6)** | −3.9 ± 0.6 (7) | −22.8 ± 1.0 (6)* |
| Na      | −2.7 ± 1.2 (6) | +1.3 ± 3.3 (7)** |                |                |                |
| Choline | +1.0 ± 4.9 (9)* |                |                |                |                |
| NMG     | +1.0 ± 4.9 (9)* |                |                |                |                |

Fig. 2. Hyperpolarizing voltage steps increase [Ca\(^{2+}\)], following MTX stimulation. Fig. 2 shows simultaneous records of membrane current (top trace) and [Ca\(^{2+}\)] (lower trace) from a HIT-T15 cell (C\(_0\), 14.0 pF) held initially at −70 mV in perforated patch voltage clamp. Membrane potential is shown as the inset. The cell was bathed in 0.8 mM glucose SES plus 1 μM TTX and was dialyzed with Cs-pipette solution. A 15-s hyperpolarizing step to −100 mV before stimulation with MTX produces a small increase in the holding current and no detectable increase of [Ca\(^{2+}\)]. The cell was then stimulated with 25 μM MTX (indicated by bar), and a step from −70 mV to −100 mV following MTX produces an increase in the holding current and a reversible rise of [Ca\(^{2+}\)]. The bath solution was then changed to a Ca\(^{2+}\)-free (Mg\(^{2+}\)-substituted + 50 μM EGTA + 1 μM TTX) solution resulting in a decrease of [Ca\(^{2+}\)], and inhibition of the hyperpolarization-induced increase of [Ca\(^{2+}\)]. On return to SES (2.6 mM Ca\(^{2+}\)), [Ca\(^{2+}\)], increases and hyperpolarization again produces a further rise of [Ca\(^{2+}\)].

Stimulation with MTX caused a small, reversible increase of the membrane current and a fall of [Ca\(^{2+}\)], (Fig. 3A). Holding currents at −70 mV increased from −0.96 ± 0.16 pA/pF in SES to −1.64 ± 0.30 pA/pF (p = 0.005) and [Ca\(^{2+}\)], decreased from 117 ± 15 nM to 99 ± 15 nM on removal of extracellular Ca\(^{2+}\); (not statistically significant, p = 0.4). In 3/18 cells the holding current showed a much larger increase on transfer to Ca\(^{2+}\)-free solution (Fig. 3B). The holding current in these cells increased by 21.8 ± 4.3 pA/pF and [Ca\(^{2+}\)], reduced from 75 ± 15 nM to 37 ± 4 nM (p = 0.1). This current inactivates rapidly, and [Ca\(^{2+}\)], is transiently elevated when SES (2.6 mM Ca\(^{2+}\)) is reintroduced to the bath (Fig. 3B). Elevation of [Ca\(^{2+}\)], and activation of a non-selective cation current were also observed in a similar subset of cells following stimulation with thapsigargin (2–10 μM, data not shown), as reported previously (10).

Table III
Ionic dependence of reversal potentials (mV) for MTX-induced currents

Table shows the effect of extracellular (bath) and intracellular (pipette) ion substitutions on the reversal potential of $I_{Ca-NS}$. The reversal potential with SES (Na\(^+\)) bath solution and Cs\(^+\)-pipette solution is taken to be the reference value and ion substitutions that produced a significant change in the reversal potential are indicated. These data confirm the non-selective nature of the MTX-sensitive channels. *p < 0.001; **, p = 0.005; ***p = 0.014.

| Cytosol | Bath | Na | K | Cs | Li | Choline |
|---------|------|----|---|----|----|---------|
| Ca      | −7.8 ± 1.0 (72) | −2.6 ± 0.7 (5) | +2.7 ± 1.7 (6)** | −3.9 ± 0.6 (7) | −22.8 ± 1.0 (6)* |
| Na      | −2.7 ± 1.2 (6) | +1.3 ± 3.3 (7)** |                |                |                |
| Choline | +1.0 ± 4.9 (9)* |                |                |                |                |
| NMG     | +1.0 ± 4.9 (9)* |                |                |                |                |

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Further evidence for the influx of divalent cations through MTX-sensitive channels is suggested by increased Mn\(^{2+}\)-quenching of intracellular fura-2 fluorescence following MTX-stimulation (Fig. 3C). Fig. 3C (i and ii) shows the raw fura-2 fluorescence emission values, application of a 60-s pulse of Ca\(^{2+}\)-free, Mn\(^{2+}\)-substituted solution before stimulation with MTX produces a gradual quenching of the fluorescence signals, a small increase in the holding current (Fig. 3C, iii), and little or no change in the 350 nm/380 nm fluorescence ratio (Fig. 3C, iv). Following activation of the inward current and rise in [Ca\(^{2+}\)], a pulse of Ca\(^{2+}\)-free, Mn\(^{2+}\)-substituted solution produces rapid quenching of fura-2 fluorescence (Fig. 3C, i and ii) with little or no effect on membrane current (Fig. 3C, iii) and reduces the fluorescence ratio (Fig. 3C, iv), consistent with a decrease in [Ca\(^{2+}\)]. Similar activation of Mn\(^{2+}\) quenching has been observed following stimulation of insulinoma cells with PACAP (6), cAMP (22), and thapsigargin (23, 24).

A role for Ca\(^{2+}\) influx through MTX-sensitive channels is further supported by the effects of applying high [Ca\(^{2+}\)] solutions. A Na\(^{+}\)-free test solution containing 100 mM Ca\(^{2+}\) was applied to a βTC6 cell prior to stimulation with MTX and produces a small increase of [Ca\(^{2+}\)], (Fig. 4), similar to the effects of applying elevated [Ca\(^{2+}\)], solutions to mouse islets (25). Application of 100 mM [Ca\(^{2+}\)], solution following stimulation with MTX produces a pronounced inhibition of $I_{Ca-NS}$ (Fig. 4) and a negative shift in the reversal potential of the current (Table I). This inhibition of $I_{Ca-NS}$ by the 100 mM Ca\(^{2+}\) solution is accompanied by a rise of [Ca\(^{2+}\)], (Fig. 4). These data could be
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Fig. 3. Effects of removing extracellular Ca\(^{2+}\) on membrane currents and changes in [Ca\(^{2+}\)]. Panel A shows simultaneous recordings of membrane current (top trace) and [Ca\(^{2+}\)] (lower trace) from a βTC6 cell (Cm, 23.5 pF) bathed in SES and held at −70 mV in perforated patch voltage clamp using K-pipette solution. Bath perfusion with Ca\(^{2+}\)-free solution (50 μM EGTA, indicated by bar) produces a small reduction of [Ca\(^{2+}\)], from 138 nM to 118 nM and a small increase in the holding current, these effects are reversed by perfusion with SES. A 10-s pulse of 50 μM MTX induces an inward current and rise of [Ca\(^{2+}\)]. The rise of [Ca\(^{2+}\)] was rapidly and reversibly reduced by bath perfusion with Ca\(^{2+}\)-free solution (bar). I\(_{\text{Ca-NS}}\) was slightly reduced by the Ca\(^{2+}\)-free solution in this cell. The βTC6 cell shown in B (Cm, 7.7 pF) was bathed in SES plus 5 mM TEA, 1 μM TTX, and 1 μM nimodipine and was held at −70 mV using Cs-pipette solution. An inward current developed, and [Ca\(^{2+}\)] fell from 52 nM to 41 nM during bath perfusion with Ca\(^{2+}\)-free solution (50 μM EGTA plus TEA, TTX, and nimodipine, indicated by bar). The reversal potential of the current was estimated from voltage ramps (r, data not shown) to be −13 mV. The current rapidly inactivates when SES is reintroduced into the bath and [Ca\(^{2+}\)]. Increases to 95 nM. A subsequent pulse of 50 μM MTX (50 s, starting at arrow) activates an inward current (reversal potential −12 mV) and a rise in [Ca\(^{2+}\)]. Panel C shows that activation of I\(_{\text{Ca-NS}}\) increases Mn\(^{2+}\)-quenching of intracellular fura-2 fluorescence. This HIT-T15 cell (Cm, 38.8 pF) was bathed in SES plus 5 mM TEA, 1 μM TTX, and 1 μM nimodipine and was held at −70 mV using Cs-pipette solution. Application of a 60-s pulse of Ca\(^{2+}\)-free, Mn\(^{2+}\)-substituted solution before MTX produces a gradual quenching of fura-2 fluorescence (i and ii), a small increase in the holding current (iii) similar to that seen in panel A, and, with little change in the fluorescence ratio (iv). Stimulation with MTX (1-s pulse, arrow) activates the inward current and rise in [Ca\(^{2+}\)], and a subsequent 60-s pulse of Ca\(^{2+}\)-free, Mn\(^{2+}\)-substituted solution produces rapid quenching of fura-2 fluorescence (i and ii) and a fall in [Ca\(^{2+}\)], (iv) with little effect on membrane current (iii).

explained by Ca\(^{2+}\) influx through a single class of non-selective cation channel with a lower permeability to Ca\(^{2+}\) than to Na\(^{+}\) under these experimental conditions. Alternatively, I\(_{\text{Ca-NS}}\) may have two (or more) components, one component being monovalent cation selective, and the other, smaller, component being selective for divalent cations.

The non-selective cation current activated by 8-bromo-cAMP in HIT-T15 cells is inhibited by whole cell dialysis with Ca\(^{2+}\)-free, EGTA-buffered solutions or by the cells with BAPTA (1,2-bis(2-aminophenoxy)ethane N\(_2\)N\(_2\)N\(_2\)N\(_2\)-tetracetic acid), a Ca\(^{2+}\)-chelator (7) leading us to test the dependence of I\(_{\text{Ca-NS}}\) activation on [Ca\(^{2+}\]) (Fig. 5). Whole cell recordings from HIT-T15 cells were performed with either normal K-pipette solution (nominally Ca\(^{2+}\)-free) or the same solution with 5 mM EGTA added (Ca\(^{2+}\)-free). Whole cell dialysis with Ca\(^{2+}\)-free intracellular solution inhibited activation of I\(_{\text{Ca-NS}}\) compared with control cells from the same platings dialyzed with nominally Ca\(^{2+}\)-free solution or compared with cells in perforated patch voltage clamp (Fig. 5). These observations suggest that physiological [Ca\(^{2+}\)] levels are required for activation of the current. It is notable that dialysis of the cells with Ca\(^{2+}\)-free solution does not induce activation of the current alone, whereas dialysis with this solution might be expected to deplete intracellular Ca\(^{2+}\) stores and thus activate store-operated currents.

Ca\(^{2+}\)-activated non-selective cation (Ca-NS) channels are expressed in β-cells that are activated at cytosolic [Ca\(^{2+}\)] > 10^−4 M and are blocked by 1 mM ATP and also by 10 mM 4AP when applied to the cytosolic face of isolated, inside-out patches (26, 27). Activation of I\(_{\text{Ca-NS}}\) is observed with physiological Ca\(^{2+}\) levels but inhibition of the current by dialysis of cells with Ca\(^{2+}\)-free solutions suggests that it may be carried through Ca-NS channels. To test this possibility, βTC6 cells were bathed in glucose-free SES with 5 mM TEA and 10 mM glycyrhrizic acid (to block Ca\(^{2+}\)-activated K\(^{+}\) channels and K\(^{+}\)-ATP channels) and dialyzed in the whole cell recording mode with K-pipette solution and 10 mM 4AP either with or without 2 mM ATP. MTX-induced currents in cells dialyzed without ATP had a mean peak amplitude of −23.6 ± 7.9 pA/pF (n = 6),
and cells from the same platings dialyzed with 2 mM ATP had peak amplitudes of $-22.5 \pm 7.5$ pA/pF ($n = 6$, not significantly different, $p = 0.9$). These data indicate that activation of ICa-NS is not glucose-dependent, consistent with previous reports of MTX-stimulated, glucose-independent insulin secretion (28). These differences in the sensitivity of ICa-NS to $[\text{Ca}^{2+}]_i$ and to block by ATP and 4AP in whole cell recordings compared with inside-out patches may indicate that ICa-NS is not carried through the Ca-NS channels reported previously (26, 27) or may reflect the different recording configurations.

The non-selective cation current activated by PACAP in βTC6 cells is inhibited by SKF 96365 (22), a blocker of depletion-activated currents (29) that inhibits MTX-induced Ca$^{2+}$ influx and insulin secretion (30). Fig. 6 shows that application of 50 μM SKF 96365 reversibly inhibits ICa-NS in βTC6 cells, similar to its effect on the PACAP-induced current in these cells (22), further supporting the suggestion that these currents are carried by the same channel type.

The activation of Mn$^{2+}$ quenching of intracellular fura-2 fluorescence by MTX (Fig. 3C) is similar to that observed following thapsigargin treatment of insulinoma cells (23, 24). Thapsigargin-sensitive Ca$^{2+}$ pools can also be depleted by econazole, which inhibits Ca$^{2+}$-ATPases (31) and thereby elevates $[\text{Ca}^{2+}]_i$, and also inhibits Mn$^{2+}$ quenching in HIT cells (23). We therefore tested the effect of econazole on the activation of ICa-NS. Fig. 7A shows that 10 μM econazole significantly reduces the amplitude of ICa-NS compared with control cells from the same platings. The basal (pre-MTX) $[\text{Ca}^{2+}]_i$ decreased from 449 ± 313 nM to 215 ± 122 nM ($n = 6$, $p = 0.01$), whereas the peak rise (increase above basal levels) of $[\text{Ca}^{2+}]_i$ was reduced from 142 ± 92 nM (control) to 44 ± 16 nM ($p < 0.001$) by econazole.

The effect of genistein, a tyrosine kinase inhibitor, on the activation of ICa-NS was tested as capacitative Ca$^{2+}$ influx activated by thapsigargin can also be inhibited by genistein (Ref. 32, Fig. 7B). The peak amplitude of ICa-NS reduces from $-20.2 \pm 3.3$ pA/pF ($n = 6$) to $-8.2 \pm 2.6$ pA/pF ($n = 8$, $p = 0.01$) following exposure of cells from the same platings to 10 μM genistein. Basal $[\text{Ca}^{2+}]_i$ (pre-MTX) is not significantly affected by genistein (88 ± 11 nM in control cells, 118 ± 19 nM in genistein, $p = 0.2$), while the peak rise of $[\text{Ca}^{2+}]_i$ during MTX responses was reduced from 1026 ± 388 nM (control) to 313 ± 92 nM (genistein, $p = 0.06$).

Protein kinase C (PKC) activates capacitative Ca$^{2+}$ entry in rat insulinoma (RINm5F) cells, and this activation of Ca$^{2+}$ entry can be blocked by 1 μM staurosporine (24). We therefore tested the effects of 1 μM staurosporine on membrane currents and on the rise of $[\text{Ca}^{2+}]_i$ following MTX stimulation of βTC6 cells (Fig. 7C). Staurosporine had no significant effect on ICa-NS, the mean peak amplitude of control currents was $-54.2 \pm 19.5$ pA/pF ($n = 5$) compared with $-51.1 \pm 7.7$ pA/pF ($n = 6$, $p = 0.9$) in cells from the same platings after addition of 1 μM staurosporine to the bathing solution. The pre-MTX basal $[\text{Ca}^{2+}]_i$ in control cells was 61 ± 11 nM and 96 ± 15 nM in cells bathed in staurosporine ($p = 0.1$), whereas the peak rise (increase above basal levels) of $[\text{Ca}^{2+}]_i$ was reduced from 1653 ± 252 nM to 142 ± 28 nM ($p < 0.001$) by staurosporine.

Activation of some types of non-selective cation current has been shown to be mediated by GTP-binding proteins (33). We therefore examined the potential role of GTP-binding proteins in the activation of ICa-NS by dialysis of βTC6 cells with Ca$^{2+}$-pipette solution supplemented with 10 mM 4AP, 2 mM Na$_2$ATP, and either 10 mM KF, 10 mM KF + 100 μM AlF$_3$, or 100 μM GTPγS. The bath contained SES with 5 mM TEA and 1 μM TTX. Whole cell dialysis of cells for 15–20 min with KF ($n = 4$), KF + AlF$_3$ ($n = 5$), or GTPγS ($n = 5$) failed to activate inward currents in cells that all subsequently responded to stimulation with MTX (data not shown).

MTX stimulates an increase in Ins(1,4,5)P$_3$ levels in β-cell lines (8), and this increase might activate ICa-NS through an intracellular Ca$^{2+}$ release mechanism. Heparin is a specific blocker of Ins(1,4,5)P$_3$ receptors that should inhibit activation of ICa-NS if Ins(1,4,5)P$_3$-gated Ca$^{2+}$ stores play an important role. βTC6 cells were dialyzed in whole cell recordings with Ca-pipette solution plus 10 mM 4AP, 2 mM Na$_2$ATP, and 0.5 mg/ml heparin for 5–4 min before stimulation with MTX. Activation of ICa-NS was not inhibited in cells dialyzed with heparin. The amplitude of the currents was not significantly different from that in control cells from the same platings, and the reversal potential of the currents was $-7.0 \pm 2.0$ mV ($n = 9$), not significantly different from control values ($p = 0.96$).

GLP-1 enhances intracellular Ca$^{2+}$ mobilization from ryanodine-sensitive stores in βTC3 cells, and ryanodine reduces the amplitude of [Ca$^{2+}$]$, spikes produced by depolarizing voltage

FIG. 6. Inhibition of ICa-NS by SKF 96365. A βTC6 cell (Cm 8.0 pF) was held at −70 mV in perforated patch voltage clamp with Cs-pipette solution and bathed in SES. A 1-s pulse of 50 μM MTX was applied (arrow) and elicited an inward current. 50 μM SKF 96365 was then applied (indicated by bar) by pressure ejection from a pipette positioned close to the cell. SKF 96365 caused a reversible inhibition of the MTX-activated current in 8 cells tested.

FIG. 7. Effects of inhibitors on ICa-NS and the associated rise of $[\text{Ca}^{2+}]_i$. Panel A shows normalized ICa-NS amplitudes (left panel, inverted scale) in βTC6 cells held at −70 mV in perforated patch voltage clamp (K-pipette solution). Control currents (Cont., $n = 5$) were obtained from cells bathed in SES, 10 μM econazole was then added to the bath solution ( Econ., $n = 5$), and currents from the same platings of cells recorded in this solution. Econazole produced a significant reduction in the current amplitude ($p = 0.01$), and the rise of $[\text{Ca}^{2+}]_i$, was also reduced ($p = 0.09$). Panel B shows control ICa-NS amplitudes (left panel, inverted scale, $n = 6$) and ICa-NS after addition of 100 μM genistein (genis., $n = 8$) to the bath solution. Genistein produces a significant reduction in current amplitude ($p = 0.01$). The equivalent reduction of the change of $[\text{Ca}^{2+}]_i$, is shown in the right panel ($p = 0.06$). Panel C (left panel) shows control ICa-NS amplitudes (inverted scale, $n = 5$) and ICa-NS amplitudes following addition of 1 μM staurosporine (Stauro.) to the bath solution. Staurosporine had no significant effect on ICa-NS current amplitudes ($p < 0.01$) but significantly reduced the rise of $[\text{Ca}^{2+}]_i$, ($p < 0.001$, right panel).
clamp steps within 1 or 2 min (11). We introduced 100 μM ryanodine into βTC6 cells by whole cell dialysis in the Cs/4AP/ATP-pipette solution (as above) and allowed 3–4 min dialysis before stimulation with MTX. Ryanodine failed to prevent activation of I_{Ca-NS} under these conditions, and the current amplitude and reversal potential (-8.0 ± 1.9 mV, n = 5) are not significantly different from control cells (p = 0.97).

**DISCUSSION**

We propose that MTX activates the same non-selective cation current as stimulation with the peptide hormones GLP-1 and PACAP (6, 7, 22). These hormones couple through GTP-binding proteins (G-proteins) to activate adenyl cyclase and elevate intracellular cAMP in β-cells (3–5), and cAMP analogs can also activate these non-selective cation currents. However, activation of G-proteins, by dialysis of cells with KF, KF + AlF₃, or GTPγS (compounds that stimulate G-protein-mediated activation of non-selective cation currents in epithelial cells (33) and activate K⁺_ATP channels in RINm5F and HIT-T15 insulinoma cells (34, 35)), neither activated nor inhibited the MTX-sensitive current.

Depletion of intracellular Ca^{2+} stores activates a MTX-sensitive current in mouse β-cells (10), and the stimulation of Ins(1,4,5)P₃ by MTX (8) raises the possibility that Ins(1,4,5)P₃ may play a role in the activation of this current. Parasympathetic, cholinergic stimulation of β-cells stimulates Ins(1,4,5)P₃ production, potentiates glucose-induced insulin secretion (36), and also activates a TTX-insensitive Na⁺-dependent depolarizing current (37) that may also be carried through Ca^{2+} release-activated non-selective cation channels (15). The role of intracellular Ca^{2+} release in the activation of this current has, however, been disputed, and cholinergic activation of this Na⁺ current is reported to be mediated by M₃-type muscarinic receptors being coupled to Na⁺ channels (38). We observed that dialysis with heparin, a blocker of Ins(1,4,5)P₃ receptors, failed to inhibit activation of I_{Ca-NS}, suggesting that Ca^{2+} release from these stores may not be critical.

The presence of a Ca^{2+} store depletion-activated current in pancreatic β-cells was proposed from studies showing: 1) that the state of filling of endoplasmic reticulum stores could regulate the membrane potential in mouse β-cells (39), 2) that thapsigargin can activate Mn^{2+} quenching of intracellular fura-2 in RINm5F cells (24), and 3) that the Mn^{2+} quenching pathway is inhibited by econazole in HIT-T15 cells (23). Activation of I_{Ca-NS} by thapsigargin and its inhibition by both econazole and SKF 96365 are consistent with the suggestion that I_{Ca-NS} may represent a Ca^{2+} release-activated current (10, 31).

A role for PKC in the activation of store-operated Ca^{2+} entry in RINm5F cells was suggested from observations that the sustained Ca^{2+} rise in response to combined stimulation with PKC-activating phorbol esters and thapsigargin is inhibited by staurosporine (24). We observed that staurosporine reduced the MTX-induced Ca^{2+} rise, but did not significantly inhibit the amplitude of I_{Ca-NS}, suggesting that PKC may not play a direct role in the activation of I_{Ca-NS} but does regulate [Ca^{2+}], responses. Such effects could be mediated through inhibition of Ca^{2+} release from intracellular Ca^{2+} stores, or could reflect the inhibition of a small, divalent cation-selective component of I_{Ca-NS} carried through a distinct set of channels other than the non-selective cation channels. The high concentration of staurosporine (1 μM) used in these experiments would also be expected to inhibit cAMP-dependent protein kinase, and further studies are required to elucidate the role(s) of PKC and cAMP-dependent protein kinase in the pathway(s) leading to the activation of I_{Ca-NS} and regulation of [Ca^{2+}], changes.

Inhibition of both I_{Ca-NS} and the MTX-induced [Ca^{2+}] rise by genistein, an inhibitor of tyrosine kinases (40) that blocks thapsigargin- and carbamol-induced Ca^{2+} entry (32, 41), suggests a role for tyrosine phosphorylation in the activation pathway of I_{Ca-NS}. However, the role of tyrosine phosphorylation remains ambiguous as only certain tyrosine kinase inhibitors (including genistein) effectively block capacitative Ca^{2+} entry (42). Thapsigargin-induced Ca^{2+} entry can also occur in the absence of detectable tyrosine phosphorylation but is still inhibited by tyrosine kinase inhibitors (43), and, therefore, the role of tyrosine kinases in activation of I_{Ca-NS} remains to be clarified.

Ca-NS channels are expressed in β-cells that are activated at cytosolic [Ca^{2+}] > 10⁻⁴ M (26, 27). Activation of I_{Ca-NS} is observed at physiological [Ca^{2+}], (approximately 100 nm) and is inhibited by dialysis of cells with Ca^{2+}-free solution, similar to the inhibition of cAMP-activated currents (7) and suggesting a Ca^{2+}-dependent step in the activation pathway of these channels. Ca-NS channels are also expressed in pancreatic acinar cells, and these channels are activated at much lower cytosolic Ca^{2+} concentrations in whole cell records than in isolated patches (44); a similar difference in Ca^{2+} sensitivity seems likely to occur for Ca-NS channels in β-cells. Ca-NS channels are blocked by 1 mM ATP and by 10 mM 4AP when applied to the intracellular face of isolated membrane patches (27); however, these two compounds did not inhibit I_{Ca-NS} when introduced into the cytosol by whole cell dialysis (at 2 mM and 10 mM, respectively). It remains to be determined whether these differences in sensitivity to [Ca^{2+}], 4AP, and ATP are a consequence of the different recording conditions or suggest that Ca-NS channels do not carry I_{Ca-NS}.

Reducing extracellular [Cl⁻] has no effect on I_{Ca-NS} amplitude or on its reversal potential, confirming the cation selectivity of the channel. Depletion of intracellular ATP levels through bathing cells in glucose-free media and dialyzing with ATP-free solutions or dialysis of cells with 2 mM ATP has no effect on I_{Ca-NS} amplitudes. This further distinguishes the MTX-induced current from the non-selective anion current described in insulin-secreting cells that increases in amplitude upon dialysis with 2 mM ATP (45).

Elevation of [Ca^{2+}] is observed following the activation of non-selective cation currents by MTX, GLP-1, or PACAP in voltage clamped cells where activation of voltage-dependent Ca^{2+} channels is prevented (6, 7, 22). This rise of [Ca^{2+}], is reversed by removal of extracellular Ca^{2+}, suggesting that Ca^{2+} influx is associated with the non-selective current, although it remains to be determined whether a single class of channel is permeant to both monovalent and divalent cations, or if two (or more) distinct conductances are involved. The physiological role of Ca^{2+} influx associated with I_{Ca-NS} in the stimulation of insulin secretion remains to be determined. It has been reported that sustained Ca^{2+} influx through L-type VDCCs is strongly coupled to insulin secretion from HIT-T15 cells, whereas more transient Ca^{2+} influx through N-type Ca^{2+} channels is only weakly coupled (46). The Ca^{2+} influx associated with I_{Ca-NS} is prolonged and may, therefore, be able to contribute to the sustained [Ca^{2+}], elevation that triggers secretion. However, the magnitude of this Ca^{2+} influx is likely to be small compared with that through L-type channels as, under physiological conditions, the cells will depolarize to a value close to that for the reversal potential of I_{Ca-NS}, and also influx through L-type VDCCs raises [Ca^{2+}], very rapidly, whereas the [Ca^{2+}], increase associated with I_{Ca-NS} develops much more slowly. This slow time course of the rise in [Ca^{2+}], is consistent with a small amplitude Ca^{2+} influx and would explain why it is difficult to resolve a decrease in I_{Ca-NS} amplitude on changing
to Ca²⁺-free solution with normal extracellular Na⁺ concentrations. It therefore seems that the main physiological role for these non-selective cation currents in the control of insulin secretion will be to depolarize the membrane potential and activate VDCCs.

The currents activated by GLP-1, PACAP, cAMP analogs, and MTX are Ca²⁺-dependent non-selective cation currents that activate over tens of seconds and persist for extended periods following removal of the stimulus. These currents are all insensitive to TTX, L-type Ca²⁺ channel blockers, TEA, and ryanodine but are inhibited by NMG, SKF96365, and La³⁺. Based upon these similarities between the temporal properties of the currents, and their associated [Ca²⁺], changes, and the pharmacology of the currents, we propose that these agents activate the same non-selective cation channels. The precise mechanism(s) controlling the activation of these non-selective cation channels remains to be determined, but a role for tyrosine kinase-induced phosphorylation is suggested by the effects of genistein. Activation of I_Ca,NS may also be controlled by the state of filling of intracellular Ca²⁺ stores, or may be partly due to Ca²⁺ release from intracellular stores raising cytosolic Ca²⁺ levels. We propose that activation of MTX-sensitive non-selective cation channels may play an important role in depolarizing β-cells in response to stimulation by GLP-1 and PACAP during feeding to initiate insulin secretion without large elevations of blood glucose. We also suggest that Ca²⁺ is permeant through the MTX-sensitive channels and suggest that spontaneous activity of these channel currents may form the depolarizing, non-selective background conductance that permits Ca²⁺ influx (25) and opposes the activity of ATP-sensitive K⁺ channels in regulating the resting potential of β-cells under both basal conditions and in response to hormonal stimulation.

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