Thiol oxidation by 2,2'-dithiodipyridine causes a reversible increase in cytoplasmic free Ca^{2+} concentration in pancreatic β -cells

Role for inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores

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2,2'-Dithiodipyridine (2,2'-DTDP), a reactive disulphide that mobilizes Ca^{2+} from ryanodine-sensitive Ca^{2+} stores in muscle, induced a biphasic increase in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in pancreatic β -cells loaded with fura 2. This increase consisted of an early transient followed by a second, slower, rise. The $[\text{Ca}^{2+}]_i$ transient was dependent on extracellular Ca^{2+} and disappeared on treatment with nimodipine. The reactive disulphide caused plasma membrane depolarization, as studied by the perforated-patch configuration of the patch-clamp technique. Hence membrane depolarization and opening of the L-type voltage-gated Ca^{2+} channels were responsible for the first transient in $[\text{Ca}^{2+}]_i$. The second slower increase in $[\text{Ca}^{2+}]_i$ was prolonged but readily reversed by the disulphide-reducing agent 1,4-dithiothreitol. This increase in $[\text{Ca}^{2+}]_i$ was not decreased by nimodipine or by omission of extracellular Ca^{2+} , but was

eliminated when the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool was first depleted by carbachol. Ryanodine or its β -alanyl analogue did not release Ca^{2+} from intracellular stores, and a high concentration of ryanodine did not inhibit Ca^{2+} release by 2,2'-DTDP. The disulphide compound suppressed glucose metabolism and decreased the mitochondrial inner-membrane potential. We conclude that thiol oxidation by 2,2'-DTDP affects Ca^{2+} homeostasis in β -cells by multiple mechanisms. However, unlike the situation in muscle, in β -cells 2,2'-DTDP releases Ca^{2+} from intracellular pools by mechanisms that do not involve activation of ryanodine receptors. Instead, in these cells the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} store comprises an alternative target for the Ca^{2+} -mobilizing action of the reactive disulphide compound.

INTRODUCTION

Pancreatic β -cells act as fuel sensors in mediating nutrient-stimulated insulin secretion. The cytoplasmic level of ATP and ADP seems to play a critical role in this process. One sensor for the cytoplasmic ATP/ADP ratio is the ATP-sensitive potassium channel (K_{ATP} channel) of the β -cell plasma membrane [1]. The activity of these channels sets the resting membrane potential in the β -cell, and their closure results in plasma membrane depolarization. According to the current model, nutrient metabolism increases the cytoplasmic ATP/ADP ratio, leading to closure of K_{ATP} channels, depolarization of the plasma membrane, opening of voltage-gated Ca^{2+} channels, an increase in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and finally exocytosis of insulin [2]. Antidiabetic sulphonylurea drugs induce closure of the K_{ATP} channels and trigger a similar cascade of events leading to insulin secretion. Recent cloning and reconstitution studies indicate that the β -cell K_{ATP} channel consists of at least two subunits, one of which is the sulphonylurea receptor [3,4]. The K_{ATP} channel of β -cells contains critical thiol groups on the cytoplasmic side, and some thiol-oxidizing agents can induce channel closure and plasma-membrane depolarization [5,6].

In β -cells an increase in $[\text{Ca}^{2+}]_i$ in response to various stimuli is brought about not only by Ca^{2+} entry through voltage-gated Ca^{2+} channels of the plasma membrane but also by release of the ion from intracellular Ca^{2+} pools. Characterization of the intracellular non-mitochondrial Ca^{2+} pools and their Ca^{2+} release channels has been the subject of several earlier studies [7–9],

which have demonstrated the presence of both $\text{Ins}(1,4,5)\text{P}_3$ -sensitive and -insensitive Ca^{2+} pools in these cells [8,9]. We have shown that Ca^{2+} from the latter pool can be mobilized by thiol reagents such as thimerosal, but not by caffeine, ryanodine or cADP-ribose [10,11]. Apart from this, little is known about the identity of the Ca^{2+} release channels that might be associated with the $\text{Ins}(1,4,5)\text{P}_3$ -insensitive Ca^{2+} pools. Numerous studies have shown that thiol oxidation releases Ca^{2+} from sarcoplasmic reticulum (SR). From SR a 160 kDa thiol-gated channel has been purified [12]. Reconstitution studies in an artificial lipid bilayer demonstrate that this channel has features of the ryanodine receptor (RyR) of SR, but the precise relation between the two channels remains to be established [13]. In non-muscle cells the most widespread Ca^{2+} release channel is the $\text{Ins}(1,4,5)\text{P}_3$ receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$]. This channel shares architectural similarity with RyR, including the presence of highly conserved cysteine residues in the C-terminal cytoplasmic tail region [14]. According to several recent studies, effects of thiol modification on $\text{Ins}(1,4,5)\text{P}_3$ binding and Ca^{2+} release vary according to the type and concentration of thiol reagents used and the predominant isoform of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ present in the cells [15]. It seems reasonable to speculate that some thiol reagents might predominantly affect the RyR, and others the $\text{Ins}(1,4,5)\text{P}_3\text{R}$.

To elucidate whether β -cells possess Ca^{2+} pools equipped with RyR-like channels, we studied Ca^{2+} release by thimerosal [16] from permeabilized cells, where the thiol reagent releases Ca^{2+} from a discrete pool distinct from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool [10,11]. Subsequently, however, some drawbacks of this approach became apparent: for instance, thimerosal activates

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; $\Delta\psi_m$, mitochondrial inner-membrane potential; 2,2'-DTDP, 2,2'-dithiodipyridine; DTT, 1,4-dithiothreitol; ER, endoplasmic reticulum; $\text{Ins}(1,4,5)\text{P}_3\text{R}$, $\text{Ins}(1,4,5)\text{P}_3$ receptor; K_{ATP} channel, ATP-sensitive potassium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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RyR, but also $\text{Ins}(1,4,5)\text{P}_3\text{R}$ [17,18]. The specificity of this mercurial compound for thiol groups is not very high, and the chemistry of its interactions might be complex [19]. Furthermore, other reports suggest that results obtained in permeabilized cell suspensions might not reflect the situation in intact cells [20,21]. These considerations made it necessary to study Ca^{2+} release from intracellular Ca^{2+} pools in intact cells and to use other Ca^{2+} -mobilizing thiol-oxidizing agents.

2,2'-Dithiodipyridine (2,2'-DTDP), a membrane-permeant reactive disulphide, oxidizes thiol groups by forming a mixed disulphide bond between the thiol group and a pyridyl ring from the compound [22]. The specificity of 2,2'-DTDP for thiol groups is absolute [22]. This disulphide seemed particularly useful because of its well-established activating effect on the SR Ca^{2+} release channel. 2,2'-DTDP-induced Ca^{2+} release from intracellular stores has not been studied in detail in non-muscle cells, although it has been reported to promote spontaneous Ca^{2+} release from intracellular pools in permeabilized hepatocytes [23]. Ryanodine- and $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores might coexist in many cells but because of limited information on the effect of 2,2'-DTDP on $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pools, it is difficult to attribute a 2,2'-DTDP-induced Ca^{2+} release to mobilization of the ion from either of these stores.

In the present study we investigated effects of 2,2'-DTDP on $[\text{Ca}^{2+}]_i$ in β -cells and the role of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} pools in this process. One objective of the study was to determine whether a RyR-like channel that is activated by 2,2'-DTDP in muscle cells is also involved in Ca^{2+} mobilization by the compound in β -cells. Furthermore, using the thiol-oxidizing agent, we studied the organization of Ca^{2+} pools in intact β -cells, which is a more physiological system than permeabilized cells, a model that we used in our previous studies [9,10].

MATERIALS AND METHODS

Isolation of islets and preparation of β -cells

Pancreatic islets from *ob/ob* mice from a local colony were isolated by digestion with collagenase and dispersed into small clusters of cells by shaking in a Ca^{2+} - and Mg^{2+} -deficient medium [24]. Human pancreatic tissues were obtained from patients undergoing pancreatotomy in a local hospital; islets were isolated and dispersed into cells by methods described previously [25]. Cells were cultured overnight, on a glass coverslip, in RPMI 1640 medium containing 11 mM glucose supplemented with 10% (v/v) fetal-calf serum, 100 i.u./ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Measurements of $[\text{Ca}^{2+}]_i$ by microfluorimetry

Cells attached to the coverslip were loaded with fura 2 by incubating for 30 min at 37 °C in basal medium containing (in mM): NaCl 125, KCl 5.9, MgCl_2 1.2, CaCl_2 1.28, Hepes 25, glucose 3, BSA 0.1%, adjusted to pH 7.4 with NaOH, in the presence of 2 μM fura 2 acetoxymethyl ester. Coverslips were gently washed with the buffer and mounted as the bottom of an open chamber placed on the stage of an inverted epifluorescence microscope (Zeiss Axiovert 35M). The perfusate volume in the chamber was 0.2 ml and the perfusion rate 0.3 ml/min. After switching of the solutions, there was a lag period of approx. 40 s before the new solution reached the chamber. The stage of the microscope was thermostatically controlled to maintain a temperature of 37 °C in the perfusate inside the chamber. The

microscope was connected to a SPEX Fluorolog-2 CM1T111 system for dual-wavelength excitation fluorimetry. The excitation wavelengths (340 and 380 nm) were generated by two monochromators and the light was directed to the cell by a dichroic mirror. The emitted light, selected by a 510 nm filter, was monitored by a photomultiplier attached to the microscope. Cells were excited at 1 Hz and the period of data collection at each point was 0.33 s. The emissions at the excitation wavelength of 340 nm (F_{340}) and 380 nm (F_{380}) were used to calculate the fluorescence ratio ($R_{340/380}$). Clusters of two to four cells, isolated optically by means of a diaphragm in the microscope, were studied by using a 100 $\times$, 1.3 numerical aperture, oil-immersion objective (Zeiss Plan Neofluar). Background fluorescence was measured after quenching fura 2 fluorescence with Mn^{2+} and was subtracted from the total emission before the calculation of $[\text{Ca}^{2+}]_i$ was performed [26]. Maximum and minimum $R_{340/380}$ values were determined by using thin films of solution containing 130 mM KCl, 5 μM fura 2 free acid, 10 mM Hepes, pH 7.20, 2 M sucrose and either 2 mM Ca^{2+} or no Ca^{2+} in the presence of 5 mM EGTA [27]. The K_d for the Ca^{2+} -fura 2 complex was taken as 225 nM. To compensate for variations in output light intensity from the two monochromators, all experiments were normalized by the inclusion of a fluorescence ratio where both monochromators were set at 360 nm.

The basal medium was used in all experiments except those involving electrophysiology.

Cell viability

Cell viability after exposure to 2,2'-DTDP was tested under experimental conditions similar to those used for the measurements of $[\text{Ca}^{2+}]_i$. Uptake of the extracellular marker ethidium bromide was used as an indicator of non-specific membrane permeability. Cells were perfused with 2 μM ethidium bromide and were excited at 490 nm. Emitted light filtered through a 520 nm long-pass filter was monitored.

Electrophysiology

The inside-out, cell-attached and perforated-patch configurations of the patch-clamp technique were used [28]. The pipettes were prepared from borosilicate glass capillary tubes, coated with Sylgard resin (Dow Corning) and finally fire-polished. The extracellular solution contained (in mM): 138 NaCl, 5.6 KCl, 1.2 MgCl_2 , 2.6 CaCl_2 and 5 Hepes/NaOH at pH 7.4. For the inside-out experiments, the intracellular solution (i.e. the bath solution) consisted of (in mM): 125 KCl, 1 MgCl_2 , 10 EGTA, 30 KOH and 5 Hepes/KOH at pH 7.15. When using the cell-attached configuration of the patch-clamp technique, both extracellular solutions and pipette solution consisted of the extracellular buffer described above, with the patch potential clamped at -70 mV. For membrane-potential measurements the pipette solution contained (in mM): 10 KCl, 76 K_2SO_4 , 10 NaCl, 1 MgCl_2 and 10 Hepes, adjusted to pH 7.35 with NaOH, in the presence of 200 mg/ml amphotericin B (dissolved in DMSO; final concentration of DMSO less than 0.1%).

For all recordings we used an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Voltage steps were generated, digitized and stored in a computer (IBM clone 386/25), with the program pClamp (Axon Instruments) and Labmaster ADC (Scientific Solutions). The current responses were filtered at 1 kHz (low-pass Bessel filter). Gigaohm seals were established at room temperature, and the experiments were then performed at 30 °C.

Measurements of the intracellular level of NAD(P)H

Small clusters of β -cells attached to the coverslip were excited at 360 nm. Light emitted through the dichroic mirror (centred at 395 nm) was filtered through a 450–490 nm band-pass filter and recorded by a photomultiplier. To limit damage to the cells and bleaching of NAD(P)H fluorescence, excitation was minimized by limiting the slits to 0.2 mm.

Measurements of mitochondrial inner-membrane potential ($\Delta\psi_m$)

β -Cells attached to the coverslip were incubated for 10 min in the basal buffer containing 32 μM rhodamine 123. Cells were excited at 500 nm, and emitted light selected by a 515–565 nm bandpass filter was measured [29].

Measurements of insulin release

Dispersed β -cells were cultured overnight in RPMI 1640 medium containing 11 mM glucose. Approx. 2×10^5 cells were mixed with Bio-Gel P4 polyacrylamide beads (Bio-Rad) in a 0.5 ml column and perfused with the basal medium containing 3 mM glucose, at 37 °C and a flow rate of approx. 200–250 $\mu\text{l}/\text{min}$. The column was perfused for 15 min, after which 2 min fractions were collected. Insulin concentrations in the collected fractions were measured by radioimmunoassay, with crystalline rat insulin as reference. 2,2'-DTDP (50 μM) did not interfere with insulin radioimmunoassay.

Materials

2,2'-DTDP and 1,4-dithiothreitol (DTT) were from Sigma (St. Louis, MO, U.S.A.). Ryanodine (99% pure) and β -alanine ryanodine (99% pure) were gifts from Dr. Henry R. Besch, Jr. (Indiana University School of Medicine, Indianapolis, IN, U.S.A.). Some ryanodine (98% pure) was from Calbiochem (San Diego, CA, U.S.A.). Nimodipine was a gift from Bayer AG (Wuppertal, Germany). Fura 2 and rhodamine 123 were from Molecular Probes (Eugene, OR, U.S.A.).

RESULTS

Exposure of β -cells to 50 μM 2,2'-DTDP in the presence of 1.28 mM extracellular Ca^{2+} induced a biphasic rise in $[\text{Ca}^{2+}]_i$, consisting of a large early transient and a subsequent smaller but prolonged increase (Figure 1A). The rapid rise in $[\text{Ca}^{2+}]_i$ occurred 2–3 min after the arrival of 2,2'-DTDP solution in the chamber. $[\text{Ca}^{2+}]_i$ then fell to a lower level before it started to rise again. During the second increase, the maximal increase in $[\text{Ca}^{2+}]_i$ occurred after 8–20 min [15 ± 2 (S.E.M.) min, $n = 9$]. Cells remained viable during this prolonged exposure to the oxidizing agent, as evidenced by exclusion of the fluorescent DNA-binding dye ethidium bromide (Figure 1B). The second prolonged increase in $[\text{Ca}^{2+}]_i$ persisted after the 2,2'-DTDP had been washed out (observed for up to 5 min) and returned to the basal level within 2 min of perfusion with the disulphide-reducing agent DTT. In some experiments the second $[\text{Ca}^{2+}]_i$ increase did not reach a true plateau, and instead continued to increase slowly after the 2,2'-DTDP had been washed out. In all cases $[\text{Ca}^{2+}]_i$ returned to a basal level after the addition of DTT. In a few experiments (two out of eight), the initial increase in $[\text{Ca}^{2+}]_i$ was not observed. A lower concentration of 2,2'-DTDP (e.g. 25 μM) did not increase $[\text{Ca}^{2+}]_i$ during the period of exposure (up to 20 min), whereas the effect of a higher concentration (e.g. 100 μM) of the oxidizing agent was no different from that of 50 μM . The reaction of 2,2'-DTDP with thiols yields 2-thio-

Figure 1 Effect of 2,2'-DTDP on $[\text{Ca}^{2+}]_i$ and viability in pancreatic β -cells

(A) Fura 2-loaded pancreatic β -cells were perfused with basal medium containing 1.28 mM Ca^{2+} and monitored by microfluorimetry. At points indicated by horizontal bars, 2,2'-DTDP (50 μM) and DTT (5 mM) were added. The trace is representative of six experiments from four preparations. (B) Cells were perfused with 2 μM ethidium bromide (E.B.), and the fluorescence from small clusters of β -cells was monitored continuously as described in the Materials and methods section. Exposure to 2,2'-DTDP (50 μM) did not increase the fluorescence, but exposure to digitonin (5 μM) increased the fluorescence within a few minutes. The trace is representative of four experiments.

pyridone. To test whether 2-thiopyridone was responsible for mediating the $[\text{Ca}^{2+}]_i$ increase by 2,2'-DTDP, we added 2,2'-DTDP (50 μM) together with DTT (2.5 mM). Under these conditions there was negligible $[\text{Ca}^{2+}]_i$ increase, indicating that the reaction product of 2,2'-DTDP with DTT, i.e. 2-thiopyridone, was not capable of increasing $[\text{Ca}^{2+}]_i$.

To test whether $[\text{Ca}^{2+}]_i$ increase after exposure to 2,2'-DTDP involved Ca^{2+} entry through the plasma membrane, we exposed β -cells to 2,2'-DTDP in the presence of nominally Ca^{2+} -free medium. The actual Ca^{2+} concentration in this medium was approx. 2.5 μM , as measured by a Ca^{2+} -selective electrode [9]. In Ca^{2+} -free extracellular medium, 2,2'-DTDP failed to elicit the initial $[\text{Ca}^{2+}]_i$ transient. However, under these conditions a slow increase in $[\text{Ca}^{2+}]_i$ was observed within 5–10 min of exposure to the disulphide compound (Figure 2A). To test whether the L-type voltage-gated Ca^{2+} -channels were involved in the Ca^{2+} entry after stimulation with 2,2'-DTDP, we first perfused the cells for 3–5 min with nimodipine (10 μM), a specific blocker of these channels. The effectiveness of this treatment is evidenced by a lack of response of $[\text{Ca}^{2+}]_i$ to KCl depolarization (results not shown). Exposure of nimodipine-treated cells to 2,2'-DTDP in the presence of 1.28 mM extracellular Ca^{2+} also failed to elicit the initial rise in $[\text{Ca}^{2+}]_i$ (Figure 2B). As shown in Figure 2(B), treatment with nimodipine did not affect the second increase in $[\text{Ca}^{2+}]_i$. In Figure 2(C), membrane potential was recorded from single β -cells, with the perforated-patch configurations of the

Figure 2 Involvement of plasma-membrane depolarization and Ca^{2+} entry through voltage (V)-gated Ca^{2+} channels in the 2,2'-DTDP-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells

(A) Cells were perfused with nominally Ca^{2+} -free medium prepared by omitting Ca^{2+} from the basal buffer. In (B), the extracellular buffer contained 1.28 mM Ca^{2+} and 10 μM nimodipine, a blocker of L-type voltage-gated Ca^{2+} channels. At the points indicated, 2,2'-DTDP (50 μM) or DTT (5 mM) was added. (A) and (B) are typical traces representing three to six similar experiments. (C) The effect of 2,2'-DTDP (50 μM) on β -cell membrane potential was measured with the perforated-patch configuration of the patch-clamp technique, as described in the text. Membrane depolarization was observed in four out of six experiments.

patch-clamp technique. Application of 2,2'-DTDP (50 μM) to the bath solution caused depolarization of the plasma membrane. The extent to which this depolarization could be accounted for by inhibition of the K_{ATP} channels, which contain reactive thiol groups [10], was tested by using the cell-attached as well as excised inside-out configuration of the patch-clamp technique. However, 2,2'-DTDP (50 μM) did not inhibit K_{ATP} channel activity in any of these experiments. 2,2'-DTDP also did not affect Ca^{2+} current through L-type voltage-gated Ca^{2+} channels as tested by the whole-cell mode of patch-clamp (results not shown).

To identify the intracellular Ca^{2+} pools from which 2,2'-DTDP released Ca^{2+} , we first challenged the cells with carbachol to deplete the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores. Such treatment by carbachol depletes the endoplasmic reticulum (ER) Ca^{2+} pool, as evidenced by a lack of Ca^{2+} release by subsequent addition of thapsigargin, a potent blocker of ER Ca^{2+} -ATPase [9,24]. In the presence of Ca^{2+} -free extracellular medium, carbachol induced a

Figure 3 2,2'-DTDP mobilizes Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool

Cells were perfused with nominally Ca^{2+} -free buffer. (A) Depletion of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool by carbachol (CCh; 200 μM) abolished the effect of 2,2'-DTDP (50 μM) on $[\text{Ca}^{2+}]_i$. In (B), Ca^{2+} was first released by 2,2'-DTDP (50 μM). Subsequent addition of carbachol (200 μM) failed to release further Ca^{2+} . The traces represent experiments that have been repeated at least three times with similar results.

Figure 4 Effect of caffeine on $[\text{Ca}^{2+}]_i$ in human pancreatic β -cells

Dispersed human pancreatic β -cells attached to coverslips were perfused with nominally Ca^{2+} -free medium during the period indicated. $[\text{Ca}^{2+}]_i$ was measured from single cells loaded with fura 2. At the points indicated, caffeine (5 mM) or glucose (11 mM) was added. The extracellular medium contained 1.28 mM Ca^{2+} during exposure to glucose. The experiment is representative of three experiments with β -cells obtained from one individual.

transient rise in $[\text{Ca}^{2+}]_i$ (Figure 3A). On recovery of $[\text{Ca}^{2+}]_i$ to basal levels, 2,2'-DTDP was added. Under these conditions, 2,2'-DTDP did not increase $[\text{Ca}^{2+}]_i$, suggesting that the 2,2'-DTDP-sensitive Ca^{2+} pool was depleted by prior application of the $\text{Ins}(1,4,5)\text{P}_3$ -forming agonist. In the reverse experiment, where

Figure 5 Effect of β -alanyl ryanodine on release of Ca^{2+} from intracellular stores

Conditions of experiments were as in the legend to Figure 1. (A) Mouse β -cells were exposed to β -alanyl ryanodine (β -Ala-Rya; $10 \mu\text{M}$) and carbachol (CCh; $200 \mu\text{M}$) for the periods indicated. The lack of an effect of β -alanyl ryanodine in increasing $[\text{Ca}^{2+}]_i$ was observed in six out of six experiments. (B) The magnitude of the peak of $[\text{Ca}^{2+}]_i$ increase by carbachol was the same irrespective of whether or not cells had been pretreated with β -alanyl ryanodine. The trace is representative of experiments that were repeated six times.

$[\text{Ca}^{2+}]_i$ was first released from intracellular stores by 2,2'-DTDP, subsequent addition of carbachol induced a small or no further increase in the intracellular concentration of the ion (Figure 3B).

Because 2,2'-DTDP releases Ca^{2+} from the SR by activating RyR, we investigated whether a RyR-like channel might be involved in mediating Ca^{2+} release by the disulphide in β -cells. Several previous studies have demonstrated that caffeine, the most commonly used pharmacological tool for activating RyR, does not induce significant Ca^{2+} release from intracellular stores in rodent insulin-secreting cells [10, 11, 24, 30]. In the present study we were able to test the effects of caffeine on $[\text{Ca}^{2+}]_i$ in β -cells obtained from human islets. As shown in Figure 4, exposure of human β -cells to caffeine (5 mM), in the presence of nominally Ca^{2+} -free extracellular medium, also failed to elicit a response of $[\text{Ca}^{2+}]_i$. Subsequent addition of glucose increased $[\text{Ca}^{2+}]_i$, indicating that the cells tested were indeed β -cells. We also investigated the effects of ryanodine, the other classical pharmacological tool for RyR studies, in mouse β -cells. Acute application of ryanodine (400 nM and $100 \mu\text{M}$) to the cells in the presence of 1.28 mM extracellular Ca^{2+} did not cause any detectable change in $[\text{Ca}^{2+}]_i$ (results not shown). High concentrations of ryanodine might block the RyR instead of activating it. For this reason we performed experiments with β -alanyl ryanodine, a ryanodine analogue that lacks the inhibitory action of the native compound even when used at high concentration [31]. As shown in Figure 5(A), $10 \mu\text{M}$ β -alanyl ryanodine also did not increase $[\text{Ca}^{2+}]_i$. It was possible that β -alanyl ryanodine had released Ca^{2+} from intracellular stores slowly without altering $[\text{Ca}^{2+}]_i$. However, prior exposure of the cells to β -alanyl ryanodine did not decrease the magnitude of the peak $[\text{Ca}^{2+}]_i$ increase by carbachol (Figure 5B), making it unlikely that the ryanodine analogue released any Ca^{2+} from a common pool also releasable by $\text{Ins}(1,4,5)\text{P}_3$. To test whether Ca^{2+} release by 2,2'-DTDP could be suppressed by ryanodine, we treated the cells with high concentration ($50 \mu\text{M}$) of the alkaloid for 1 h. Cells were then exposed to 2,2'-DTDP in Ca^{2+} -free extracellular medium. The basal $[\text{Ca}^{2+}]_i$ values in control cells ($140 \pm 25 \text{ nM}$, $n = 5$) and ryanodine-treated cells ($135 \pm 6 \text{ nM}$, $n = 5$) were not significantly different. Moreover, 10 min after exposure to 2,2'-DTDP, the $[\text{Ca}^{2+}]_i$ in ryanodine-treated cells was comparable with that in the control cells

Figure 6 Lack of inhibitory effect of ryanodine on Ca^{2+} release by 2,2'-DTDP

The extracellular medium was nominally Ca^{2+} -free. In (A), cells were incubated in basal buffer with $50 \mu\text{M}$ ryanodine for 1 h before starting the experiment. In (B), control cells were incubated in the same buffer without ryanodine. On average, $[\text{Ca}^{2+}]_i$ before and after exposure to 2,2'-DTDP ($50 \mu\text{M}$) and the rate of rise of $[\text{Ca}^{2+}]_i$ were not significantly different in the ryanodine-treated cells from those in the control cells. The traces are representative of five experiments for each condition.

Figure 7 Effect of 2,2'-DTDP on glucose metabolism and mitochondrial inner-membrane potential

(A) The intracellular level of reduced nicotinamide nucleotide was estimated by monitoring NAD(P)H-specific fluorescence from β -cells by microfluorimetry. In control cells, increasing the glucose concentration in the medium from 3 to 16 mM caused a prompt increase in the NAD(P)H fluorescence. In (B), cells were first incubated with 50 μ M 2,2'-DTDP for 3 min. Exposure of these cells to 16 mM glucose in the continued presence of 2,2'-DTDP failed to increase the intracellular NAD(P)H level. (C) β -Cells loaded with rhodamine 123 were perfused with basal buffer and were excited at 500 nm. The emitted light selected by a 515–565 nm bandpass filter was measured by photomultiplier. Depolarization of $\Delta\psi_m$ is indicated by an increase in rhodamine 123 fluorescence. For the period indicated, 2,2'-DTDP (50 μ M) was added (solid trace). Sodium azide (3 mM) depolarized $\Delta\psi_m$ further. In control cells, to which 2,2'-DTDP was not added, there was a progressive decline in rhodamine 123 fluorescence (dotted trace), probably representing leakage of dye. The trace is representative of five different experiments.

(350 $\pm$ 90 nM compared with 325 $\pm$ 80 nM; $P = 0.87$; $n = 10$) (Figure 6). In addition, the rate of increase in $[Ca^{2+}]_i$ after exposure to 2,2'-DTDP was not significantly different between the control and ryanodine-treated cells.

Persistent elevation of $[Ca^{2+}]_i$ by 2,2'-DTDP suggested the possible impairment of $[Ca^{2+}]_i$ homeostatic mechanisms. This could be due to compromised cellular energy status because of increased consumption of ATP or inhibition of nutrient metabolism. We investigated the latter by examining the effect of 2,2'-DTDP on glucose metabolism, as measured by monitoring the glucose-induced change in the level of intracellular NAD(P)H. When concentration of glucose in the medium was raised from 3 to 16 mM, an increase in NAD(P)H-specific fluorescence was observed within 40 s (Figure 7A). The glucose-induced increase in NAD(P)H fluorescence was almost completely inhibited by prior exposure of β -cells to 2,2'-DTDP for 3 min (Figure 7B). It has been reported that pro-oxidants might also decrease mitochondrial $\Delta\psi_m$ [32] and thereby decrease mitochondrial synthesis

of ATP [33]. To investigate whether treatment by 2,2'-DTDP impaired mitochondrial function, we tested the effect of the oxidizing agent on $\Delta\psi_m$ by monitoring the fluorescence of the $\Delta\psi_m$ -sensitive dye rhodamine 123. An increase in rhodamine 123 fluorescence indicates depolarization of $\Delta\psi_m$, as seen in Figure 7(C), after the addition of NaN_3 , an inhibitor of cytochrome *c* oxidase [29]. In cells loaded with the dye and perfused with basal buffer only, rhodamine 123 fluorescence decreased slowly during the period of observation (approx. 20 min) (Figure 7C, dotted line), possibly indicating dye leakage. Exposure of rhodamine 123-loaded β -cells to 50 μ M 2,2'-DTDP reversed this trend and induced a progressive increase in fluorescence, indicating a decrease in $\Delta\psi_m$ (Figure 7C, solid line). In some experiments (two out of five), this slow depolarization of $\Delta\psi_m$ was preceded by a transient rapid depolarization (results not shown). 2,2'-DTDP or its reaction products did not interfere with rhodamine 123 fluorescence as tested in a cell-free system.

An increase in $[Ca^{2+}]_i$ might induce the exocytosis of insulin. Moreover, several thiol-oxidizing agents have been reported to stimulate insulin secretion [34,35]. For these reasons, we examined the effect of 2,2'-DTDP on insulin secretion from β -cells in a perfusion system. In these experiments the addition of 2,2'-DTDP (50 μ M) to the perfusate did not increase secretion at any time point during a 15 min observation period. Instead a slow decline in insulin secretion was noted, such that 6 and 12 min after the addition of 2,2'-DTDP the average insulin secretions were 73 % and 51 % respectively of those before the addition of 2,2'-DTDP.

DISCUSSION

In SR preparations, 2,2'-DTDP releases Ca^{2+} at concentrations as low as 5–10 μ M [12]. The effective concentration of 2,2'-DTDP in the present study was higher, reflecting difficulties involved in delivering the lipophilic substance to attached cells and the reducing cytoplasmic environment [36]. Our study shows that in β -cells thiol oxidation by 2,2'-DTDP induces a biphasic pattern of increase in $[Ca^{2+}]_i$, which can be readily reversed by the disulphide-reducing agent DTT. The initial increase in $[Ca^{2+}]_i$ was transient and not observed in approx. 25 % of the experiments. This increase in $[Ca^{2+}]_i$ was due to the entry of Ca^{2+} through the L-type voltage-gated Ca^{2+} channels as a result of plasma membrane depolarization induced by 2,2'-DTDP. In a previous study we showed that oxidation by 2,2'-dithiobis-(5-nitropyridine) of critical thiol groups associated with the K_{ATP} channel causes channel closure and plasma membrane depolarization [5]. However, although structurally related, 2,2'-DTDP did not inhibit K_{ATP} channel activity. Other K^+ channels in β -cells do not contain critical thiol groups and do not determine the cells' resting membrane potential. The substance also did not directly affect the activity of L-type voltage-gated Ca^{2+} channels. Identification of other possible mechanisms that could mediate 2,2'-DTDP-induced membrane depolarization was beyond the scope of the present study.

Because 2,2'-DTDP releases Ca^{2+} from intracellular Ca^{2+} stores in muscle cells and is a well-known activator of the SR Ca^{2+} release channel, we investigated whether 2,2'-DTDP-induced Ca^{2+} release could be attributed to the activation of an RyR-like channel in the β -cell. Several lines of evidence from the present study suggest that this was not so. First, we were unable to induce Ca^{2+} release from intracellular stores of human β -cells by caffeine and from mouse β -cells by ryanodine. Ryanodine can have both stimulatory and inhibitory effects on RyR, which sometimes make interpretation of ryanodine data difficult [31].

In this respect, lack of a Ca^{2+} -releasing effect of β -alanyl ryanodine was of particular significance, because this membrane-permeant analogue of ryanodine is a potent activator of RyR and most importantly lacks any inhibitory effect on the receptor. Secondly, pretreatment of the cells with a high concentration of ryanodine did not affect the magnitude or the rate of Ca^{2+} release by 2,2'-DTDP.

Early suggestions that β -cells might contain RyRs arose from the fact that caffeine, the classical pharmacological tool for RyR studies, induces a pronounced increase in $[\text{Ca}^{2+}]_i$ in these cells [37]. In a recent study we have demonstrated that caffeine can increase $[\text{Ca}^{2+}]_i$ in β -cells by mechanisms unrelated to its action on the RyRs [24]. Our previous suggestion that insulin-secreting cells might possess RyR relied heavily on the use of thimerosal as an activator of the RyR channel [7,10,11]. However, the lack of a notable Ca^{2+} release by caffeine, ryanodine [10] or cADP-ribose [30], as demonstrated under a variety of experimental conditions, tends to support the notion that the expression of Ca^{2+} -release channels, having pharmacological properties of the known RyRs, must be very low in insulin-secreting cells. The issue nevertheless remains controversial. Recent studies have implicated RyRs in glucagon-like peptide 1- [38] and nitric oxide-induced [39] release of Ca^{2+} in β -cells, on the evidence that ryanodine inhibits such responses. Although this has been a conventional interpretation of ryanodine data, one caveat to this concerns the possible non-specific effects of high concentrations of the alkaloid used in the experiments. By default, therefore, it can be assumed that 2,2'-DTDP released Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pools in pancreatic β -cells. This interpretation is supported by our finding that 2,2'-DTDP did not release Ca^{2+} when applied after emptying the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool by carbachol. Hence one mechanism of this Ca^{2+} release could be the activation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ by thiol oxidation [23]. However, our results also suggest that treatment with 2,2'-DTDP compromises cellular energy status, an effect that could contribute to the release of Ca^{2+} from intracellular stores. Such a compromise of cellular metabolism in β -cells by 2,2'-DTDP is consistent with the impairment of glycolysis by the compound described in Ehrlich ascites cells [40]. Nevertheless, despite demonstrable adverse effects of 2,2'-DTDP on cellular energy status, the compound did not seem to have major toxic effects, as evidenced from the rapid return of $[\text{Ca}^{2+}]_i$ to the basal level after the addition of DTT, the exclusion of ethidium bromide and the lack of leakage of insulin from the cells. Thus although it seems likely that cellular energy depletion might partly account for Ca^{2+} -release by 2,2'-DTDP, our study falls short of implicating or excluding the possibility that thiol oxidation can directly activate $\text{Ins}(1,4,5)\text{P}_3\text{R}$ in β -cells. The latter possibility proved difficult to test because of the lack of a potent cell-permeant inhibitor of $\text{Ins}(1,4,5)\text{P}_3\text{R}$. Nevertheless, results from hepatocytes reported previously suggest that 2,2'-DTDP can stimulate $\text{Ins}(1,4,5)\text{P}_3\text{R}$, although it requires the presence of $\text{Ins}(1,4,5)\text{P}_3$ [23]. One can speculate that 2,2'-DTDP-induced Ca^{2+} release from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool and the suppressing effect of the compound on metabolism might, in fact, be closely associated. Thus it is conceivable that once 2,2'-DTDP opens an $\text{Ins}(1,4,5)\text{P}_3\text{R}$ Ca^{2+} -release channel, the Ca^{2+} -ATPases will turn over rapidly and hydrolyse cellular ATP until it is depleted. It should be noted that, in the present study, Ca^{2+} release by 2,2'-DTDP was not due to release of the ion from mitochondria, because Ca^{2+} release was completely blocked after depletion of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool by carbachol. It also seems unlikely that a direct inhibition of sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase by thiol oxidation played a significant role in the Ca^{2+} release, because low concentrations of 2,2'-DTDP, as used in our study, have previously been shown

not to inhibit ATP hydrolysis and Ca^{2+} translocation by sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase [12].

Under physiological conditions a rise in $[\text{Ca}^{2+}]_i$ is associated with, and is a causative factor in, initiating exocytosis of secretory granules [2]. However, in the present study, despite a modest increase in $[\text{Ca}^{2+}]_i$, 2,2'-DTDP did not stimulate insulin secretion. This is consistent with previous reports that in β -cells, membrane-permeant thiol reagents usually do not stimulate secretion and might instead inhibit it [35,41]. Multiple factors might account for such a dissociation between increase in $[\text{Ca}^{2+}]_i$ and secretion, one of them being cellular energy depletion, as discussed above. These results imply that insulin secretion can be triggered by an increase in $[\text{Ca}^{2+}]_i$ only when the latter takes place in the correct cellular compartment and is accompanied by appropriate cellular energy status.

Our previous results in permeabilized insulin-secreting cells showed that the intracellular Ca^{2+} pool mobilized by thiol-oxidizing agents represents a discrete pool separate from the $\text{Ins}(1,4,5)\text{P}_3$ - and thapsigargin-sensitive ones [10]. In contrast, in intact β -cells, as shown in the present study, the two pools overlap completely. This suggests that in intact cells the ER Ca^{2+} pools exist as one compartment with luminal continuity and are equipped with $\text{Ins}(1,4,5)\text{P}_3\text{R}$ and possibly other Ca^{2+} -release channels on the limiting membrane [42]. The reason for such differences in results obtained from permeabilized cells and intact cells respectively might be due to fragmentation of the ER, as has been reported to take place under conditions of experiments with the former model [15,21].

We have characterized effects of 2,2'-DTDP, an activator of RyRs in muscle, on $[\text{Ca}^{2+}]_i$ in the pancreatic β -cell. In contrast with the situation in muscle, in β -cells a ryanodine-sensitive Ca^{2+} pool does not seem to be the target for Ca^{2+} mobilization by 2,2'-DTDP. Our findings document two mechanisms by which the reactive disulphide can promote a distinct pattern of rise in $[\text{Ca}^{2+}]_i$ in these cells. The first involves plasma-membrane depolarization and Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels. The second involves the release of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} pools. It seems reasonable to speculate that modification of critical thiol groups, associated with the intracellular Ca^{2+} -release channels, by cellular redox state might modulate $[\text{Ca}^{2+}]_i$ in β -cells under physiological or pathophysiological conditions.

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REFERENCES

- Ashcroft, F. M., Harrison, D. E. and Ashcroft, S. J. H. (1984) *Nature* (London) **311**, 271–273
- Berggren, P.-O., Arkhammar, P., Islam, M. S., Juntti-Berggren, L., Khan, A., Kindmark, H., Köhler, M., Larsson, O., Nilsson, T., Sjöholm, Å., Szecowka, J. and Zhang, Q. (1993) *Adv. Exp. Med. Biol.* **334**, 24–45
- Inagaki, N., Gono, T., Clement, IV, J. P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S. and Bryan, J. (1995) *Science* **270**, 1166–1170
- Phillipson, L. H. and Steiner, D. F. (1995) *Science* **268**, 372–373
- Islam, M. S., Berggren, P.-O. and Larsson, O. (1993) *FEBS Lett.* **319**, 128–132
- Lee, K., Ozanne, S. E., Hales, C. N. and Ashford, M. L. (1994) *J. Membr. Biol.* **139**, 167–181

- 7 Islam, M. S. (1994) Thesis, Karolinska Institute, Stockholm
- 8 Biden, T. J., Prentki, M., Irvine, R. F., Berridge, M. J. and Wollheim, C. B. (1984) *Biochem. J.* **223**, 467–473
- 9 Islam, M. S. and Berggren, P.-O. (1993) *Biochem. J.* **293**, 423–429
- 10 Islam, M. S., Rorsman, P. and Berggren, P.-O. (1992) *FEBS Lett.* **296**, 287–291
- 11 Islam, M. S., Larsson, O. and Berggren, P.-O. (1993) *Science* **262**, 584–586
- 12 Zaidi, N. F., Lagenaur, C. F., Abramson, J. J., Pessah, I. and Salama, G. (1989) *J. Biol. Chem.* **264**, 21725–21736
- 13 Hilbert, R., Zaidi, N., Shome, K., Nigam, M., Lagenaur, C. and Salama, G. (1992) *Arch. Biochem. Biophys.* **292**, 1–15
- 14 Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- 15 Joseph, S. K., Ryan, S. V., Pierson, S., Renard-Rooney, D. and Thomas, A. P. (1995) *J. Biol. Chem.* **270**, 3588–3593
- 16 Swann, K. (1991) *FEBS Lett.* **278**, 175–178
- 17 Galione, A., McDougall, A., Busa, W. B., Willmott, N., Gillot, I. and Whitaker, M. (1993) *Science* **261**, 348–352
- 18 Tanaka, Y. and Tashjian, Jr., A. H. (1994) *J. Biol. Chem.* **269**, 11247–11253
- 19 Pintado, E., Baquero-Leonis, D., Conde, M. and Sobrino, F. (1995) *Biochem. Pharmacol.* **49**, 227–232
- 20 Renard-Rooney, D. C., Hajnóczky, G., Seitz, M. B., Schneider, T. G. and Thomas, A. P. (1993) *J. Biol. Chem.* **268**, 23601–23610
- 21 Menniti, F. S., Bird, G. St. J., Takemura, H., Thastrup, O., Potter, B. V. L. and Putney, Jr., J. W. (1991) *J. Biol. Chem.* **266**, 13646–13653
- 22 Brocklehurst, K. (1979) *Int. J. Biochem.* **10**, 259–274
- 23 Missiaen, L., Taylor, C. W. and Berridge, M. J. (1992) *J. Physiol. (London)* **455**, 623–640
- 24 Islam, M. S., Larsson, O. and Berggren, P. O. (1995) *Biochem. J.* **306**, 679–686
- 25 Kindmark, H., Köhler, M., Arkhammar, P., Efendic, S., Larsson, O., Linder, S., Nilsson, T. and Berggren, P.-O. (1994) *Diabetologia* **37**, 1121–1131
- 26 Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- 27 Poenie, M. (1990) *Cell Calcium* **11**, 85–91
- 28 Hamill, O. P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F. J. (1981) *Pflügers Arch.* **391**, 85–100
- 29 Duchen, M. R., Smith, P. A. and Ashcroft, F. M. (1993) *Biochem. J.* **294**, 35–41
- 30 Rutter, G. A., Theler, J.-M., Li, G. and Wollheim, C. B. (1994) *Cell Calcium* **16**, 71–80
- 31 Humerickhouse, R. A., Bidasse, K. R., Gerzon, K., Emmick, J. T., Kwon, S., Sutko, J. L., Ruest, L. and Besch, Jr., H. R. (1994) *J. Biol. Chem.* **269**, 30243–30253
- 32 Fagian, M. M., Pereira-da-Silva, L., Martins, I. S. and Vercesi, A. E. (1990) *J. Biol. Chem.* **265**, 19955–19960
- 33 Zoratti, M., Pietrobon, D. and Azzone, G. F. (1982) *Eur. J. Biochem.* **126**, 443–451
- 34 Hellman, B., Lernmark, Å., Sehlin, J., Söderberg, M. and Täljedal, I.-B. (1973) *Arch. Biochem. Biophys.* **158**, 435–441
- 35 Ammon, H. P. T., Hägele, R., Youssif, N., Eujen, R. and El-Amri, N. (1983) *Endocrinology (Baltimore)* **112**, 720–726
- 36 Buxser, S. (1993) *Neuroprotocols* **3**, 165–174
- 37 Herchuelz, A. and Lebrun, P. (1993) *Biochem. Pharmacol.* **45**, 7–11
- 38 Gromada, J., Dissing, S., Bokvist, K., Renström, E., Frøkjær-Jensen, J., Wulff, B. S. and Rorsman, P. (1995) *Diabetes* **44**, 767–774
- 39 Willmott, N. J., Galione, A. and Smith, P. A. (1995) *FEBS Lett.* **371**, 99–104
- 40 Grassetti, D. R. and Murray, Jr., J. F. (1968) *Biochem. Pharmacol.* **17**, 2281–2290
- 41 Landgraf-Leurs, M. M. C., Mayer, L. and Landgraf, R. (1978) *Diabetologia* **15**, 337–342
- 42 Bird, G. St. J., Obie, J. F. and Putney, Jr., J. W. (1992) *J. Biol. Chem.* **267**, 18382–18386