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Loperamide mobilizes intracellular Ca^{2+} stores in insulin-secreting HIT-T15 cells

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1 We have investigated the effects of loperamide on intracellular Ca^{2+} stores and membrane K^+ channels in insulin-secreting hamster insulinoma (HIT-T15) cells.

2 In cell-attached patch-clamp mode, loperamide ($3-250\,\mu$M) activated large single-channel currents. The loperamide-activated currents were tentatively identified as Ca^{2+}-activated K^+ channel (K_{Ca}) currents based on their single-channel conductance (145 pS), apparent reversal potential, and insensitivity to tolbutamide. Smaller single-channel currents with a conductance (32 pS) indicative of adenosine triphosphate-sensitive K^+ channels (K_{ATP} channels) were also recorded, but were insensitive to loperamide.

3 Surprisingly, the loperamide-activated currents persisted in the absence of extracellular Ca^{2+}. Yet under these conditions, we still measured loperamide-induced Ca^{2+} increases. These effects are dose dependent. Loperamide had no effects in the inside-out patch configuration, suggesting that loperamide does not directly activate the channels with large conductance, but does so secondarily to release of Ca^{2+} from intracellular stores.

4 Carbachol ($100\,\mu$M), an agonist of muscarinic receptors, which mediates IP_3-dependent intracellular Ca^{2+} release, enhanced the effects of loperamide on K_{Ca} channels.

5 Both the putative K_{Ca} currents and Ca^{2+} signals induced by loperamide (with '0' $[Ca^{2+}]_o$) were abolished when the intracellular Ca^{2+} stores had been emptied by pretreating the cells with either carbachol or thapsigargin, an endoplasmic reticulum Ca^{2+}-ATPase inhibitor that blocks reuptake of calcium.

6 These data indicate that loperamide in insulin-secreting β-cells evokes intracellular Ca^{2+} release from IP_3-gated stores and activates membrane currents that appear to be carried by K_{Ca}, rather than K_{ATP} channels.

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; K_{ATP} channel, adenosine triphosphate-sensitive K^+ channel; K_{Ca} channel, Ca^{2+}-activated K^+ channel; SOC channel, store-operated Ca^{2+} channel

Introduction

In this paper, we examine the effect of loperamide on single-channel K^+ currents and intracellular Ca^{2+} signals in insulin-secreting hamster insulinoma (HIT-T15) cells.

Loperamide is a widely used antidiarrheal opioid receptor agonist, but at higher concentrations it is thought also to modulate the activity of ionic channels either directly or as a result of altered Ca^{2+} signaling. Thus, colonic mucosal secretion may be blocked by loperamide by a mechanism involving Cl^- channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, and the calmodulin system (Diener *et al*., 1988). In hippocampal neurons, loperamide at micromolar concentrations blocks a broad spectrum of high-voltage-activated Ca^{2+} channels, and, less effectively, NMDA-activated currents (Church *et al*., 1994). Furthermore, in a wide variety of cell types, loperamide has been found to increase Ca^{2+} influx, but only after targeting IP_3-sensitive intracellular Ca^{2+} stores with the likely consequence of mobilizing store-operated Ca^{2+} (SOC) channels (Daly *et al*., 1995; Harper *et al*., 1997; Daly & Harper, 2000). To clarify the effects of loperamide on ionic channels and Ca^{2+} signaling, we studied single-channel currents and intracellular Ca^{2+} transients in individual clonal pancreatic β cells (HIT-T15 cells).

Pancreatic β cells respond to intermediate glucose concentrations (~ 8 mM) with a reduction in K^+ permeability leading to membrane depolarization followed by volleys of Ca^{2+}-dependent action potentials that elevate the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and trigger the release of insulin (Atwater *et al*., 1978; Wollheim & Sharp, 1981; Henquin & Meissner, 1984; Prentki & Matschinsky, 1987). At least three types of K^+ channels play a role in β cells. First, an ATP-sensitive channel is blocked by intracellular ATP (Cook & Hales, 1984) or by glucose metabolism (Ashcroft *et al*., 1984; Misler *et al*., 1986) and has been linked to the depolarization of

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the membrane from −70 mV (in the absence of glucose) to −60 mV (in the presence of glucose), which is the threshold for activation of Ca^{2+}-current-mediated action potentials. Second, a delayed rectifier K^+ channel is rapidly activated during each action potential spike (Rorsman & Trube, 1986; Zunkler *et al*., 1988). Third, different K^+ channels (K_{Ca}) with high conductance, activated by $[Ca^{2+}]_i$ and membrane depolarization, are conspicuously present in pancreatic β cells from rodents and insulin-secreting cell lines (Atwater *et al*., 1979; Marty & Neher, 1982; Atwater *et al*., 1983; Cook *et al*., 1984; Eddlestone *et al*., 1989; Tabcharani & Misler, 1989; Ämmälä *et al*., 1991; Bordin *et al*., 1995; Göpel *et al*., 1999). However, the specific roles of each of the subtypes of β cell K_{Ca} channels have not been determined.

The role of intracellular Ca^{2+} stores in stimulus-secretion coupling is also uncertain. In addition to directly affecting exocytosis, sequestration and release of Ca^{2+} could affect membrane potential via Ca^{2+}-activated channels or store-operated channels. Such mechanisms likely underlie the enhancement of glucose-induced electrical activity and insulin secretion by muscarinic agonists (Bertram *et al*., 1995; Bordin *et al*., 1995), which stimulate IP_3-dependent mobilization of stored Ca^{2+}. Glucose causes an initial uptake of Ca^{2+} into the endoplasmic reticulum (Roe *et al*., 1994; Chow *et al*., 1995), possibly followed by voltage- or Ca^{2+}-dependent release of the ion (Roe *et al*., 1993; Worley *et al*., 1994). These observations suggest that intracellular Ca^{2+} stores and the ionic currents coupled to them may also be involved in glucose-induced electrical signaling in the pancreatic β cell.

The primary purpose of this study was to examine the effects of loperamide on HIT cells, which are known to possess ionic channels and Ca^{2+} stores of the types implicated in the mode of action of the drug. Contrary to what might be expected from the literature, we found that loperamide appeared to activate the large conductance K_{Ca} channel, yet did not require extracellular Ca^{2+}. With this insight, we proceeded to demonstrate that loperamide mobilized Ca^{2+} from intracellular stores, and that it therefore may serve as a means to explore the regulatory role of intra- and extracellular Ca^{2+} in the control of insulin secretion in pancreatic β cells.

Methods

Cell preparation

Experiments were performed on pancreatic β cells from a cell line derived from HIT-T15 cells. The stock was purchased from American Type Culture Collection (Manassas, VA, U.S.A.) and maintained in F-12 K medium supplemented with 10% dialyzed horse serum, 2.5% fetal bovine serum, 100 U ml^{-1} penicillin, and 0.05 mg ml^{-1} streptomycin in a 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days and the cells were subcultured once a week. The passage range of the HIT cells used was 59 – 70.

Cells were plated on 35-mm dishes and maintained in culture for 2 – 3 days. Before experiments, the culture medium was replaced with extracellular Krebs-Ringer (KR) solution containing (mM): 140 NaCl, 4 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 2.8 or 5.6 glucose, and 10 HEPES at pH 7.4. In many of the experiments, we used a 'Ca^{2+}-free' extracellular KR solution, which contained no added Ca^{2+}, but included 5 mM of the Ca^{2+}-chelator EGTA to bind trace contaminants. The glucose concentration was 2.8 or 5.6 mM in different experiments, which rendered most cells quiescent.

Loperamide, carbachol, tobutamide, thapsigargin, and thimerosal were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Charybdotoxin was from RBI, which is now owned by Sigma. F-12 Nutrient Mixture medium, fetal bovine serum, dialyzed horse serum, trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA), and penicillin-streptomycin (10,000 U ml^{-1} penicillin G sodium and 10,000 μg/ml streptomycin sulfate) were from GIBCO/BRL, (Rockville, MD, U.S.A.) and Biofluids Inc. (Rockville, MD, U.S.A.).

Recording of single-channel and whole-cell membrane currents

Patch-clamp experiments were conducted at room temperature (20 – 25°C). For single-channel recording, we used mainly the cell-attached configuration. In a few experiments, we used excised patches in the inside-out configuration (Figure 5). Electrodes were made from microhematocrit capillary glass (Blue-Tip) using a BB-CH-PC microcomputer-controlled multistep puller (Mecanex, Geneva, Switzerland). Electrodes were coated to near the tip with Sylgard (Dow Corning, Midland, MI, U.S.A.). For cell-attached measurements, pipettes were filled with a solution of the following composition (mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 5 Na-HEPES and had tip resistance from 8 to 10 MΩ. Recordings started after a high resistance seal (> 5 GΩ) was formed between the pipette tip and the cell membrane. Single-channel currents were recorded using an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt-Eberstadt, Germany), and stored continuously in digital form on VCR tapes using a pulse code modulator (Sony PCM 501-ES). Single-channel current records were later analyzed using commercially available software packages (pCLAMP, IPROC and LPROC, Axon Instrument, Foster City, CA, U.S.A.).

Ca^{2+}-imaging

Two-dimensional confocal Ca^{2+} imaging was used to estimate the intracellular Ca^{2+} concentration in multiple nondialyzed cells loaded with 5 μM Fluo-3 AM for 20 – 60 min at 37°C. The experiments were conducted following previously published methods (Cleemann *et al*., 1998). In Ca^{2+}-imaging experiments, rapid switching (< 50 ms) between different external solutions was accomplished using an electronically controlled multibarrelled perfusion system (Cleemann & Morad, 1991).

Static images of Ca^{2+}-dependent fluorescence (Figures 3, 8) show a change in the average fluorescence intensity (ΔF) calculated from several frames recorded before and during exposure to loperamide. Changes in the intracellular Ca^{2+} concentration were estimated pseudoratiometrically (F/F_0) by measuring the time course of fluorescence (F) relative to the initial fluorescence prior to interventions (F_0) (Wang *et al*., 2000). Considering the affinity of Fluo-3 ($K_d \cong 350$ nM) and the approximate resting $[Ca^{2+}]_i$ (50 – 100 nM), it is estimated that measured fluorescence values ($F/F_0 < 5$) do not produce saturation. Bleaching was assessed by measuring for extended periods under control conditions and was minimized by using

signal averaging in combination with a low intensity of the scanning excitation beam.

Results

Loperamide activates a high-conductance channel and enhances intracelluar Ca^{2+} even in the absence of extracellular Ca^{2+}

Figure 1 shows traces from a cell-attached patch recording from a HIT cell. Loperamide (250 μM) greatly increased the frequency of single-channel openings at both 0 and +20 mV. The superfusing KR solution contained 2.5 mM Ca^{2+} throughout the experiment. Loperamide decreased the mean closed time from 819 to 29 ms so that an expanded time scale had to be used (insets) to resolve individual openings after the addition of the drug. The measured unitary currents were of small amplitude (<2 pA) under control conditions (panel a) while numerous single-channel currents of larger amplitude (2–10 pA) were dominant (panel b) after switching to the solution with loperamide. Analysis of the records obtained at different holding potentials (−20 to +20 mV) suggests that loperamide induced openings of a high-conductance channel of 145 pS (Figure 1c, channel 1) without affecting the unitary properties of the low-conductance channel of 32 pS (Figure 1c, channel 2) seen before the addition of the drug (Figure 1c, control). The large single-channel currents were rarely observed under control conditions. They appeared with increasing frequency but constant amplitude when the superfusion control solution was exchanged with one containing loperamide over a period of ~1 min. The patch pipette contained a KCl-based solution, so that it is plausible that the measured inward currents were carried mainly by K^+. Notice that we aimed to preserve physiological Ca^{2+} fluxes and therefore did not take any measures, such as KCl depolarization, to stabilize the potential of the cell membrane outside the patch. It is possible therefore that the unclamped membrane potential of the cell, and thereby the potential difference across the patch, may have changed somewhat after activation of single-channel activity by loperamide. The recordings shown in Figure 1 suggest that loperamide activates a large conductance channel in HIT cells.

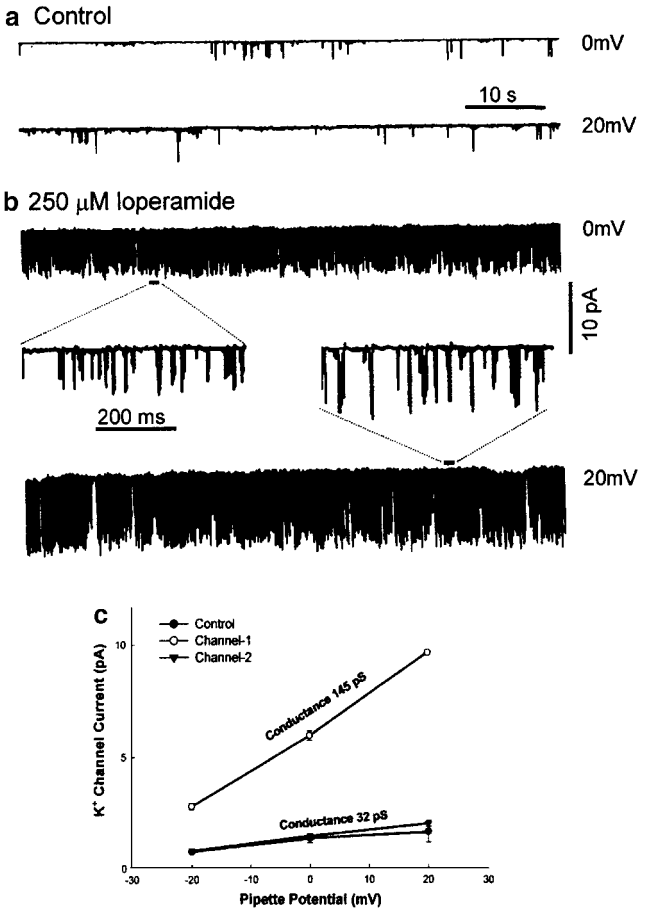

Figure 1 Loperamide activates channels with high conductance in HIT cells. The tracings show single-channel currents recorded at different holding potentials (0, +20 mV) from a cell-attached patch of a HIT cell under control conditions (panel a) and in the presence of 250 μM loperamide (panel b). Loperamide caused a large increase in the open probability of the channels so that recording with an expanded time-scale was required to resolve single-channel openings (insets of panel b). Panel c illustrates the single-channel slope conductances of two channels (145 and 32 pS) determined from recordings at −20, 0, and 20 mV. The superfusing solutions used in this experiment all contained 2.5 mM Ca^{2+} and 2.8 mM glucose.

The following experiments were carried out to test the hypothesis that the high-conductance channel activated by loperamide is a K_{Ca} channel, while the low-conductance channel is an ATP-dependent K^+ channel that does not respond to loperamide. The activation of a Ca^{2+}-activated K^+ current might depend on the influx of Ca^{2+} through Ca^{2+} channels in the cell membrane or on the release of Ca^{2+} from intracellular Ca^{2+} stores. To distinguish these possibilities we carried out experiments in which loperamide was applied in a Ca^{2+}-free solution to which 5 mM EGTA had been added to bind trace contaminants (Figure 2).

In the normal KR solution with 2.5 mM $CaCl_2$, only a few openings of the high-conductance channel were observed (panel a). Removing extracellular Ca^{2+} and adding 5 mM EGTA to the superfusion solution (Ca^{2+}-free solution) abolished all channel activity (Figure 2b). Yet under these conditions, exposure to 30 μM loperamide still produced the characteristic large single-channel currents (Figure 2c), indicating that extracellular Ca^{2+} is not required to activate the loperamide-sensitive current.

Using 2-D confocal Ca^{2+} imaging of multiple cultured HIT cells, we explored whether loperamide might mobilize Ca^{2+} from an intracellular store or whether its effect was independent of Ca^{2+} signaling. Figure 3 illustrates changes in the intracellular Ca^{2+} activity measured pseudoratiometrically with fluo-3 (Cleemann *et al*., 1998). In the presence of 2.5 mM Ca^{2+}, rapid exposure to loperamide (250 μM) caused a substantial rise in intracellular Ca^{2+}. The Ca^{2+} signal reached a steady level in ~30 s and returned to the basal level 2–5 min after removal of loperamide. When loperamide was applied again after first switching to a Ca^{2+}-free solution and adding the drug 1 min later, it still produced an increase in intracellular Ca^{2+}, although the signal was generally of somewhat reduced amplitude (Figure 3a and b). To examine the reason for this decline we reversed the sequence so that loperamide was applied first in the absence and then in the presence of 2.5 mM Ca^{2+} (Figure 3c and d). The results were nearly identical suggesting that the decreasing response

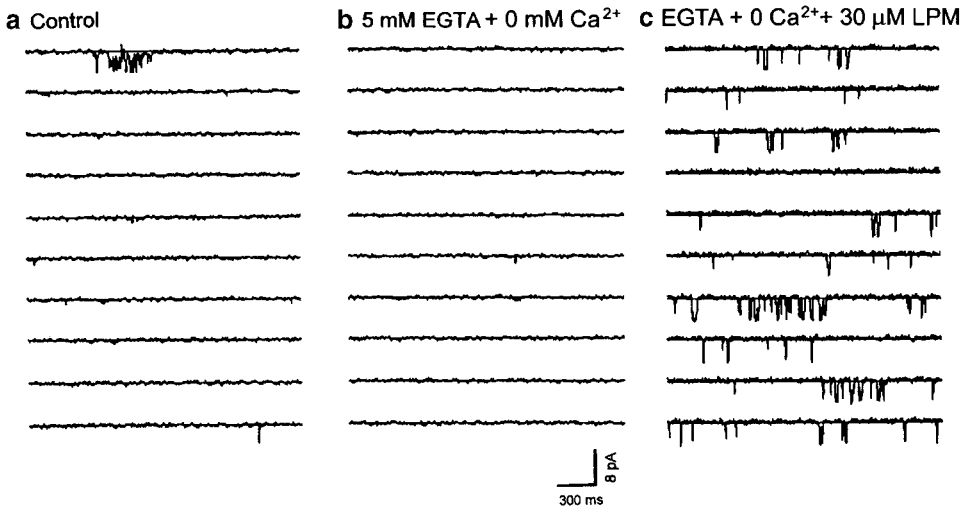

Figure 2 Activation of large single-channel currents by loperamide in the absence of extracellular Ca^{2+}. Single-channel currents recorded in the cell-attached configuration from a HIT cell superfused with KR solution containing 2.5 mM Ca^{2+} (panel a), 3 min after changing to a Ca^{2+}-free solution to which 5 mM EGTA had been added (panel b), and 5 min after changing to a Ca^{2+}-free solution which, in addition to 5 mM EGTA, also contained 30 μM loperamide (panel c). The glucose concentration was 5.6 mM throughout the experiment.

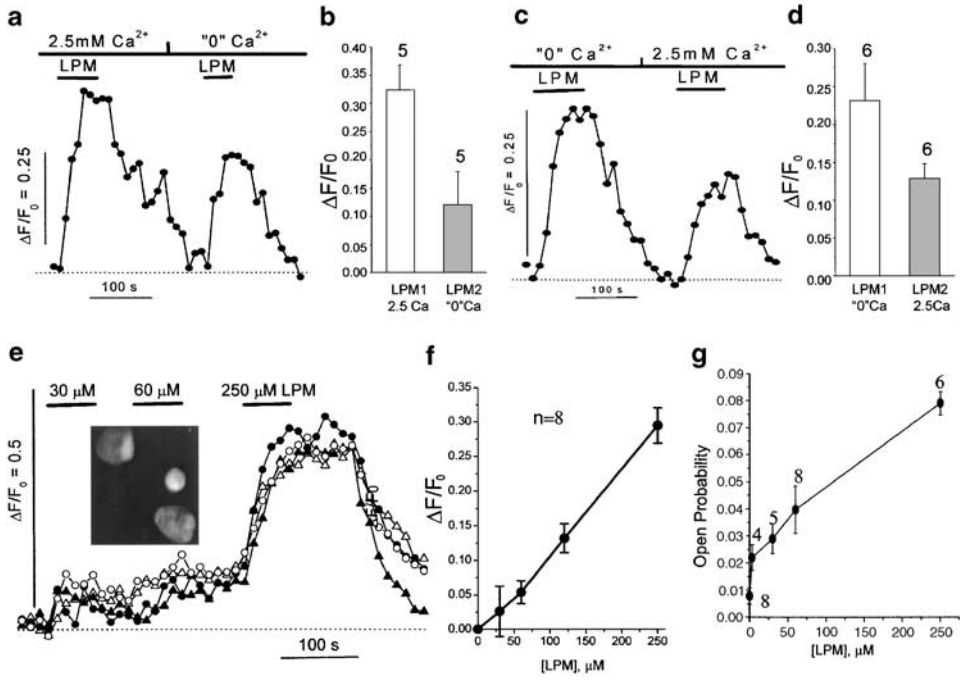

Figure 3 The loperamide-induced increase in intracellular Ca^{2+} is independent of extracellular Ca^{2+}. Panels (a, c, and e) show the change in the intracellular Ca^{2+} concentration measured with Fluo-3 in representative cells. The increase in the intensity of Ca^{2+}-induced fluorescence (ΔF) was normalized relative to the fluorescence (F_0) measured at the beginning of the recordings. The additions of loperamide and the change of extracellular Ca^{2+} concentration are indicated by bars over the traces. The upper panels show comparisons of rise in intracellular Ca^{2+} evoked by 250 μM loperamide when the extracellular Ca^{2+} concentration was first 2.5 and then 0 mM (panels a, b; $n=5$), or *vice versa* (panels c, d, $n=6$). Panel f shows the relation between the increase in intracellular Ca^{2+} and the concentration of loperamide based on experiments with successive increases in the loperamide concentration (panel e). The solvent for loperamide (up to 0.8% ethanol) produced no detectable change in the Ca^{2+} signal. The inset in Panel (e) shows a confocal image of Fluo-3 loaded HIT cells, often clustered 2 or 3 together. Brightly fluorescent Ca^{2+}-overloaded cells, and nonresponsive cells were excluded from numerical analysis. Panel g shows the dose-response curve for the open probability of the single-channel current. The threshold of detection was set at $\sim$0.5 pA, so that smaller single-channel currents were also included in the analysis giving rise to an open probability of 0.008 ± 0.003 in the absence of the drug.

observed in most cells during repeated exposure to loperamide was not related to absence or presence of extracellular Ca^{2+}. The more significant finding is therefore that loperamide, independent of extracellular Ca^{2+}, may activate a Ca^{2+}-dependent K^+ current by releasing Ca^{2+} from intracellular stores.

Dose dependence of the loperamide effects

The dose dependence of loperamide was tested by quantifying both single-channel and intracellular Ca^{2+} measurements. Successive applications of increasing concentrations of loperamide (30, 60, and 250 μM) evoked Ca^{2+} transients of increased amplitude that settled at a steady level within 30–60 s after each solution change (Figure 3e). The effect of loperamide on intracellular Ca^{2+} was significant at 30 μM, and increased nearly linearly with increasing concentration up to 250 μM (Figure 3f). In single-channel recordings, the different concentrations of loperamide (3, 30, 60, and 250 μM in the Ca^{2+}-free external solution) caused a similar increase in the open probability (Figure 3g). These results indicate that the effects of loperamide on both Ca^{2+} transients and open probability increased nearly linearly up to 250 μM.

Tolbutamide does not suppress the current activated by loperamide

The experiment illustrated in Figure 4 shows that the current activated by loperamide is not blocked by tolbutamide, a cell-permeant specific blocker of K_{ATP} channels. No single-channel activity was observed in normal KR solution with 2.5 mM $CaCl_2$ and 0 mV holding potential (Figure 4a). Switching to 30 μM loperamide in Ca^{2+}-free KR solution elicited prominent single-channel openings. In the presence of loperamide, tolbutamide (100 μM in Ca^{2+}-free KR solution) appeared not to reduce the channel activity (Figure 4c). This observation was confirmed by quantitative off-line analysis in which the detection threshold was set low (0.25 pA) to include both the large- and small-conductance channels. With loperamide alone (cf. Figure 4b), 1185 events in $n=3$ records yielded a mean open time of 14.4 ± 4.5 ms (s.d.), open probability 10 ± 10% (s.d.), and mean amplitude 1.1 ± 0.1 pA (s.d.). Analysis of matched records obtained after addition of tolbutamide (cf. Figure 4c) produced 830 events with mean open time 14.0 ± 1.2 ms (s.d.), open probability 7 ± 5% (s.d.), and mean amplitude 1.6 ± 0.1 pA (s.d.). The similarity of single-channel data in the absence and presence of tolbutamide suggests that the K_{ATP} channel does not contribute significantly to the loperamide-activated current.

Interestingly, we found that when charybdotoxin (ChTX), a specific antagonist of the 'maxi' K_{Ca} channel, was added to the bath solution, loperamide subsequently did not activate noticeable single-channel currents (data not shown). However, the implications of this finding for the identification of the loperamide-activated channel are not clear, since even a large concentration of the highly basic peptide ChTX (100 nM) may not gain access to the cell-attached membrane patch unless perfused through the pipette.

Experiments of the type illustrated in Figure 4 were performed with the drugs (loperamide, tolbutamide, and charybdotoxin) added not only to 'Ca^{2+}-free' KR solution, but also to KR solution with the normal 2.5 mM Ca^{2+}. Although the experiments with normal Ca^{2+} concentration were not chosen for illustration, they gave rise to loperamide-induced currents similar to those seen in Ca^{2+}-free solution. Thus, it is neither the Ca^{2+}-free solution (Figure 2b), nor loperamide in combination with 'Ca^{2+}-free' solution that activates the large-conductance current.

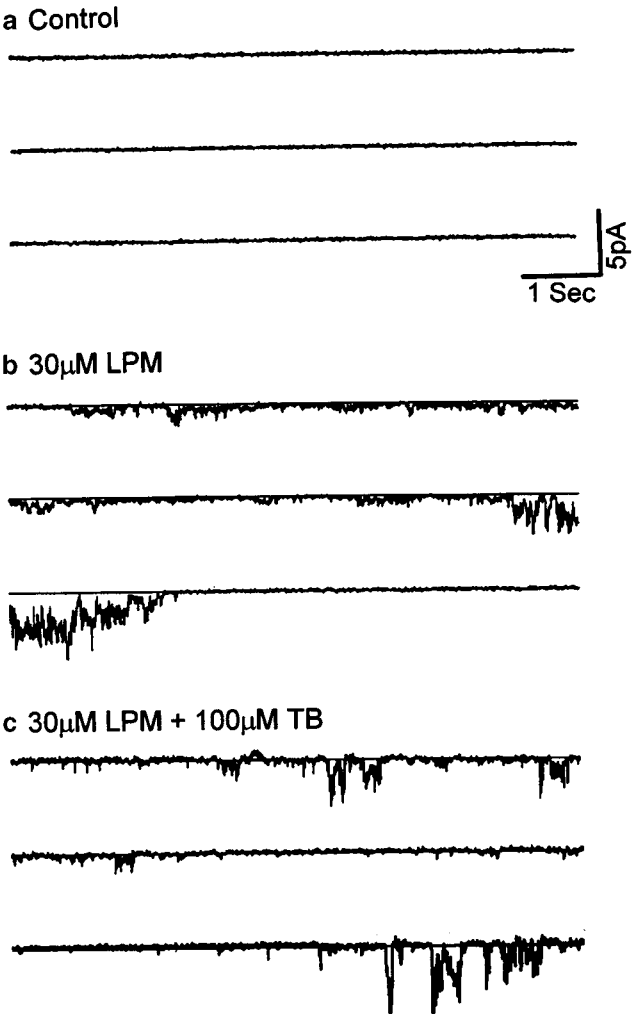

Figure 4 The single-channel currents activated by loperamide are insensitive to tolbutamide. Single-channel currents were measured in the cell-attached configuration under control conditions with 2.5 mM Ca^{2+} (panel a) after 3 min exposure to 30 μM loperamide (LPM) in '0' Ca^{2+} solution (panel b), and after 100 μM of the specific K_{ATP} channel blocker tolbutamide had been added to the loperamide-containing, but Ca^{2+}-free solution (panel c). The records are representative of three similar experiments conducted in different cells. The glucose concentration was 5.6 mM.

Loperamide has no effects in an inside-out patch configuration

To investigate whether loperamide directly affects the large conductance channel, we measured the single-channel currents from cultured HIT cells in the inside-out patch configuration. The internal membrane of the cell was exposed to the KR solution containing 0 mM $CaCl_2$ and 5 mM EGTA. The holding potential was 0 mV. Under these conditions, numerous channel openings appeared (Figure 5a). Mean open time was 18.5 ± 3 ms ($n=3$), close time 176.1 ± 10 ms ($n=3$), open probability 25 ± 6% ($n=3$), close probability 75 ± 8% ($n=3$), and current amplitude 4.6 ± 0.1 pA ($n=3$) (Figure 5c). Applying loperamide (30 μM) to the bath solution (internal side of cell membrane) did not increase the channel activity (Figure 5b). Mean open time was 18.2 ± 3 ms ($n=3$), close time 170 ± 6 ms ($n=2$), open probability 20 ± 4% ($n=3$), close

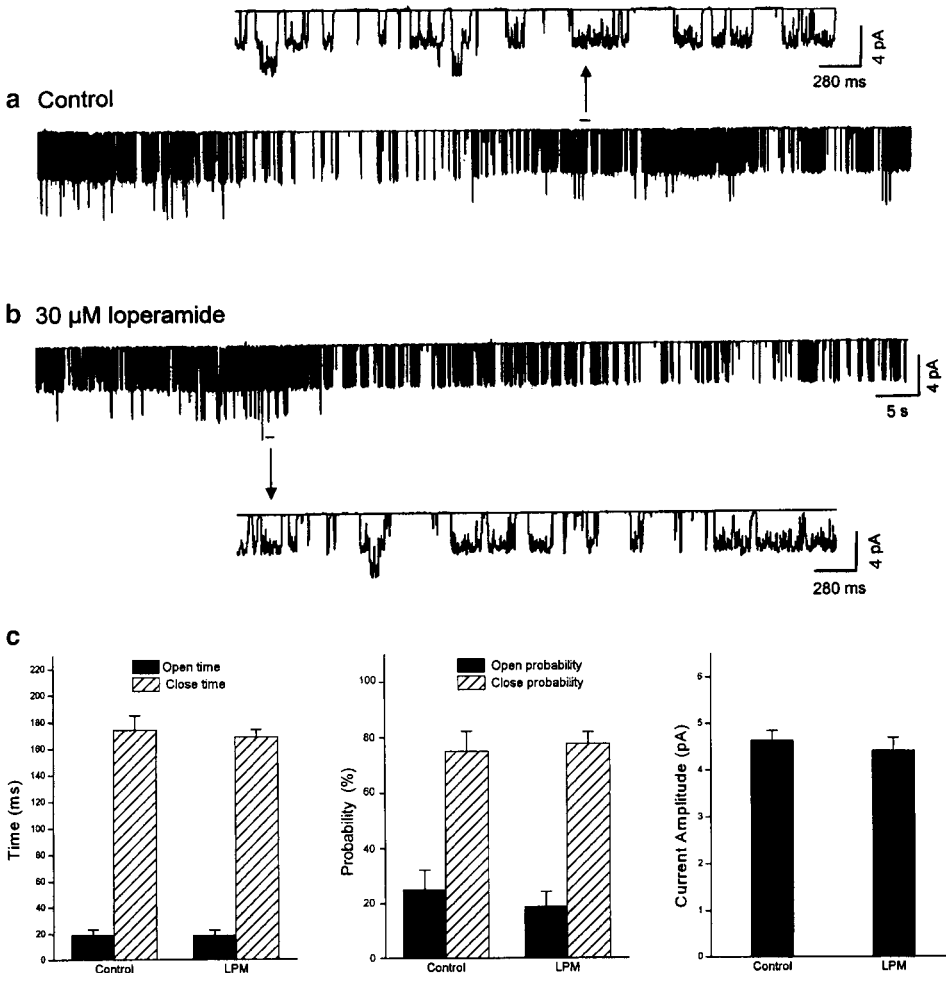

Figure 5 Loperamide has no direct effect on the large single-channel currents measured in excised patches from HIT cells. Recording from an inside-out patch configuration before (panel a) and after (panel b) addition of 30 μM loperamide to the solution superfusing the cytoplasmic side of the cell membrane. Insets show details on an expanded time scale. The graphs in panel c illustrate the mean open time (left), open probability (middle), or amplitude (right) of the single-channel current before (control) and after (LPM) addition of loperamide. Vertical bars show s.e.m., $n=3$. (Holding potential = 0 mV; all solutions were Ca^{2+} free and contained 5 mM EGTA; records are representative of five similar experiments conducted in different cells.)

probability $78 \pm 4\%$ ($n=3$), and current amplitude 4.4 ± 0.2 pA ($n=3$) (Figure 5c). Compared to control, loperamide had no significant effect on channel activity when applied to excised patches (Figure 5c).

Carbachol enhanced the loperamide-stimulated K_{Ca} channel activity

Carbachol is a muscarinic receptor agonist, which causes release of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores. It stimulates K_{Ca} channel activity in human submandibular gland (HSG) cells (Liu *et al*., 1998). The calcium-mobilizing effects of loperamide motivated comparison with muscarinic agonists. Figure 6 shows the activity of the large conductance channel in a cell-attached recording from a single cultured HIT cell. In normal KR solution containing 2.5 mM Ca^{2+}, the channel was almost always closed (Figure 6a). Application of loperamide (60 μM) activated the large single-channel currents (Figure 6b) and the addition of carbachol (100 μM) to the loperamide-containing, but Ca^{2+}-free, solution increased the activity without changing the amplitude of the single-channel current (Figure 6c). This enhancement of the loperamide effect by carbachol is consistent with the idea that the intracellular Ca^{2+} stores are the source of the Ca^{2+} that activates the high-conductance channel.

The loperamide effects were abolished by thapsigargin and carbachol

The effect of loperamide on single-channel currents and $[Ca^{2+}]_i$ was also examined in experiments in which thapsigargin was used to empty intracellular Ca^{2+} stores. Thapsigargin inhibits the Ca^{2+}-ATPase of the endoplasmic reticulum and thereby the reuptake required to maintain the Ca^{2+} stores over a period of minutes. Figure 7a shows the activity of the large-conductance channel recorded immediately after incubation with thapsigargin (1 μM, 10 min) in Ca^{2+}-free bath solution. Notice that numerous channel openings were observed when thapsigargin was first added suggesting an increase in $[Ca^{2+}]_i$ as thapsigargin blocked the Ca-ATPase (Figure 7a) that is required to maintain the stores by counteracting their small continuous leak of Ca^{2+}. The channel activity disappeared

over a period of several minutes and after 20 min the patch was essentially quiet (Figure 7b). Under these conditions, the application of 30 μM loperamide in Ca^{2+}-free extracellular solution failed to stimulate large single-channel currents (Figure 7c). These results indicate that loperamide is ineffective after thapsigargin-sensitive intracellular Ca^{2+} stores have been emptied.

Figure 8 shows intracellular Ca^{2+} measurements from cultured HIT-T15 cells loaded with 5 μM Fluo-3AM (Figure 8a). Exposure to 250 μM loperamide in Ca^{2+}-free perfusion solution caused a marked increase in $[Ca^{2+}]_i$ (Figure 8b, left traces; $\Delta F/F_0 = 0.5$). After 5 min application of thapsigargin (2 μM in Ca^{2+}-free solution) to empty intracellular stores, loperamide failed to produce an increase in intracellular Ca^{2+} (Figure 8b, middle traces). After washout of thapsigargin with KR solution containing 2.5 mM Ca^{2+} for 20 min, loperamide recovered the ability to raise intracellular Ca^{2+} (Figure 8b, right traces).

To ascertain if 5 min incubation with zero Ca^{2+} would prevent the agonist from subsequently eliciting a response, whether or not thapsigargin was present, the same experiment was carried out with the cells incubated in Ca^{2+}-free KR solution for 5 min without thapsigargin. The second application of loperamide now gave nearly the same rise in $[Ca^{2+}]_i$ (Figure 8b middle, dot line trace) as control (Figure 8b left, dotted line trace), indicating that 5 min in Ca^{2+}-free solution does not deplete the stores in the absence of thapsigargin.

To further compare the effects of loperamide and carbachol, we also tested their combined effects on $[Ca^{2+}]_i$ (Figure 8c and d). In the Ca^{2+}-free extracellular solution, carbachol (100 μM) caused a rapid increase of $[Ca^{2+}]_i$. The following application of carbachol plus loperamide (250 μM) caused no detectable elevation of $[Ca^{2+}]_i$ (Figure 8c), indicating that 100 μM carbachol had effectively emptied the loperamide-sensitive Ca^{2+} store. In the presence of 2.5 mM extracellular Ca^{2+}, this emptying apparently did not take place since the addition of loperamide to the second application of carbachol, now produced an elevation of $[Ca^{2+}]_i$ (Figure 8d).

The results in Figures 7 and 8 indicate that both the activation of large-conductance channels and the rise in $[Ca^{2+}]_i$ normally seen with loperamide are blocked when intracellular Ca^{2+} stores have been depleted by prior application of thapsigargin or carbachol.

Since carbachol is known to target IP_3-sensitive stores, we tested the effects of thimerosal to sensitize the IP_3 receptors to ambient levels of IP_3 (Mihai *et al*., 1999). Rapid application of 100 μM thimerosal caused a significant rise in $[Ca^{2+}]_i$ that continued to climb as long as the drug was present ($\Delta F/F_0 = 0.2 - 0.4$ after 1 min) and was cumulative during repeated exposures, but did not decline noticeably during washout periods (2 – 5 min; data not shown). This sustained effect of thimerosal was clearly different from the rapidly reversible effects of loperamide, but it was difficult to evaluate the possible synergistic effects of the two drugs. Nevertheless, the experiments with thimerosal support the presence of an IP_3-releasable pool of intracellular Ca^{2+}.

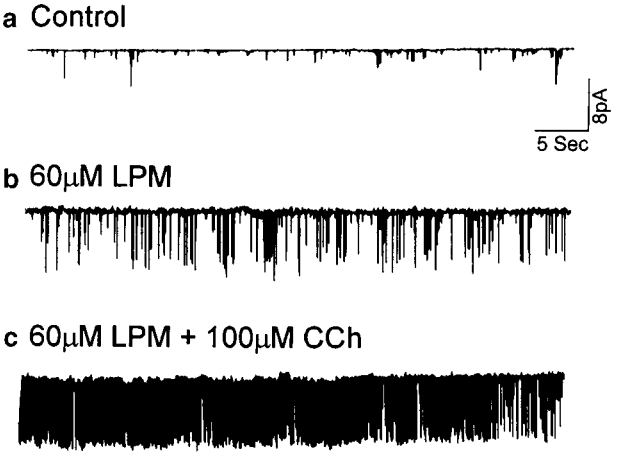

Figure 6 Carbachol increased the open probability of the loperamide-stimulated channel in HIT cells. Recordings under control conditions with 2.5 mM Ca^{2+} (panel a), when the cell was exposed to 60 μM loperamide in Ca^{2+}-free solution (panel b) and when 100 μM carbachol was also added to the loperamide-containing but Ca^{2+}-free solution (panel c). (Recording in the cell-attached configuration; holding potential = 0 mV; glucose concentration was 2.8 mM; records are representative of three similar experiments conducted in different cells.)

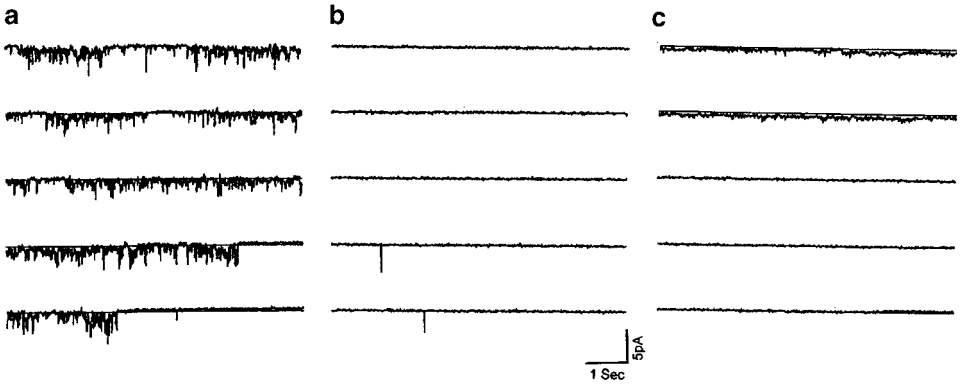

Figure 7 Loperamide shows no stimulatory effects at the single-channel level after pretreatment with thapsigargin. panel a: Single-channel currents recorded immediately after incubation with 1.0 μM thapsigargin in Ca^{2+}-free solution. Panel b: Recording after continuous exposure to thapsigargin for 20 min. Panel c: after 3 min exposure to 30 μM loperamide in Ca^{2+}-free solution. (Recording in the cell-attached configuration; holding potential 0 mV; glucose concentration was 5.6 mM; this record is representative of three similar experiments conducted in different cells.)

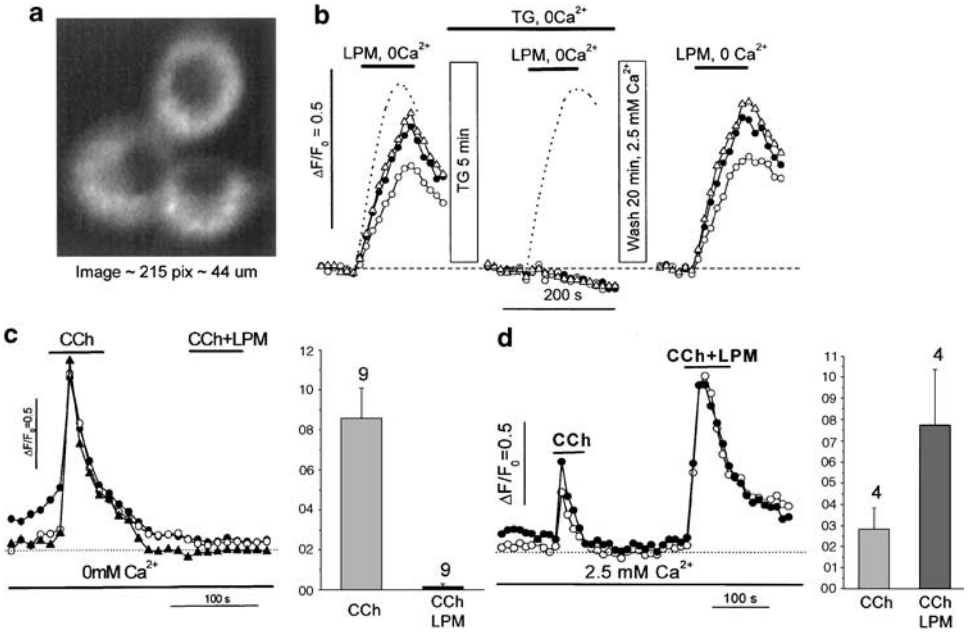

Figure 8 Thapsigargin (panels a, b) and carbachol (panels c, d) abolished the loperamide-induced rise in intracellular Ca^{2+}. Panel a: confocal image of three HIT cell clusters producing the records in panel b. Panel b shows changes in intracellular Ca^{2+} signals produced by loperamide (250 μM) under control conditions (left), following 5 min exposure to thapsigargin (2 μM) in Ca^{2+}-free solution (middle), and after 20 min washout and reequilibration with standard KR solution (2.5 mM Ca^{2+}; right). The traces in a dashed line are from a control experiment with the same timing and exposure to Ca^{2+}-free solution, but without thapsigargin. The lower panes show Ca^{2+}-transients evoked first by carbachol alone (CCh, 100 μM) and then together with 250 μM loperamide (CCh + LPM) in experiments with Ca^{2+}-free solution (panel c) and standard KR solution with 2.5 mM Ca^{2+} (panel d). The bar graphs show average signals.

Discussion and conclusions

Loperamide is an agonist of opioid receptors (Awouters *et al*., 1983; Ooms *et al*., 1984; Burleigh, 1988) and is usually used as an antidiarrheal agent. In addition loperamide has nonopioid effects such as inhibition of calmodulin (Zavecz *et al*., 1982; Diener *et al*., 1988) and calcium channels (Reynolds *et al*., 1984; Burleigh, 1988; Church *et al*., 1994; Daly *et al*., 1995). Loperamide has also been shown to enhance Ca^{2+} influx in various types of cells when SOC channels had been previous activated (Daly *et al*., 1995; Harper *et al*., 1997; Daly & Harper, 2000). Here we report for the first time the effect of loperamide on K_{Ca} channel activity in insulin-secreting cells and the mechanism underlying the effect.

Does loperamide stimulate charybdotoxin-sensitive K_{Ca} channel activity?

In our study loperamide stimulated openings of a large-conductance (145 pS) K^+ channel and had no effects on a small conductance (32 pS) K^+ channel (Figure 1). The conductance of the latter channel is consistent with that of the K_{ATP} channel, which is formed by complexes of an inward rectifying K^+ channel (Kir6.2) and a sulfonylurea receptor (SUR1) (Ashcroft *et al*., 1984; Cook & Hales, 1984; Rorsman & Trube, 1985; Ashcroft *et al*., 1988; Inagaki *et al*., 1995). K_{ATP} channels play an important role in glucose and sulfonylurea-induced depolarization of the β cell, and may also be involved in driving membrane potential oscillations (bursts) at intermediate glucose concentrations (7 – 10 mM; Ding *et al*., 1996; Detimary *et al*., 1998; He *et al*., 1998). However, our results with the K_{ATP} channel agonist tolbutamide, which had no effect on the K^+ channel activated by loperamide (Figure 4), show that loperamide acts on a different K^+ channel. The conductance of the K^+ channel activated by loperamide (145 pS) is similar to that of the charybdotoxin-sensitive 'maxi' K_{Ca} channel which has been identified in both rodent pancreatic β cells and insulin-secreting cell lines (Cook *et al*., 1984; Li *et al*., 1999). Surprisingly, loperamide-induced activation of the 145 pS K^+ channel did not require the presence of Ca^{2+} in the superfusing KR solution (Figure 2). (In such experiments we did not also remove Ca^{2+} from the pipette solution, but we estimate that the minute amounts of Ca^{2+} that might have entered through the cell-attached patch would be rapidly distributed on the timescale of the loperamide response and therefore insignificant compared to the Ca^{2+} that might enter though the rest of the cell membrane, when it was present in the bath.) Our further studies showed that loperamide-induced activation of the channel is most likely Ca^{2+} dependent, with activation of the channel occurring subsequent to release of stored Ca^{2+} (Figure 3). Thus, loperamide had no direct effect on K^+ channel activity when applied to the intracellular side of inside-out membrane patches (Figure 5), but did produce increases in intracellular Ca^{2+}, even when applied in a Ca^{2+}-free medium (Figure 3). The dose dependences of single-channel open probability and intracellular Ca^{2+} rise were nearly linear up to 250 μM loperamide (Figure 3e, f, and g). The effects were barely detectable at concentrations below 3 μM, indicating that they are not mediated by opioid receptors, which have IC_{50}

values in the submicromolar range (Giagnoni *et al*., 1983). At 250 μM the effects of loperamide showed no sign of saturation and developed more slowly than the effects of carbchol (100 μM; Figure 8c and d), suggesting that the Ca^{2+} release mechanism had not been fully activated.

While this evidence is consistent with activation of the large conductance or 'maxi' Ca^{2+}-activated K^+ channel, K_{Ca}, we cannot entirely exclude the contribution of other ionic channels, for example, a Ca^{2+}-activated Cl^- channel or nonselective cation channel, to the current activated by loperamide. A more definitive identification of the K_{Ca} channel might involve ascertaining block by charybdotoxin, verifying that the reversal potential is governed by the K^+ concentrations, and performing simultaneous measurements of loperamide-induced current and $[Ca^{2+}]_i$.

Activation of the K_{Ca} current by loperamide vs the rise in intracellular Ca^{2+}

The release of Ca^{2+} by loperamide is closely correlated with the activation of the K_{Ca} current as indicated by the dose–response curves (Figure 3f and g), and the finding that the timing and amplitude of this current consistently reflects $[Ca^{2+}]_i$: (a) The responses of K_{Ca} current and $[Ca^{2+}]_i$ to carbachol are stronger (and faster) than those to loperamide (Figure 6 vs Figure 8c and d), (b) thapsigargin gives rise to relatively modest transient responses of both K_{Ca} current (Figure 7a and b) and $[Ca^{2+}]_i$ (not shown), and (c) loperamide does not activate K_{Ca} when the releasable Ca^{2+} stores have been depleted by thapsigargin (Figure 7c vs 8b) or charbachol (Figure 7c vs 8c). Furthermore, 30 μM loperamide, which generally produced a significant response (Figure 3g), did not activate K_{Ca} current when applied (in Ca^{2+}-free solution) to inside-out patches. Of course, such experiments are always subject to the criticism that some essential component (e.g. ATP/ADP) may be missing from the cytosolic aspect of the membrane after excising the patch. Yet, on balance the evidence indicates that loperamide does not directly modulate the K_{Ca} channel, but activates it by raising $[Ca^{2+}]_i$ as observed also with excised patches (Findlay *et al*., 1985).

Loperamide releases Ca^{2+} from a thapsigargin-sensitive store

In order to characterize the nature of the loperamide-sensitive intracellular Ca^{2+} store, we performed experiments in the presence of thapsigargin. Thapsigargin causes depletion of Ca^{2+} from the endoplasmic reticulum by inhibiting the sarco-endoplasmic reticulum Ca^{2+} ATPase, blocking uptake of the ion into the store (Thastrup *et al*., 1989). Following application of thapsigargin, loperamide (in Ca^{2+}-free solution) was no longer able to activate K_{Ca} channels (Figure 7) or increase intracellular Ca^{2+} (Figure 8). In this context, we notice that the patch-clamp experiments performed without Ca^{2+} in the bathing solution were not compromised by the Ca^{2+} remaining in the patch pipette, since in that case the depletion of intracellular Ca^{2+} stores by thapsigargin would not have abolished the activation of current by loperamide. We conclude that loperamide releases Ca^{2+} from thapsigargin-sensitive intracellular stores in insulin-secreting cells. Interestingly, depletion of thapsigargin-sensitive Ca^{2+} stores has been associated with activation of store-operated currents in both β cells and insulin-secreting cell lines (Bode & Goke, 1994; Worley *et al*., 1994; Bertram *et al*., 1995; Liu & Gylfe, 1997; Miura *et al*., 1997; Roe *et al*., 1998), and loperamide has been reported to be a positive modulator of such channels (Harper *et al*., 1997). However, the K^+ channel activated by loperamide in this study is unlikely to be a store-operated channel *per se*, since application of thapsigargin caused only a transient opening of the channel (Figure 7a and b), most likely resulting from the transient increase in intracellular Ca^{2+} produced by the SERCA inhibitor (Figure 8). Furthermore, loperamide did not enhance the activity of the channels when applied after thapsigargin, as has been reported for SOC channels (Harper *et al*., 1997).

IP_3-gated Ca^{2+} stores are targeted by both loperamide and muscarinic agonists

Direct measurements of ER Ca^{2+} dynamics in rodent β cells have shown that IP_3 acts on a subset of the thapsigargin-sensitive Ca^{2+} store (Tengholm *et al*., 1999; 2000; Maechler *et al*., 1999). We therefore targeted IP_3-gated Ca^{2+} stores using carbachol and thimerosal.

Carbachol enhanced the stimulatory effect of loperamide on K_{Ca} channels (Figure 6). Without extracellular Ca^{2+}, carbachol rapidly and completely emptied IP_3-sensitive stores and abolished the loperamide effect (Figure 8c). On the other hand, with normal extracellular Ca^{2+} (2.5 mM) the Ca^{2+} stores remained intact, and loperamide enhanced the carbachol-induced Ca^{2+} release (Figure 8d). These results suggest that loperamide in HIT cells (i) may mobilize Ca^{2+} stores similar to those that respond to muscarinic receptor agonists, (acetyl choline and carbachol), in mouse (Nenquin *et al*., 1984) and rat (Mathias *et al*., 1985; Morgan *et al*., 1985) pancreatic islets, (ii) are insensitive to mitochondrial poisons (Gylfe & Hellman, 1986), and (iii) produce Ca^{2+} efflux that correlates with the rise in the islet IP_3 concentration (Morgan *et al*., 1985). Using thimerosal to sensitize IP_3 receptors to basal IP_3 levels (Mihai *et al*., 1999), we observed a rise in Ca^{2+} that confirmed the presence of IP_3-gated stores, but did not explore whether thimerosal enhanced the effects of loperamide.

Our results indicate that loperamide caused release of Ca^{2+} from intracellular stores that can be emptied by thapsigargin and carbachol and therefore may be located in the endoplasmic reticulum, maintained by SERCA, and gated by IP_3.

In summary, we have found that loperamide releases Ca^{2+} from a thapsigargin-sensitive intracellular store. The augmentation of the intracellular Ca^{2+} produced by this action of loperamide is sufficient to activate an ion channel with biophysical properties similar or identical to those of the 'maxi' K_{Ca} channel. Loperamide may therefore serve as a useful tool for further studies of the coupling between intracellular Ca^{2+} stores and membrane potential in physiological regulation of insulin secretion.

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