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Inhibition of the ATP-sensitive potassium channel from mouse pancreatic β-cells by surfactants

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1 We have used patch-clamp methods to study the effects of the detergents, Cremophor, Tween 80 and Triton X100 on the K_{ATP} channel in the pancreatic β-cell from mouse.

2 All three detergents blocked K_{ATP} channel activity with the following order of potency: Tween 80 ($K_i < \sim 83$ nM) > Triton X100 ($K_i = 350$ nM) > Cremophor. In all cases the block was poorly reversible.

3 Single-channel studies suggested that at low doses, the detergents act as slow blockers of the K_{ATP} channel.

4 Unlike the block produced by tolbutamide, that produced by detergent was not affected by intracellular Mg^{2+}-nucleotide, diazoxide or trypsin treatment, nor did it involve an acceleration of rundown or increase in ATP sensitivity of the chanel.

5 The detergents could block the pore-forming subunit, Kir6.2ΔC26, which can be expressed independently of SUR_1 (the regulatory subunit of the K_{ATP} channel). These data suggest that the detergents act on Kir6.2 and not SUR_1.

6 The detergents had no effect on another member of the inward rectifier family: Kir1.1a (ROMK1).

7 Voltage-dependent K-currents in the β-cell were reversibly blocked by the detergents with a far lower potency than that found for the K_{ATP} channel.

8 Like other insulin secretagogues that act by blocking the K_{ATP} channel, Cremophor elevated intracellular Ca^{2+} in single β-cells to levels that would be expected to elicit insulin secretion.

9 Given the role of the K_{ATP} channel in many physiological processes, we conclude that plasma borne detergent may have pharmacological actions mediated through blockage of the K_{ATP} channel

Keywords: ATP-sensitive K-channel; Kir6.2; SUR1; β-cell; sulphonylurea; Cremophor EL; Tween 80; Triton X100; surfactant; detergent

Introduction

Closure of the adenosine 5′-triphosphate (ATP)-sensitive potassium channel (K_{ATP} channel) is a key component in the mechanism by which glucose and other metabolites stimulate insulin secretion from pancreatic β-cells (Ashcroft & Rorsman, 1989). K_{ATP} channels constitute the dominant potassium permeability of the β-cell plasma membrane, the extent of K_{ATP} channel activity controlling the membrane potential of the β-cell (Smith *et al*., 1990). It is widely thought that the activity of the K_{ATP} channel in the pancreatic β-cell is primarily controlled by the ratio of cytosolic ATP/ADP (Ashcroft & Rorsman, 1989). At low, subthreshold, glucose concentrations (< 5 mM) the cytosolic ATP/ADP ratio is low and the activity of the K_{ATP} channel results in a hyperpolarized membrane potential (~ -70 mV). As the glucose concentration is raised, the concomitant increase in cell metabolism and the ATP/ADP ratio inhibits the K_{ATP} channels which results in depolarization of the membrane potential. If this depolarization is sufficiently large (> -50 mV), Ca^{2+}-dependent action potentials are elicited, promoting influx of Ca^{2+} into the β-cell and stimulation of insulin secretion (Ashcroft & Rorsman, 1989; Duchen *et al*., 1993). This vital role of the K_{ATP} channel in the stimulus-secretion of insulin therefore makes this ion channel an important target for therapeutic drugs (Ashcroft, 1996). It is well established that sulphonylureas, such as glibenclamide, which are administered to control non-insulin-dependent diabetes mellitus, directly inhibit the activity of the K_{ATP} channel (Ashcroft, 1996). In doing so they mimic the action of glucose and promote insulin secretion. Clearly, any circulating agent which can inhibit the activity of K_{ATP} channels might be expected to stimulate insulin secretion and perturb both insulin and glucose homeostasis, thereby making the K_{ATP} channel an important target for environmental agents.

The molecular structure of the ATP-sensitive potassium channel consists of four inward-rectifier K^+ channel pore-subunits (Kir6.2), and four high affinity sulphonylureas receptor subunits (SUR_1, Inagaki *et al*., 1995; Sakura *et al*., 1995; Clement *et al*., 1997). SUR_1 belongs to the ATP-binding cassette family of proteins (Aguilar-Byran *et al*., 1995) which also contains the multidrug resistance gene (MDR) product, P-glycoprotein (P-gp) (Bradley *et al*., 1988).

Cremophor is a polyethoxylated castor oil and is a surfactant commonly used as a vehicle for the parenteral administration of water insoluble drugs and vitamins. It has been shown that low doses of Cremophor can reverse the MDR phenotype by blocking the ability of P-gp to transport lipophilic substances (Friche *et al*., 1990; Woodcock *et al*., 1992). The improvement in the effectiveness of anti-cancer drugs against MDR-expressing cells when administered with Cremophor has suggested that this surfactant may have possible therapeutic potential (Woodcock *et al*., 1992; Webster *et al*., 1993). Two other surfactants (Helenius *et al*., 1979), Triton X100 (non-ionic) and Tween 80 (anionic) can also inhibit P-gp at concentrations that are too low to disrupt membrane integrity (Friche *et al*., 1990; Woodcock *et al*., 1992;

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Zordan-Nudo *et al*., 1993). Considering the structural homology between SUR_1 and P-gp, one might expect them to possess a similar pharmacology. For example Tween 80 and Cremophor have both been shown to stimulate smooth muscle contraction in aortic rings, results that may be interpreted in terms of an inhibition of K_{ATP} channels by the detergents (Zengil *et al*., 1995). Using patch-clamp techniques, we have therefore investigated whether detergents can block K_{ATP} channels in pancreatic β-cells and therefore act as insulin secretagogues. The possible consequences of surfactants on insulin release and other processes that involve K_{ATP} channels are discussed.

Methods

β-cell preparation

For this study pancreatic β-cells were prepared from NMRI mice as previously described (Duchen *et al*., 1993). Cells were plated onto glass cover slips or directly onto plastic petri dishes and maintained for 1–4 days in RPMI 1640 tissue culture medium supplemented with 10% foetal calf serum, 50 μu ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Molecular biology

We used techniques identical to those described by Gribble *et al*. (1997). Xenopus oocytes were defolliculated and injected with 2 ng of mRNA encoding either a truncated form of Kir6.2 (Kir6.2ΔC26) or Kir1.1a. Currents were measured in giant inside-out patches excised from oocytes 1–4 days after injection. Mock-injected oocytes had no such currents.

Electrophysiology

Solutions For whole-cell recordings of K-ATP currents in pancreatic β-cells the standard extracellular (bath) solution contained (in mM): NaCl 140, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 10 (pH 7.4 with NaOH). The pipette was filled with either a MgATP or MgATP-free solution. The MgATP solution contained (in mM): KCl 107, $MgCl_2$ 1-2 (free $Mg^{2+} \sim 0.6-1.3$ mM), $CaCl_2$ 1 (free $Ca^{2+} \sim 20$ nM), EGTA 10 and HEPES 10 (pH 7.15 with KOH; total $K \sim 137$ mM) and 0.3 mM K_2ATP. The MgATP-free solution contained (in mM): KCl 110, $CaCl_2$ 4.6 (free $Ca^{2+} \sim 30$ nM), EDTA 10, HEPES 10 (pH 7.15 with KOH; total $K^{2+} \sim 138$ mM). For whole-cell recordings of voltage-gated K^+ currents the bath contained the standard extracellular solution and pipettes were filled with the MgATP solution supplemented with a total of 3 mM K_2ATP to block K-ATP currents.

In studies on cloned channels in oocytes, the pipette solution contained (in mM): KCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6 and HEPES 10 (pH 7.4 with KOH). The internal (bath) solutions were similar to those used in the studies on β-cells; either the MgATP-free solution or one that contained (in mM): KCl 110, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 10 and HEPES 10 (pH 7.2 with KOH). In the studies on the cloned inward-rectifier K^+ channel, Kir1.1a (ROMK1), 1 mM ATP was included in the internal solution to maintain channel activity. For these experiments the detergents were only tested on the cytoplasmic face of the channel by addition of the compounds directly to the bath solution.

For outside-out single-channel recordings, pipettes were filled with the MgATP-free solution and the bath contained the standard extracellular solution, for inside-out single-channel recordings these solutions were reversed such that pipettes were filled with the standard extracellular solution and the bath contained the MgATP-free solution.

Recording methods Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard at their tips and fire-polished immediately before use. Membrane currents were recorded with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The zero-current potential of the pipette was adjusted with the pipette in the bath just before establishment of the seal. No corrections have been made for the liquid junction potentials (<4 mV). Only one cell was studied from each petri dish. All electrophysiological experiments were conducted at room temperature, 23–25°C.

Whole-cell current analysis Whole-cell K_{ATP} channel activity was monitored as the current flowing in response to alternate 10 mV depolarizing and hyperpolarizing pulses of 200 ms in duration, applied at 0.5 Hz (Trube *et al*., 1986). Drugs were tested only after the whole-cell currents had reached a steady-state level (i.e. normally within 5 min after the standard whole-cell configuration had been obtained following the equilibration of the cytosolic solution with the pipette solution). Drug-containing solutions were alternated with control solutions. Due to the slow time course and poor reversibility of the block by the surfactants, the K_{ATP} currents in the presence of surfactant were measured as the fraction of that obtained in control before exposure to the drug. In the majority of experiments only one concentration of detergent was tested per cell, dose-response relationships were constructed by pooling the data from individual cells. In experiments where rundown of the K_{ATP} currents was present, the value for the amplitude of the control current was estimated by a linear extrapolation of the control current to the point at which the current amplitude in detergent was measured.

Cloned K-ATP channel analysis Macroscopic currents of cloned channels expressed in oocytes were recorded from giant inside-out patches in response to a 3 s voltage ramp from −110 to 100 mV (Hilgemann *et al*., 1991). The slope conductance of the expressed currents was measured by fitting a straight line to the linear portion of the data between −20 and −100 mV; the average of 3–5 consecutive ramps was calculated for each solution.

Single-channel analysis of K-ATP currents The membrane potential was held at 0 mV for both outside-out and inside-out patch studies. Although 0 mV is above the threshold for activation of both the outward delayed-rectifier and Ca^{2+}-activated K-channels, both channel types are substantially inactivated by the continuous depolarization. Furthermore, the free Ca^{2+} concentration in the MgATP free solution (<30 nM) is well below that required for activation of Ca^{2+}-activated K-channels.

Single-channel data were filtered with an 8 pole Bessel (−3 dB at 2 kHz) and digitized at 10 kHz. Single-channel currents were analysed with the half-amplitude threshold technique following the methods detailed by Colquhoun & Sigworth (1995) and were essentially the same as those described in Smith *et al*. (1994).

In most cases the activity of the K-ATP channel was stable for the lifetime of the patch, with little or no rundown due to the absence of Mg^{2+} in the cytoplasmic solution (Kozlowski & Ashford, 1990). The relationship between the concentration of detergent and channel activity was determined as described

below. We did not attempt to discriminate between the number of available channels, N, and their open probability, P_o.

Due to the use of identical electrochemical gradients and ionic conditions the kinetic and biophysical properties of the K_{ATP} channel were independent of whether they were recorded in the inside-out or outside-out patch configurations. The effects of the detergents on the kinetic parameters of the single-channels were indistinguishable whether they had been applied intracellularly to inside-out patches, or extracellularly to outside-out patches: because the parameters overlapped considerably. For these reasons, data from the two patch configurations were pooled.

Quantification of inhibition To quantify the degree of inhibition of currents produced by detergent, the fractional block was calculated as follows; whole-cell recordings, the amplitude of the native K_{ATP} current in the presence of a drug (I) was expressed as fraction of its amplitude in control solution (I_c), i.e. I/I_c; macroscopic recordings, the slope-conductance of the cloned K_{ATP} current in the presence of a drug (G) was expressed as fraction of its amplitude in control solution (G_c), i.e. G/G_c; single channel recordings, channel activity in the test solution (NP_o) was normalized to its value in control solution (NP_{o_c}) before exposure to the surfactant i.e. NP_o/NP_{o_c}.

Mean dose-response curves were fitted with the modified Hill equation:

$$\text{Fractional block} = I_o + \frac{I - I_o}{1 + ([D]/K_i)^{n_H}} \qquad \text{(Equation 1)}$$

Where D is the drug concentration, K_i is the concentration of drug which produces half-maximal inhibition of K_{ATP}, I_o is the fractional, drug-insensitive, component and n_H is the Hill coefficient.

For the display of whole-cell K_{ATP} current records data were filtered with an 8 pole Bessel (−3 dB at 10 Hz) and digitized at 200 Hz.

Voltage-dependent K^+ currents

Whole-cell voltage-dependent K^+ currents ($I_{K(V)}$) were evoked from a holding potential of −70 mV by incremental 10 mV voltage steps of 200 ms duration applied at a frequency of 0.5 Hz. To correct for linear leak currents and uncompensated capacity transients, the current responses of negative voltage steps to −100, −90 and −80 mV were scaled, averaged and subtracted from the raw current traces. The percentage outward current remaining in the presence of surfactant was measured as a fraction of that obtained in control solution before and after exposure to the drug. The K^+ conductance-voltage relationship, which is an estimate of the voltage-dependence of activation, was calculated by dividing $I_{K(V)}$ by the estimated electrochemical driving force: $Vm - E_K$, where Vm is the membrane potential and E_K is the Nernst equilibrium potential for potassium (~−80 mV), assuming the currents measured are carried solely by K^+ ions. No corrections were made for the voltage errors (<4 mV) arising from the series resistance (3.4±0.2 MΩ, $n=7$).

Ca^{2+} measurements

Microphotometry with the calcium fluorophore, fluo-3, was used to monitor $[Ca^{2+}]_i$ as described previously (Duchen *et al*., 1993). Cells were incubated with 1 μM fluo-3-AM ester (Molecular Probes, Oregon, U.S.A.) at room temperature (23–25°C) for 20–30 min in Hank's solution supplemented with 2.8 mM glucose. The dye was excited at a wavelength of 480 nm and the fluorescence monitored at 530 nm. The fluorescence signal was filtered at 1–10 Hz with a 4-pole Bessel filter and stored on DAT tape pending analysis. The fluorescence signals have not been calibrated in terms of $[Ca^{2+}]_i$ but are presented as percentage change in signal. As Fluo-3 has a K_d for Ca^{2+} of ~1 μM (Molecular Probes) changes in fluorescence are expected to depend linearly on the $[Ca^{2+}]_i$ across the physiological range (~100–500 nM).

For these experiments the bath was continuously perfused with a modified Hank's solution which contained (in mM): KCl 5.6, NaCl 137, NaH_2PO_4 1.2, $NaHCO_3$ 4.2, $MgCl_2$ 1.2, $CaCl_2$ 2.6 and HEPES 10 (pH 7.4 with ~6 mM NaOH) and 2.8 mM glucose. Experiments were conducted at 31–33°C to promote cellular metabolism and quasi-physiological Ca^{2+} handling by the β-cells (Duchen *et al*., 1993).

Reagents

Tolbutamide, Cremophor-EL, Triton X100 and Tween-80 (Polysorbate-80) were dissolved in dimethylsulphoxide (DMSO; final concentration <0.1%). Diazoxide (68 mM) was dissolved in 0.1 mM NaOH (final added OH equivalents <0.1 mM). All other chemicals, unless stated otherwise, were obtained from Sigma.

The action of trypsin on the efficacy of the detergents was tested by the application of either 20 or 50 μg ml^{-1} of trypsin (type IX, Sigma) for 5 or 2 min, respectively, to the inner face of inside-out patches (Proks & Ashcroft, 1993).

Statistical analysis

Data are expressed as mean±s.e.mean (n), where n is the number of samples. Statistical significance was determined by the Wilcoxon U-statistic for paired data and Mann–Whitney with correction for ties for unpaired data. $P<0.05$ was considered significant.

Results

We first compared the effects of detergents on standard whole-cell K_{ATP} currents, recorded with MgATP-containing pipette solution, with that of the sulphonylurea, tolbutamide, a well characterized blocker of this channel (Ashcroft, 1996). Application of 5 μM tolbutamide to the bath caused a rapid and reversible block of the K_{ATP} current to 46±4% ($n=12$) of its control value (Figure 1a). The time course for both the onset and reversal of the tolbutamide block was limited by the rate of bath perfusion and reflects the time course of the drug concentration in the bath. Subsequent perfusion of 5×10^{-4}% v/v Cremophor caused a slow inhibition of the K_{ATP} current and produced a steady-state block within 5 min of application (Figure 1a). Cremophor 5×10^{-4}% v/v reduced the K_{ATP} current to 37±6% ($n=8$) of its control value. In the majority of whole-cell experiments, the block produced by Cremophor was poorly reversible (see later).

It is conceivable that our measurement of the block produced by Cremophor is due to, or enhanced by, rundown of the K_{ATP} channel: a spontaneous decay in K_{ATP} current amplitude which is accelerated by intracellular Mg^{2+} (Kozlowski & Ashford, 1990). To check for this possibility the experiments were repeated with a Mg^{2+}-free pipette solution, conditions under which rundown is absent (Kozlowski & Ashford, 1990).

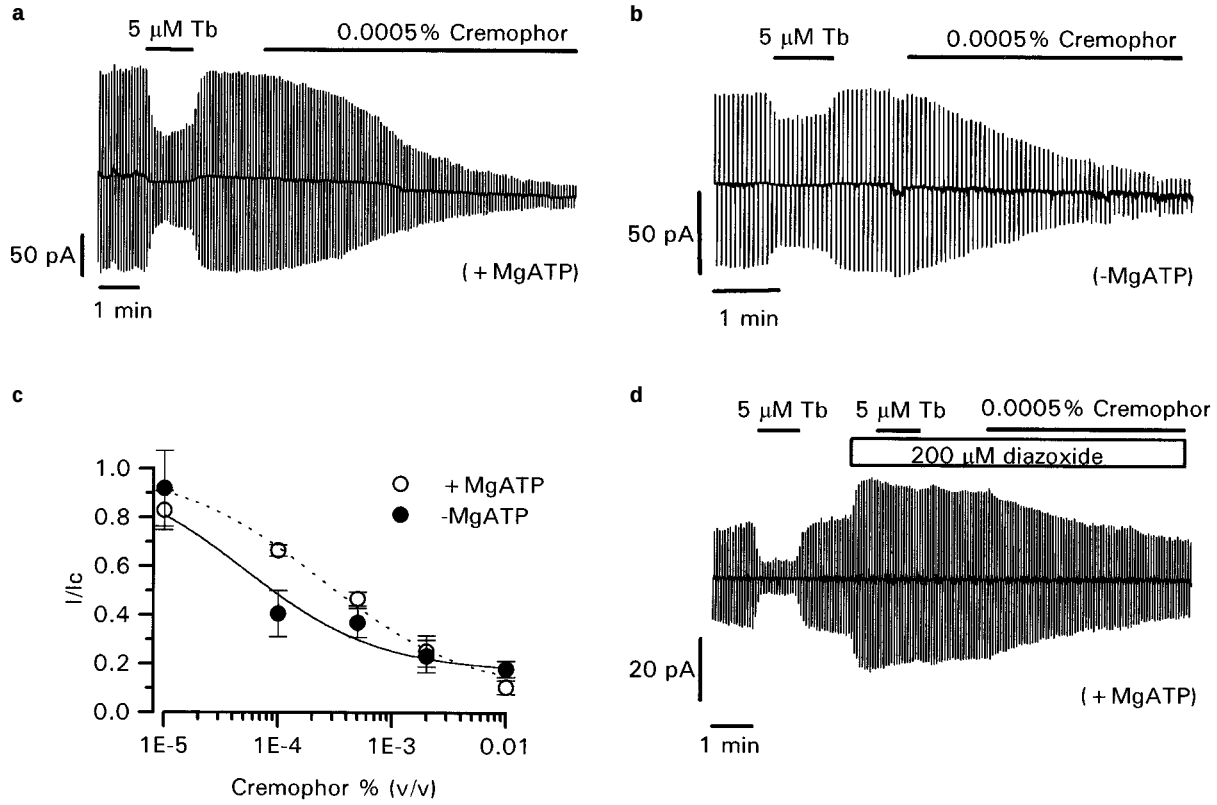

Figure 1 Representative effects of tolbutamide (Tb) and Cremophor on standard whole-cell K_{ATP} currents recorded as the response to ± 10 mV pulses from a holding potential of -70 mV. Tolbutamide and Cremophor (v/v) were applied for the duration of the horizontal bars as indicated. (a) Pipette contained 0.3 mM MgATP. (b) As in (a) but the pipette contained EDTA with no added Mg^{2+} or ATP. (c) Relationships between Cremophor concentration and K_{ATP} current (I), normalized to the control current (Ic), measured in the presence and absence of MgATP. Solid and dotted lines are best fits equation 1 to the mean data ($n=3-25$) with the parameters given in text. (d) Representative effect of diazoxide on the actions of tolbutamide (Tb) and Cremophor on whole-cell K_{ATP} currents. Pipette contained 0.3 mM MgATP.

Figure 1b illustrates a typical whole-cell recording of K_{ATP} currents made with a Mg^{2+}- and nucleotide-free pipette solution. Clearly both tolbutamide and Cremophor can still block the K_{ATP} current.

The time course of the block produced by Cremophor was dose-dependent. In 1×10^{-5}% v/v Cremophor, the lowest dose of Cremophor tested, the steady-state block of the K_{ATP} current was reached within 5 min of application. At a higher dose ($>2\times10^{-3}$% v/v) of detergent the steady-state block caused by Cremophor was almost immediate in onset and, like that produced by tolbutamide, was limited by the rate of bath perfusion (data not shown). Figure 1c displays the dose-response relationships for the steady-state block of the K_{ATP} channel produced by Cremophor. The best fit of the mean data with the Hill equation was obtained with a K_i of 5×10^{-5}% v/v and n_H of 0.7.

Although the data in Figure 1c suggest that Cremophor appears to be more potent at blocking K_{ATP} currents in the absence of MgATP than in its presence, this difference was not significant for any of the concentrations of detergent tested. The K_i for Cremophor in the absence of MgATP was 2.5×10^{-4}% v/v ($n_H=0.7$).

The drug diazoxide is a specific opener of the K_{ATP} channel, it also acts non-competitively to inhibit the block of the K_{ATP} channel produced by tolbutamide (Kozlowski & Ashford, 1992; Schwanstecher *et al*., 1992). Cloning-expression studies have demonstrated that, like tobutamide, diazoxide only exerts its major action on the K_{ATP} channel in the presence of SUR_1 and not by a direct effect on the inward rectifier subunits (Tucker *et al*., 1997). We therefore investigated whether the block of K_{ATP} by Cremophor, like that obtained with tolbutamide, was affected by diazoxide. Bath application of 200 μM diazoxide increased the K_{ATP} current by 140 ± 14% ($n=4$, Figure 1d). In agreement with previous work (Trube *et al*., 1986), the K^+-channel opener abolished the block of K_{ATP} channels produced by a low concentration of sulphonylurea; 5 μM tolbutamide blocked the K_{ATP} current by 54 ± 4% and 2 ± 1% ($n=4$) in the absence and presence of 200 μM diazoxide, respectively. However, diazoxide did not prevent the inhibition of the K_{ATP} current caused by a concentration of Cremophor of a similar potency to that of 5 μM tolbutamide (Figure 1d), although it did reduce the amount the block caused by the surfactant: 5×10^{-4}% v/v Cremophor blocked the K_{ATP} current by 66 ± 6% ($n=6$) and 43 ± 3% ($n=4$) in the absence and presence of 200 μM diazoxide, respectively. Due to cell to cell variation in the rate of rundown of the K_{ATP} currents the significance of this difference ($P=0.04$) is difficult to assess.

On the basis of these results we investigated the idea that the inhibition of the K_{ATP} current by Cremophor is not mediated via the sulphonylurea receptor, SUR_1, but is via a direct action of the detergent on the inward rectifier subunit, Kir6.2. This was achieved by testing the surfactant on modified ATP-sensitive channels (ΔK_{ATP}) which were assembled from Kir6.2ΔC26. ΔK_{ATP} possesses biophysical properties similar to those of wild-type K_{ATP} channels, but can be expressed independently of the SUR_1 subunit and is therefore insensitive to tolbutamide and diazoxide (Tucker *et*

al., 1997). Figure 2a shows that application of 0.01% v/v Cremophor to the cytoplasmic face of a giant patch completely inhibited the current flowing through ΔK_{ATP} channels. At this concentration, Cremophor inhibited ΔK_{ATP} currents by $94 \pm 3\%$ ($n=3$). This is a similar degree of block to that produced by the same concentration of detergent on the wild-type K_{ATP} channel (82%). Likewise the block was irreversible over the time course of the experiment. Cremophor 0.01% v/v applied in the absence of internal MgATP also blocked ΔK_{ATP} by a similar amount ($92 \pm 1\%$, $n=3$) to that found for the native channel (89%). A 10 fold lower concentration of Cremophor (1×10^{-3}% v/v) applied in the presence of MgATP inhibited the current flowing through ΔK_{ATP} channels by $75 \pm 3\%$ ($n=6$, data not shown), an amount similar to the inhibition of native K_{ATP} channels produced by 2×10^{-3}% v/v Cremophor (77%).

To determine if Cremophor could affect other members of the inward-rectifier family, Cremophor was tested on Kir1.1a (ROMK1). Cremophor 0.01% v/v, a dose that produces near maximal block of K_{ATP} and ΔK_{ATP}, had no effect on currents carried by Kir1.1a (data not shown): in the presence of Cremophor the slope conductance for Kir1.1a was $101 \pm 0.24\%$ ($n=3$) of the control value. The similarity of the time course and efficacy of the block of ΔK_{ATP} produced by Cremophor to that observed on the native K_{ATP} channel supports the idea that the surfactant acts directly on the inward-rectifier subunits and not on SUR_1, furthermore the block by this surfactant is not shared by the Kir1.1a inward-rectifier subunit.

Cremophor is composed of a complex mixture of hydrophilic (17% wt/wt) and hydrophobic (83% wt/wt) compounds. The hydrophilic component consists mainly of glycerin polyglycol ethers (10% wt/wt) and polyglycols (7% wt/wt). Whereas the hydrophobic component consists predominantly of esters of ricinoleic acid with glycerin polyglycol ethers and polyglycols, it also contains a little unreacted ricinoleic acid (3% wt/wt, Muller, 1996). It is possible that one or several of the compounds present in Cremophor account for its inhibition of the K-ATP channel. Diacylglcyerols have been shown to block K_{ATP} channels at micromolar concentrations (Plant, 1989), we therefore tested if the monoacylglycerol component of Cremophor, ricinoleic acid, can also block K_{ATP} channel activity. Ricinoleic acid has an estimated concentration of $\sim 10\ \mu$M in a 1×10^{-2}% v/v Cremophor solution.

Addition of 8 μM ricinoleic acid to that bath blocked whole-cell K_{ATP} currents, recorded with a MgATP-containing pipette solution, by only $6 \pm 4\%$ ($n=5$), in fact two of these cells failed to show any response to the fatty acid. Ricinoleic acid is therefore unlikely to be responsible for the majority of the block of the K_{ATP} channel produced by Cremophor.

Triton X100 (TX100), non-ionic) and Tween 80 (TW80, anionic) are surfactants which, unlike Cremophor, have a more homogeneous composition. They were investigated for their ability to block native K_{ATP}, ΔK_{ATP} and Kir1.1a-channel activity. TX100 and TW80 inhibited the current flowing through the K_{ATP} channel. Both TX100 (Figure 2c, d) and TW80 (data not shown) at 1×10^{-4}% v/v blocked currents flowing through the ΔK_{ATP} channel irrespective of the presence of MgATP: ΔK_{ATP} current was blocked $73 \pm 2\%$ ($n=3$) by TX100 and $87 \pm 4\%$ ($n=3$) by TW80. The recovery of ΔK_{ATP} from block by these surfactants was also poorly reversible (Figure 2). TX100, at a concentration that completely inhibited the activity of K_{ATP} and ΔK_{ATP} (1×10^{-4}% v/v), was without effect on currents carried by Kir1.1a: in the presence of TX100

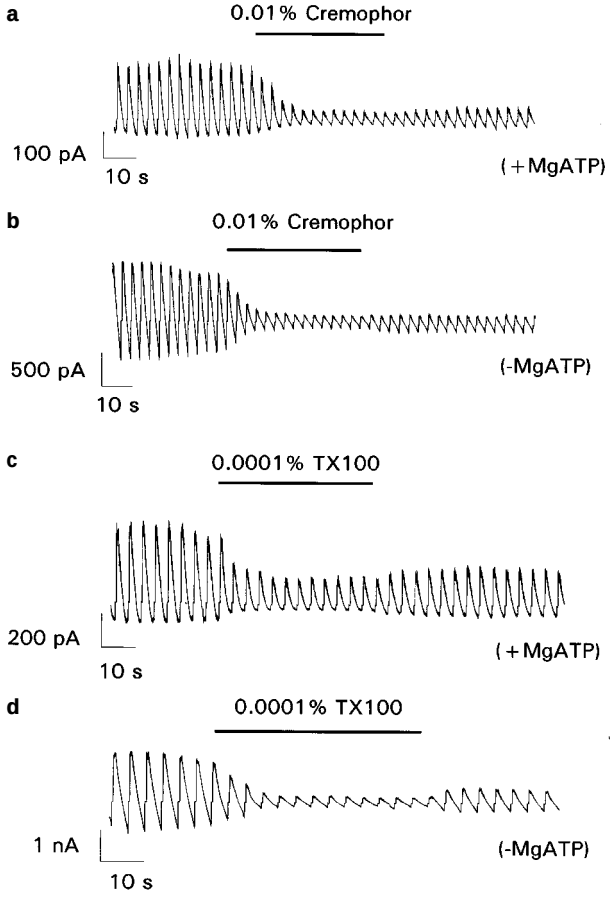

Figure 2 Representative effects of detergents on macroscopic currents recorded from giant inside-out patches pulled from oocytes expressing Kir6.2ΔC26. Currents were elicited by 3 s voltage ramps from −110 to 100 mV. Detergents (v/v) were applied for the duration of the bars. Currents shown were recorded in the presence or absence of MgATP in the intracellular solution as indicated.

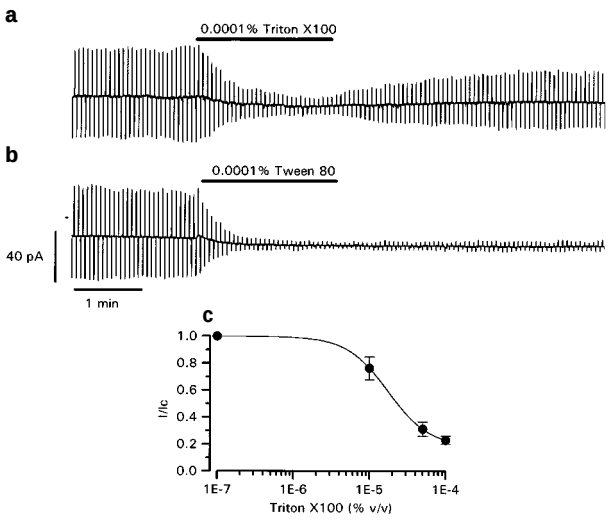

Figure 3 Representative effects of Triton X100 (a) and Tween 80 (b) on standard whole-cell K_{ATP} currents recorded as the response to ±10 mV pulses from a holding potential of −70 mV. The detergents (v/v) were applied for the duration of the bars. The pipette contained EDTA with no MgATP. (c) Relationship between Triton X100 and fractional block of K_{ATP} current, measured in the absence of MgATP. The solid line is the best fit of equation 1 to the mean data ($n=3-7$) with the parameters given in text.

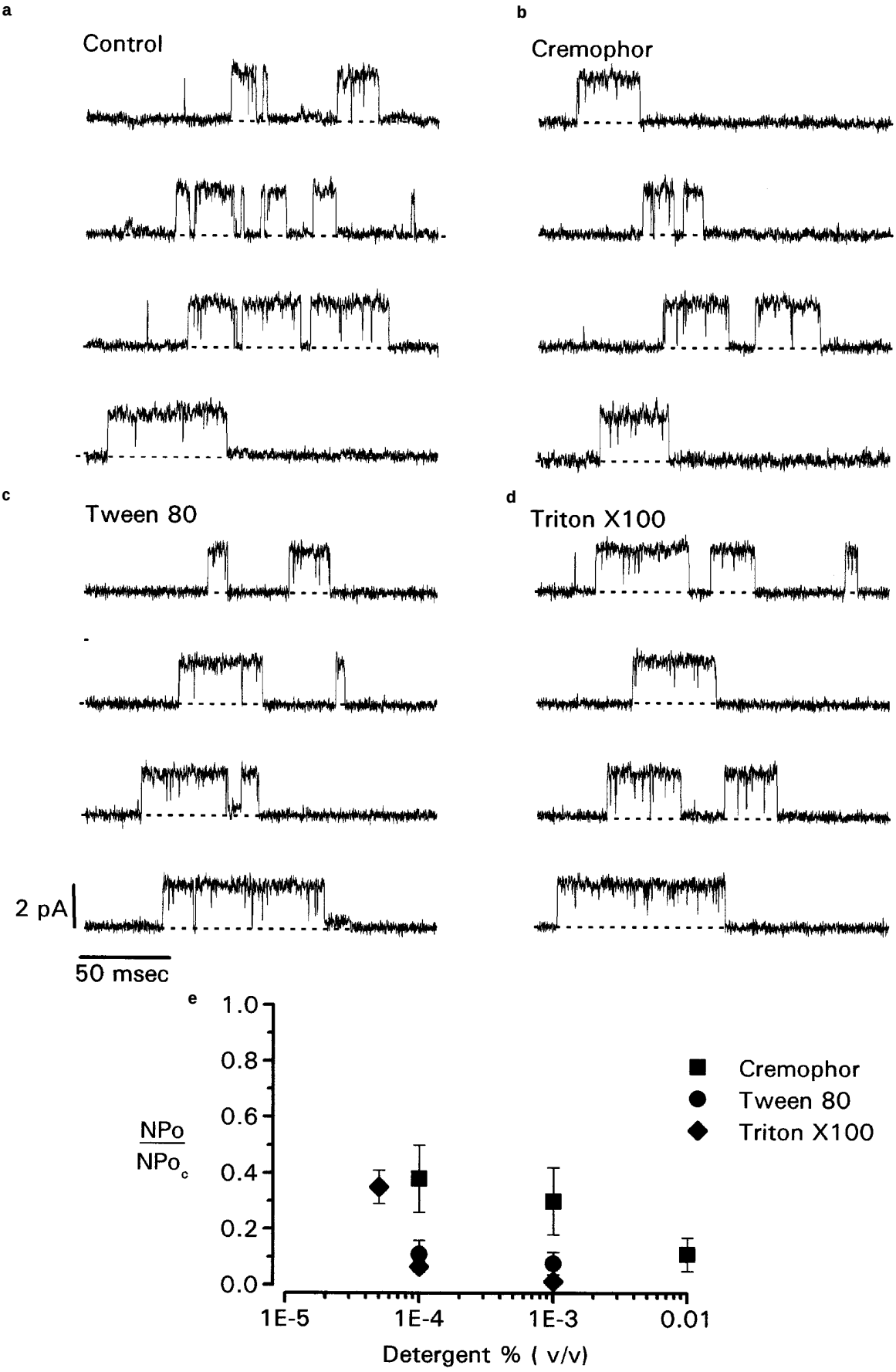

Figure 4 Representative single-channel recordings of β-cell K_{ATP} channel activity measured at a holding potential of 0 mV. Currents are from inside-out patches under; (a) control conditions, or in the presence of either (b) Cremophor, (c) Tween 80 or (d) Triton X100, as indicated. All surfactants were applied at 0.0001^4% v/v. (e) Relationship between detergent concentration (v/v) and single-channel activity (NP_o), normalized to that measured under control conditions (NP_{o_c}). Data are means ($n=4-7$).

the slope conductance of Kir1.1a was $100 \pm 0.1\%$ ($n = 3$) of control values (data not shown).

Figure 3a and b demonstrates that bath application of either TX100 or TW80 at 1×10^{-4}% v/v, produced a rapid inhibition of the native whole-cell K_{ATP} current. At all concentrations tested, the onset of block produced by TX100 and TW80, like that observed with tolbutamide, was almost immediate in onset and was governed by the rate of bath perfusion, steady-state inhibition being reached within 2 min of detergent addition.

The dose-dependency of the K_{ATP} channel block by TX100 in the absence of MgATP is shown in Figure 3c. The best fit of equation 1 to this data gave a K_i of 2×10^{-5}% v/v ($n_H = 1.6$). This affinity for TX100 is $\sim$12 fold higher than that fitted for Cremophor under similar conditions (2.5×10^{-4}%). Comparison of the block produced by 1×10^{-4}% v/v TW80 with the other surfactants suggests that the K_i for TW80 is $< 1 \times 10^{-5}$% v/v. Maximal block of the K_{ATP} current was observed at TX100 and TW80 concentrations in excess of 2×10^{-3}% v/v, values 10 fold lower than that determined for Cremophor under similar conditions (1×10^{-2}% v/v). However, the maximal amount of inhibition of K_{ATP} produced by TX100 or TW80 was similar to that produced by Cremophor ($\sim$80–90%). The inhibition produced by 1×10^{-4}% v/v TW80 or TX100 was, like that produced by Cremophor, unaffected by MgATP.

All three surfactants showed poor reversibility which was independent of whether MgATP was present or absent. In the absence of MgATP, a condition in which decay of the K_{ATP} current due to rundown is minimized, the K_{ATP} current blocked by 1×10^{-4}% v/v TX100 (77% block), 1×10^{-4}% v/v TW80 (81% block) or 2×10^{-3}% v/v Cremophor (75% block), recovered after 5 min of wash in control solution by only $48 \pm 4\%$ ($n = 3$), $22 \pm 14\%$ ($n = 3$) and $30 \pm 7\%$ ($n = 5$), respectively. Little further improvement in recovery was seen after a further 5 min of washing.

Surfactants, such as TX100, are known to alter ion channel function: by decreasing the stiffness of the plasmamembrane they can affect the stability of the different channel states (Lundbaek *et al.*, 1996). We therefore studied the single-channel behaviour of the K_{ATP} channel to investigate whether a similar mechanism could explain the block of this channel by surfactants (see methods). Figure 4 shows representative records of single-channel K_{ATP} currents recorded in inside-out patches in control (a) and in the presence of 1×10^{-4}% v/v Cremophor (b), TW80 (c) or TX100 (d). All three detergents decreased channel activity (NP_o) in a dose-dependent manner (Figure 4e). The order of potency of block for 1×10^{-4}% v/v detergent in the excised patches was TX100 > TW80 > Cremophor. In two patches tested, a 10 min wash in control solution after application of either TW80 or Cremophor recovered NP_o to only $\sim$50% of control values, this poor reversibility is comparable to the whole-cell situation.

The single-channel recordings shown in Figure 4 clearly demonstrate that the inhibition of K_{ATP} channel activity by the surfactants was not due to an open-channel rapid block mechanism, nor a reduction in the single-channel conductance, because neither the open channel current noise or the single-channel current amplitude (2 pA, Table 1) was affected by the surfactant.

The kinetic properties of the K_{ATP} channel were not obviously affected by detergent (Figure 4 and Table 1), channel openings still occurred both singularly and clustered within bursts. The failure of the detergents to affect either the burst length or open-channel lifetime significantly, indicate that they do not act by an open channel block mechanism. The lack of an effect of detergent on the lifetime for gaps within the burst and the absence of additional fast closed states further indicate that the inhibition of K_{ATP} channel activity by detergent was not via a flickery, open-channel, block mechanism. The slowest close-channel lifetime component, $\tau_{c,4}$, was significantly lengthened by all three detergents, as was the proportion of closures that fell into this class (Table 1).

Trypsin treatment of the intracellular membrane surface has been demonstrated to increase K_{ATP} channel activity and remove the regulatory affects of SUR_1 (Proks & Ashcroft, 1993). After trypsin treatment, the channel is unaffected by sulphonylureas or MgADP. We observed that trypsin increased K_{ATP} channel activity by $350 \pm 132\%$ ($n = 10$, data not shown). However, it did not affect the ability of either 1×10^{-3}% v/v Cremophor or 1×10^{-4}% v/v TX100 to inhibit channel activity: in trypsin-treated patches Cremohor blocked by $76 \pm 12\%$ ($n = 4$) and TX100 blocked by $97 \pm 1\%$ ($n = 7$) compared to 70% and 93%, respectively, in untreated patches (Table 1).

To investigate whether detergents affected other types of K^+-channels, they were tested on the voltage-dependent outward K^+ currents ($I_{K(V)}$) present in the β-cell. $I_{K(V)}$ in the β-cell is principally carried by Kv2.1 (Roe *et al.*, 1996). Figure 5 illustrates that membrane depolarization to potentials greater than $\sim$40 mV elicits outward K^+-currents which were rapidly and reversibly blocked by 0.01% v/v Cremophor. This concentration of detergent, which inhibits K_{ATP} channel activity by $\sim$90%, blocked the peak and the steady-state $I_{K(V)}$ by $18 \pm 5\%$ and $41 \pm 5\%$, respectively ($n = 3$, Figure 5ci). 2×10^{-3}% v/v Cremophor, which inhibits K_{ATP} channel activity by $\sim$75%, blocked the peak and the steady-state $I_{K(V)}$ by only $8 \pm 2\%$ and $5 \pm 5\%$, respectively ($n = 3$, data not shown). Application of 2×10^{-3}% v/v TW80, which maximally blocks the K_{ATP} channel (>90%),

Table 1 Kinetic parameters of K_{ATP} channel in the absence and presence of detergent

Detergent (v/v)	i (pA)	NP_o/NP_{o_c}	τ_o (ms)	$\tau_{c,1}$ (μs)	$\tau_{c,2}$ (ms)	$\tau_{c,3}$ (ms)	$\tau_{c,4}$ (s)	$t_{b,1}$ (ms)	$n_{b,1}$	$t_{b,2}$ (ms)	$n_{b,2}$
Control	2 ± 0.03	1	6.5 ± 0.3	140 ± 2	3.7 ± 0.64	35 ± 5.7	0.2 ± 0.04	40 ± 4.8	6.6 ± 0.9	7.1 ± 0.7	1.5 ± 0.06
1×10^{-4}% TX100	2 ± 0.07	*0.07 ± 0.02	7.1 ± 0.3	140 ± 3	*2.1 ± 0.22	35 ± 5.7	*0.30 ± 0.05	54 ± 13	7.2 ± 1.7	4.9 ± 0.8	1.3 ± 0.06
1×10^{-4}% TW80	2 ± 0.01	*0.11 ± 0.05	*7.5 ± 0.2	130 ± 4	2.9 ± 0.57	36 ± 9.9	*0.46 ± 0.02	62 ± 17	9.4 ± 2.6	3.9 ± 1.1	1.2 ± 0.05
1×10^{-4}% CRM	2 ± 0.07	*0.38 ± 0.12	6.4 ± 0.3	140 ± 4	*2.0 ± 0.34	38 ± 9.1	0.25 ± 0.09	28 ± 3.9	6.0 ± 2.0	5.0 ± 1.4	1.3 ± 0.11
1×10^{-3}% TX100	2 ± 0.05	*0.01 ± 0.01	6.9 ± 0.4	150 ± 7	*1.5 ± 0.18	32 ± 3.5	*0.59 ± 0.01	37 ± 10	6.3 ± 1.4	4.7 ± 1.3	1.4 ± 0.11
1×10^{-3}% TW80	2 ± 0.01	*0.08 ± 0.04	*7.5 ± 0.3	130 ± 2	*2.2 ± 0.59	26 ± 5.8	*0.32 ± 0.1	60 ± 17	13 ± 3.9	3.6 ± 1.5	1.2 ± 0.09
1×10^{-3}% CRM	2 ± 0.07	*0.3 ± 0.12	6.8 ± 0.5	130 ± 5	2.9 ± 0.50	43 ± 11	*0.34 ± 0.09	52 ± 26	8.4 ± 4.5	5.6 ± 0.2	1.3 ± 0.10

Key: CRM, cremophor, *i* mean single channel current amplitude, NP_o/NP_{o_c} channel activity normalized to control, τ_o mean open channel lifetime, $\tau_{c,i}$ mean closed channel lifetime of ith component, $t_{b,i}$ mean burst lifetime of ith component, $n_{b,2}$ mean number of openings per burst of ith component. *Indicates data that is significant at the 5% level relative to control value. Number of experiments was control, $n = 12$, TX100, $n = 7$, Tw80, $n = 5$ and CRM, $n = 4$.

inhibited the peak and the steady-state $I_{K(V)}$ by $27 \pm 5\%$ and $43 \pm 5\%$. respectively ($n = 3$, Figure 5b and di). Comparisons of the peak current-voltage relationships for $I_{K(V)}$ in control, in detergent and that of the detergent-sensitive current (Figure 5ci and di), suggest that the inhibition of $I_{K(V)}$ by detergent is voltage-independent. This idea is also supported by the percentage block produced by the detergent being independent of voltage (Figure 5ei). The conductance-voltage relationships for the activation of $I_{K(V)}$ in control and detergent are shown in Figure 5cii and dii. There was no obvious affect of detergent on the voltage-dependence of activation of $I_{K(V)}$ (Figure 5cii and dii). Figure 5eii demonstrates that both Cremophor and TW80 enhanced the inactivation of *I*-KV. Inspection of the whole-cell current records (Figure 5a and b) suggests that the increase in inactivation by detergent is caused by an acceleration of the rate of inactivation.

In the intact β-cell, inhibition of the K_{ATP} channel resulted in membrane depolarization and calcium influx. Figure 6 illustrates that in sub-threshold 2.8 mM glucose, bath application of 10 μM tolbutamide elicited a rapid and reversible rise in intracellular $[Ca^{2+}]_i$. Subsequent application of 2×10^{-3}% v/v Cremophor, which is expected to block K_{ATP} channel activity by a similar amount to that caused by 10 μM tolbutamide ($\sim$75%, Figure 1), also produced a rise in intracellular $[Ca^{2+}]_i$. Removal of Cremophor was followed by a slow recovery of the intracellular $[Ca^{2+}]$ back to basal values. Addition of 11 mM glucose stimulated a reversible increase in intracellular $[Ca^{2+}]$. The increases in intracellular $[Ca^{2+}]$ elicited by tolbutamide, Cremophor and glucose were all associated with an increased root mean square noise in the fluorescence signal (Figure 6b): the noise (% fluorescence) was 2.4% in control, 5% in tolbutamide, 8% in Cremophor and 5.2% in glucose. These data are consistent with Ca^{2+} entry associated with Ca^{2+}-dependent action potential activity elicited by membrane depolarization on block of K_{ATP} channels. The ability of glucose to increase the intracellular $[Ca^{2+}]$ confirms that the cell is a glucose-responsive β-cell. Similar results were obtained in four other cells.

Discussion and conclusions

The potency of detergent block of K-ATP

In this study we have demonstrated that the surfactants, Cremophor, Triton X100 and Tween 80 are all potent blockers of the K_{ATP} channel in pancreatic β-cells. The order of potency of block based on the fitted K_i for whole-cell data is; Tween 80 ($< 10^{-5}$% v/v) > Triton X100 (2×10^{-5}% v/v) > Cremohor (2.5×10^{-4}% v/v). Assuming that Tween 80 (mw $\approx$ 1310,

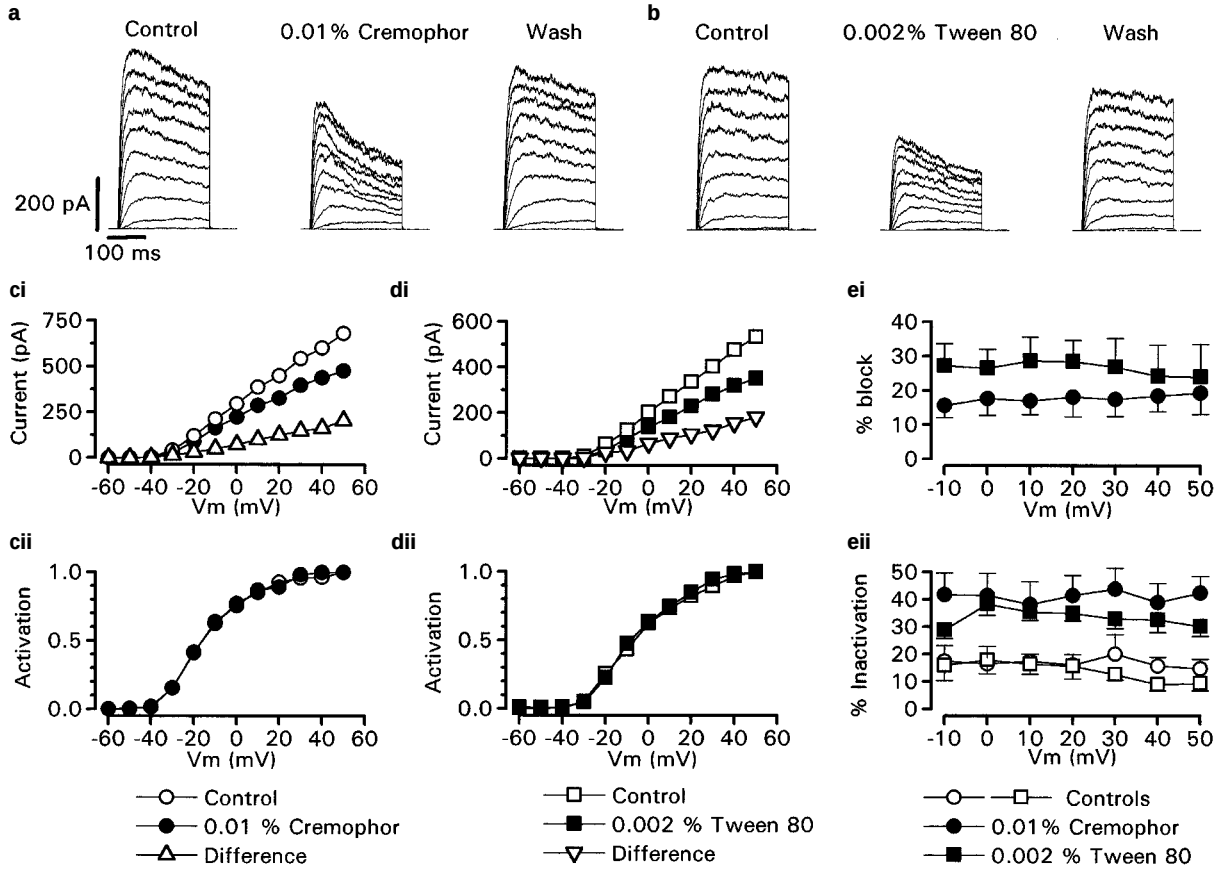

Figure 5 Representative whole-cell records of voltage-dependent outward K^+ currents ($I_{K(V)}$) recorded in control solution, in the presence of the indicated detergent (v/v) and then after wash in control solution. Currents were elicited from a holding potential of -70 mV by $+10$ mV incremental voltage steps to $+60$ mV. (a) Effect of Cremophor, (b) effect of Tween 80. (ci) Peak-current-voltage relationships for $I_{K(V)}$ measured in the absence and presence of 0.01% v/v Cremophor, also shown is the Cremophor-sensitive, difference, current. (cii) Conductance-voltage relationships for the *I*-Vs shown in (ci) (di) Peak current-voltage relationships for $I_{K(V)}$ measured in the absence and presence of 0.002% v/v Tween 80, also shown is the Tween 80-sensitive, difference, current. (dii) Conductance-voltage relationships for the *I*-Vs shown in (di). (ei) Percentage block of the peak $I_{K(V)}$ current, produced by Cremophor and Tween 80. (eii) Percentage steady-state inactivation of $I_{K(V)}$ measured in controls and in the presence of 0.01% v/v Cremophor and 0.002% v/v Tween 80 as indicated ($n = 3$). In all cases the lines solely link the symbols.

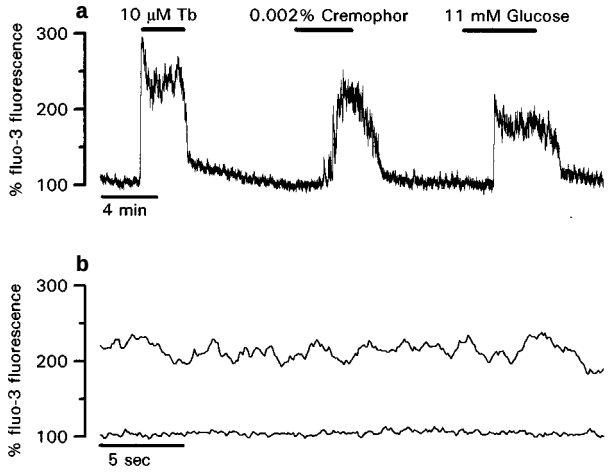

Figure 6 (a) Effects of 10 μM tolbutamide (Tb), 0.002[3]% v/v Cremophor and 11 mM glucose on $[Ca^{2+}]_i$ monitored as the change in fluo-3 fluorescence. This result is representative of 5 cells. (b) Time expansion and superimposition of 30 s of fluorescence signal taken during the period when $[Ca^{2+}]_i$ was either basal (lower trace) or elevated (upper trace) in the presence of Cremophor. Note the increased noise of the signal when $[Ca^{2+}]_i$ is elevated.

$p \approx 1.08$) and Triton X100 ($mw \approx 628$, $p \approx 1.1$) are homogeneous compounds, conversion of units (% v/v to nM), give a K_i of $\sim$350 nM for Triton X100 and $< \sim$83 nM for Tween 80. The polydisperse nature of Cremophor prevents this calculation (Muller, 1996). Tween 80 is obviously a very potent blocker of K_{ATP} channels.

The Hill coefficients varied slightly between the different surfactants. The low Hill coefficient of Cremophor ($\sim$0.6) may be explained if the detergent undergoes self association in aqueous solution and exists predominantly as a dimer (Kessel, 1992). If the dimer cannot block the channel but one or both of its constituent parts can, Cremophor would need to dissociate before it could inhibit the K_{ATP} channel, thereby producing the observed square root dependency ($n_H = 0.5$) of detergent concentration to block the channel. The high Hill coefficient of Triton X100 ($\sim$1.6) has several possible explanations: (1) at least two molecules of detergent must bind to the channel to inhibit its activity. (2) Triton X100 associates in the media and then binds as a dimer. (3) Triton X100 has a high level of positive cooperativity, binding of one detergent molecule favouring further binding before block ensues.

The block of K_{ATP} by detergent is independent of MgATP

The ability of the detergents to block irrespective of the presence of MgATP supports the notion that the reduction in K_{ATP} current produced by the detergents is due to a direct block of the channel and is not due to an acceleration of channel rundown. The ability of the detergents to block in the absence of ATP show that they do not inhibit the K_{ATP} channel by increasing its sensitivity to ATP.

The block of K_{ATP} is mediated via Kir6.2 and does not involve SUR_1

The ability of the detergents to block ΔK_{ATP}, in the absence of SUR_1, by a comparable amount to the native channel, strongly supports the idea that the surfactants do not act through SUR_1 but act directly on the Kir6.2 pore subunit. The observation that trypsin treatment of the inner membrane surface appears to uncouple SUR_1 from Kir6.2 (Proks & Ashcroft, 1993) but has no effect on the ability of detergents to inhibit K_{ATP} supports a lack of involvement of SUR_1. Furthermore, the failure of detergent to block currents flowing through inward-rectifier channels composed solely of Kir1.1a pore-subunits suggest that Kir6.2 has a specific detergent-sensitive site that is absent from Kir1.1a. The effect of detergent on the behaviour of the K_{ATP} channel can be explained by it either binding directly to this site or acting indirectly though alterations in the interactions between the Kir6.2 pore subunit and its membrane lipid-environment e.g. annular lipids. If the topology of the octameric model proposed for the quartenary structure of the K_{ATP} channel by Clement *et al.* (1997) is correct, namely four SUR_1 molecules forming a ring around the channel pore, effectively shielding the Kir6.2 subunits from the membrane environment, then the model of a direct action of detergent on Kir6.2 is favoured. The slow rate of reversal of the block of the K_{ATP} channel produced by the different detergents are entirely consistent with their nanomolar affinities.

Mechanism of the block of K_{ATP} channels by detergent

Single-channel analysis of the kinetics of the K_{ATP} channel suggests all the surfactants tested act as slow channel blockers (Smith *et al.*, 1994), promoting or stabilizing closed state/s that proceed those that are involved in the open-channel burst behaviour. This is manifest as a reduction in NP_o, with little or no effect on the kinetic behaviour of the channel during a burst, but causing elongation of the longest closures between bursts. A decrease in NP_o would increase both the length and proportion of periods during which channel openings are absent, i.e. increasing the likelihood of the longest close time component, $\tau_{c,4}$.

The similar potency of block of the K_{ATP} channel in excised patch and whole-cell, for which the holding potentials differ by $\sim$70 mV, indicates that the block produced by detergent is voltage-independent, also supported by the finding that the block of the cloned channels by detergent is independent of the ramp voltage. The high affinity of block of the K_{ATP} channel produced by Tween 80 ($K_i < 83$ nM) is more consistent with a pharmacological rather than physicochemical action of this detergent on the K_{ATP} channel.

Block of $I_{K(V)}$ by detergents

The mechanism of block of $I_{K(V)}$ produced by Cremophor and TW80 clearly differs to that of K_{ATP} channel being of a far lower potency and easily reversible, the latter consistent with their low affinity. Moreover, the detergents appear to act by causing an acceleration of inactivation and not by a tonic block. A similar phenomenon has been described for TX100 on the cloned voltage-gated K-channel, Kv2.1 (Höllerer & Heinemann, 1996). The molecular identity of the voltage-gated K-channels which underlie the bulk of $I_{K(V)}$ in the pancreatic β-cell have been shown to be Kv2.1 (Roe *et al.*, 1996). Although, under certain experimental conditions, K^+ flux through large Ca^{2+}-activated K^+-channels may contribute to the outward K^+-current (Ashcroft & Rorsman, 1989), the absence of both a voltage-dependent biphasic activation of the whole-cell current and a bell-shaped hump in the current-voltage relationship of $I_{K(V)}$, suggest that these channels were not very active in this study and $I_{K(V)}$ is indeed carried mainly by Kv2.1. Whether Cremophor and TW80 block $I_{K(V)}$ in β-cells by a similar mechanism to that proposed for the action of TX100

on Kv2.1 by Höllerer and Heinemann (1996), namely a lowering of the energy barrier between the resting channel and its C-type inactivated conformation promoting inactivation, is unknown.

Comparison of detergent effects on MDR

From this study it appears that any similarities in the pharmacology of P-glycoprotein and K_{ATP} channels are unlikely to be achieved by the channel protein containing a structural homologue of P-glycoprotein: SUR_1. Instead it appears that the channel pore-subunit itself, Kir6.2, may possess a lipophilic binding site. Other lipophilic compounds have already been shown to block directly the Kir6.2 subunit, e.g. phentolamine on ΔK_{ATP} (Proks & Ashcroft, 1997). Kir6.2 is not unique in the possession of a modulatory lipophilic binding site/s, since other ion channels, e.g. the nicotinic acetylcholine receptor, have also been described that are modulated by lipophilic signalling molecules (Barrentes, 1993). It is tempting to speculate that the putative lipophilic binding site on Kir6.2 proposed in this study may be an alternative route by which β-cell metabolism could effect the activity of the K_{ATP} channel and insulin secretion.

Physiological consequences of detergent

Expectedly, low doses of Cremophor can depolarize the β-cell and elicit Ca^{2+} entry. Similar effects were observed with low doses of TX100 and TW80 (data not shown). Furthermore, we have demonstrated that in intact cells this effect is reversible and does not appear to destroy normal β-cell function; therefore ruling out the general disruption of the membrane by low doses of detergent. The increase in intracellular Ca^{2+} produced by the detergents are comparable to those produced by other insulin secretagogues, namely glucose and tolbutamide. Therefore it is possible that plasma borne detergent, at a sufficient concentration, may elevate levels of circulating insulin. Whether this is the case is not known.

Plasma measurements of Cremophor in patients receiving the surfactant, either as a vehicle for lipophilic drugs or as a therapy for cancer, are typically around 0.1% v/v (Webster *et al*., 1993; Buckingham *et al*., 1996). Assuming 99% of this is bound to plasma protein, the free concentration is estimated to be 1×10^{-3}% v/v (Webster *et al*., 1993), a concentration well in excess of the K_i for block (3×10^{-5}% v/v) of K_{ATP} channel activity. It is interesting to speculate about the clinical and pathological impact of detergents in our environment on physiological process which involve K_{ATP} channels composed of Kir6.2. For example both Tween 80 and Cremophor have been found to stimulate smooth muscle contraction in aortic rings at concentrations as low as those employed here (1×10^{-4}% v/v for Tween 80 and 1×10^{-3}% v/v for Cremophor, Zengil *et al*., 1995). It is conceivable that these effects may simply be due to detergent-mediated inhibition of K_{ATP} channels in the smooth muscle.

Conclusion

In conclusion we have demonstrated that surfactants are potent blockers of the K_{ATP} channel and appear to act via the Kir6.2 pore-subunit. The concentrations of unbound surfactant in the plasma of patients receiving drug administrations which contain detergent are expected to affect the activity of K_{ATP} channels and associated physiological functions.

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