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Inhibition of heterologously expressed cystic fibrosis transmembrane conductance regulator Cl^- channels by non-sulphonylurea hypoglycaemic agents

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1 Hypoglycaemia-inducing sulphonylureas, such as glibenclamide, inhibit cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels. In search of modulators of CFTR, we investigated the effects of the non-sulphonylurea hypoglycaemic agents meglitinide, repaglinide, and mitiglinide (KAD-1229) on CFTR Cl^- channels in excised inside-out membrane patches from C127 cells expressing wild-type human CFTR.

2 When added to the intracellular solution, meglitinide and mitiglinide inhibited CFTR Cl^- currents with half-maximal concentrations of 164 ± 19 μM and 148 ± 36 μM, respectively. However, repaglinide only weakly inhibited CFTR Cl^- currents.

3 To understand better how non-sulphonylurea hypoglycaemic agents inhibit CFTR, we studied single channels. Channel blockade by both meglitinide and mitiglinide was characterized by flickery closures and a significant decrease in open probability (P_o). In contrast, repaglinide was without effect on either channel gating or P_o, but caused a small decrease in single-channel current amplitude.

4 Analysis of the dwell time distributions of single channels indicated that both meglitinide and mitiglinide greatly decreased the open time of CFTR. Mitiglinide-induced channel closures were about 3-fold longer than those of meglitinide.

5 Inhibition of CFTR by meglitinide and mitiglinide was voltage-dependent: at positive voltages channel blockade was relieved.

6 The data demonstrate that non-sulphonylurea hypoglycaemic agents inhibit CFTR. This indicates that these agents have a wider specificity of action than previously recognized. Like glibenclamide, non-sulphonylurea hypoglycaemic agents may inhibit CFTR by occluding the channel pore and preventing Cl^- permeation.

Keywords: CFTR; chloride channel; channel blockade; non-sulphonylurea hypoglycaemic agents; glibenclamide; meglitinide

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulphonic acid; DPC, diphenylamine-2-carboxylate; K_{ATP} channels, ATP-sensitive K^+ channels; Kir, inwardly rectifying K^+ channel; NBD, nucleotide-binding domain; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; SUR, sulphonylurea receptor

Introduction

In contrast to cation-selective channels, the pharmacology of Cl^--selective channels is less well defined. The search for modulators of Cl^- channels is important in at least two respects. First, high affinity inhibitors of Cl^- channels might be useful as agents for distinguishing Cl^- channels and as probes of the mechanism of ion permeation. Second, dysfunction of Cl^- channels causes a number of genetic diseases including, cystic fibrosis (CF), kidney stone diseases, and myotonia (Welsh *et al.*, 1995; Jentsch & Günther, 1997). This suggests that novel pharmacological modulators of Cl^- channels may be of value in the treatment of disease.

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR; Riordan *et al.*, 1989). CFTR is a Cl^- channel regulated by cyclic AMP-dependent phosphorylation and intracellular ATP (for review see Hanrahan *et al.*, 1994; Gadsby *et al.*, 1995; Welsh *et al.*, 1995). Intracellular ATP also regulates a class of K^+ channels (ATP-sensitive K^+ channels; K_{ATP} channels; for review see Ashcroft & Ashcroft, 1990; Ashcroft & Gribble, 1998). K_{ATP} channels have a distinct pharmacology: they are inhibited by sulphonylureas, a group of hypoglycaemia-inducing drugs used to treat non-insulin dependent diabetes mellitus and activated by a novel class of drugs called K^+ channel openers (Gerich, 1989; Edwards & Weston, 1993). In search of modulators of CFTR Cl^- channels, we previously demonstrated that the sulphonylureas, tolbutamide and glibenclamide, and the K^+ channel openers, diazoxide, BRL 38227 (levcromakalim), and minoxidil sulphate inhibit cyclic AMP-stimulated whole-cell Cl^- currents in cells expressing wild-type human CFTR (Sheppard & Welsh, 1992). The data suggested that glibenclamide (half-maximal inhibitory concentration (K_i) ~ 20 μM) was a potent inhibitor of CFTR Cl^- currents. Subsequent studies showed that glibenclamide produces a concentration-dependent decrease in the open time of single channels, suggesting that glibenclamide is an open-channel blocker of the CFTR Cl^- channel (Schultz *et al.*, 1996; Sheppard & Robinson, 1997).

The treatment of non-insulin dependent diabetes mellitus with sulphonylureas is associated with a number of adverse

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side-effects (Gerich, 1989). To circumvent these problems, several non-sulphonylurea hypoglycaemic agents have recently been developed. These include the benzoic acid derivative repaglinide (Gromada *et al*., 1995) and the 3-phenylpropionic acid derivatives A4166 and KAD-1229 (Akiyoshi *et al*., 1995; Mogami *et al*., 1994). The chemical structures of these agents are related to meglitinide, a benzoic acid derivative of the non-sulphonylurea moiety of glibenclamide (Panten *et al*., 1996). Like the sulphonylureas tolbutamide and glibenclamide, meglitinide stimulates insulin secretion from pancreatic β-cells by binding to the sulphonylurea receptor (SUR) to cause the inhibition of K_{ATP} channels (Sturgess *et al*., 1988; Panten *et al*., 1989). When compared to tolbutamide and glibenclamide, non-sulphonylurea hypoglycaemic agents inhibit K_{ATP} channels in pancreatic β-cells with a rank order of potency of glibenclamide = repaglinide > KAD-1229 $\geqslant$ meglitinide $\geqslant$ A4166 > tolbutamide (Sturgess *et al*., 1988; Gromada *et al*., 1995; Mogami *et al*., 1994; Akiyoshi *et al*., 1995).

In search of modulators of the CFTR Cl^- channel, the aim of this study was to investigate the effect of non-sulphonylurea hypoglycaemic agents on CFTR. To address this aim, we examined the effect of meglitinide, repaglinide, and mitiglinide (KAD-1229) on CFTR Cl^- channels using excised inside-out membrane patches from cells expressing wild-type human CFTR.

Methods

Cells and cell culture

For this study, we used mouse mammary epithelial (C127) cells that had been stably transfected with wild-type human CFTR using a bovine papilloma virus-based expression vector (Marshall *et al*., 1994). These cells were a generous gift of Drs S.H. Cheng, C.R. O'Riordan, and A.E. Smith (Genzyme, Framingham, MA, U.S.A.). C127 cells were cultured as previously described (Sheppard & Robinson, 1997). For experiments using excised inside-out membrane patches, cells were seeded onto glass coverslips and used within 48 h.

Electrophysiology

CFTR Cl^- channels were recorded in excised inside-out membrane patches using an Axopatch 200A patch-clamp amplifier (Axon Instruments Inc.) and pCLAMP data acquisition and analysis software (version 6.03, Axon Instruments Inc.) as previously described (Hamill *et al*., 1981; Sheppard & Robinson, 1997). The established sign convention was used throughout; currents produced by positive charge moving from intra- to extracellular solutions (anions moving in the opposite direction) are shown as positive currents.

The pipette (extracellular) solution contained (mM): N-methyl-D-glucamine (NMDG) 140, aspartic acid 140, $CaCl_2$ 5, $MgSO_4$ 2, and N-tris[Hydroxymethyl]methyl-2-aminoethane-sulphonic acid (TES) 10, adjusted to pH 7.3 with TRIS ($[Cl^-]$, 10 mM). Patch pipettes had resistances of 10–20 MΩ when filled with this solution. The bath (intracellular) solution contained (mM): NMDG 140, $MgCl_2$ 3, CsEGTA 1, and TES 10, adjusted to pH 7.3 with HCl, ($[Cl^-]$, 147 mM; free $[Ca^{2+}]$, $<10^{-8}$ M). The intracellular solution was maintained at 37°C using a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL, U.S.A.). To avoid significant evaporation of the intracellular solution, experiments were designed to include multiple interventions each of 4 min duration; between interventions the intracellular solution was replaced with fresh solution.

After excision of inside-out membrane patches from C127 cells, CFTR Cl^- channels were activated by the addition of the catalytic subunit of protein kinase A (PKA; 75 nM) and ATP (1 mM) to the intracellular solution within 5 min of patch excision. In most experiments, the ATP concentration was subsequently reduced to 0.3 mM ($\sim EC_{50}$ for CFTR Cl^- channel activation by intracellular ATP (Anderson *et al*., 1991)) before the addition of non-sulphonylurea hypoglycaemic agents to the intracellular solution; PKA was added to all intracellular solutions. Unless otherwise indicated, membrane patches were voltage-clamped at -50 mV.

To determine whether non-sulphonylurea hypoglycaemic agents inhibit CFTR, we studied CFTR Cl^- currents. To investigate how these agents inhibit CFTR, we studied single-channels using membrane patches that contained five or less active channels. The number of channels in a membrane patch was determined from the maximum number of simultaneous channel openings observed during the course of an experiment, as previously described (Lansdell *et al*., 1998). An experiment typically lasted 30–90 min and included multiple interventions each of 4 min duration that significantly stimulate single-channel open probability (P_o) (e.g. ATP (0.3–1 mM) + PKA (75 nM)). To compensate for any channel run-down during an experiment, specific interventions were bracketed by control periods made with similar concentrations of ATP and PKA, but without the test compound; the intervention data were then compared with the average data of pre- and postintervention control periods. To investigate whether channel blockade was voltage-dependent, membrane patches were bathed in symmetrical 147 mM Cl^- solutions and voltage was stepped from -80 to $+60$ mV in 20 mV increments of 30 s duration in the absence and presence of drugs, and P_o measured at each step.

CFTR Cl^- currents were initially recorded on digital audiotape using a digital tape recorder (Biologic Scientific

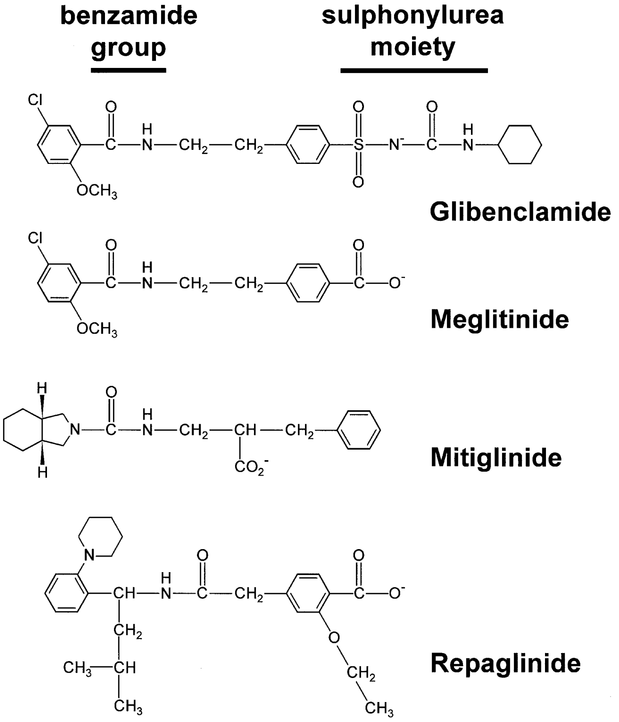

Figure 1 Chemical structures of the agents used in this study. The anionic forms of the compounds are shown. The sulphonylurea moiety and benzamide group of glibenclamide are indicated.

Instruments, model DTR-1204; Intracel Ltd, Royston, U.K.) at a bandwidth of 10 kHz. On playback, records were filtered with an eight-pole Bessel filter (Frequency Devices^{TM}, model 902LPF2; SCENSYS Ltd, Aylesbury, U.K.) at a corner frequency of 500 Hz and acquired using a Digidata 1200 interface (Axon Instruments, Inc.) and pCLAMP at sampling rates of either 2.5 kHz (time-course studies) or 5 kHz (single-channel studies).

In time-course studies, each data point is the average current for a 4 s period with data points collected continuously; no data were collected while solutions were changed. Average current (I) for a specific intervention was determined as the average of all the data points collected during the intervention. Concentration-response relationships were fit to the Hill equation:

$$I_{Drug}/I_{Control} = 1/\{1 + ([Drug]/K_i)^n\} \quad (1)$$

where [Drug] is the concentration of drug, $I_{Drug}/I_{Control}$ is the fractional current at the indicated drug concentration relative to that in the same solution in the absence of added drug, K_i is

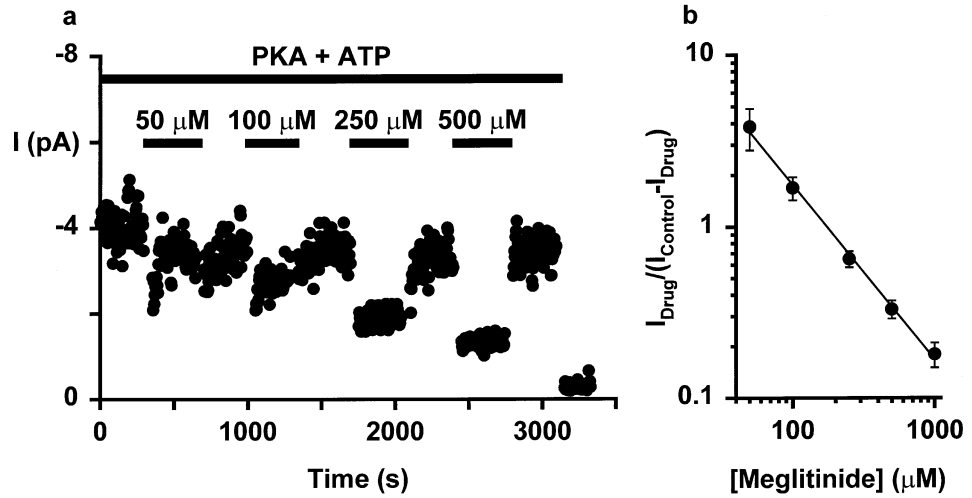

Figure 2 Meglitinide inhibits CFTR Cl$^-$ currents. (a) Time-course of CFTR Cl$^-$ current in an excised inside-out membrane patch. ATP (0.3 mM), PKA (75 nM), and meglitinide (50–500 μM) were present in the intracellular solution during the times indicated by the bars. Voltage was −50 mV, and there was a large Cl$^-$ concentration gradient across the membrane patch (internal [Cl$^-$] = 147 mM; external [Cl$^-$] = 10 mM). Each point is the average current for a 4 s period and no data were collected while solutions were changed. For the purpose of illustration, the time-course has been inverted so that an upward deflection represents an inward current. (b) Hill plot of the effect of meglitinide concentration on CFTR Cl$^-$ currents. Data are means ± s.e.mean; $n = 3-6$ observations at each concentration. The continuous line is the fit of a first-order regression to the data. Other details as in (a).

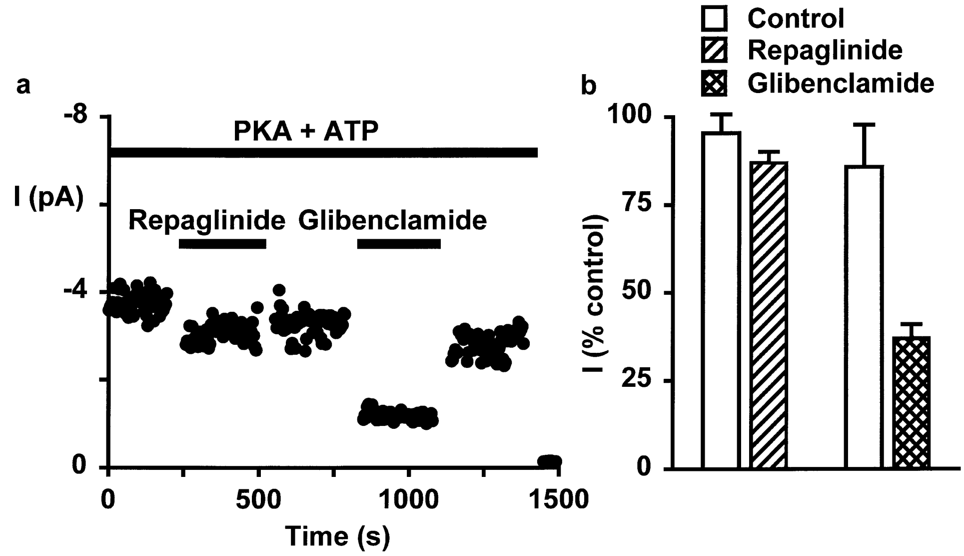

Figure 3 When compared to glibenclamide, repaglinide only weakly inhibits CFTR Cl$^-$ currents. (a) Time-course of CFTR Cl$^-$ current in an excised inside-out membrane patch. ATP (1.0 mM), PKA (75 nM), repaglinide (50 μM), and glibenclamide (50 μM) were present in the intracellular solution during the times indicated by the bars; other details as in Figure 2a. (b) Comparison of the effects of repaglinide (50 μM) and glibenclamide (50 μM) on CFTR Cl$^-$ currents. Columns and error bars indicate means ± s.e.mean ($n = 5$) for each concentration. Values represent the average current recorded during an intervention normalised to that measured under control conditions at the start of the experiment. Repaglinide (50 μM) reduced CFTR Cl$^-$ current to 87 ± 3% of control ($n = 5$; $P < 0.05$), whereas glibenclamide (50 μM) reduced CFTR Cl$^-$ current to 37 ± 4% of control ($n = 5$; $P < 0.001$). Other details as in (a). In the presence of both glibenclamide (50 μM) and repaglinide (50 μM) CFTR Cl$^-$ currents were reduced to 37 ± 3% of control ($n = 5$), a value not significantly different from that observed in the presence of glibenclamide (50 μM; $n = 5$; $P > 0.05$).

the drug concentration causing half-maximal inhibition, and n is the slope factor (Hill coefficient). Data from individual experiments were fitted to a linear form of equation (1) using linear least-squares regression to yield K_i and n values. Single-channel current amplitude (i) was determined from the fit of Gaussian distributions to current amplitude histograms. For P_o and kinetic analyses, lists of open and closed times were created using a half-amplitude crossing criterion for event detection. Transitions < 1 ms in duration were excluded from the analyses. Single-channel open and closed time histograms were plotted with logarithmic x-axes with 10 bins decade^{-1}, and the maximum likelihood method was used to fit a one-, two-or three-component exponential to the data. To determine which component function fitted best, the log-likelihood ratio test was used and considered statistically significant at a value of 2.0 or greater (Winter *et al.*, 1994). Only membrane patches that contained a single active channel were used for single-channel kinetic analyses. P_o was calculated using the equation:

$$P_o = (T_1 + T_2 + \ldots + T_N)/(NT_{tot}) \qquad (2)$$

where N is the number of channels, T_{tot} is the total time analysed, and T_1 is the time that one or more channels are open, T_2 is the time two or more channels are open and so on.

Reagents

The catalytic subunit of PKA was obtained from Promega Ltd. ATP (disodium salt) and TES were purchased from Sigma-Aldrich Company Ltd (Poole, U.K.). All other chemicals were of reagent grade.

The chemical structures of the agents studied are shown in Figure 1. They include glibenclamide (Sigma-Aldrich Company Ltd), meglitinide (a generous gift of Hoechst Marion Roussel, Frankfurt am Main, Germany), mitiglinide (a generous gift of the Institut de Recherches Internationales Servier, Courbevoie, France), and repaglinide (a generous gift of Novo Nordisk A/S, Bagsvaerd, Denmark). Mitiglinide is the international non-proprietary name for KAD-1229. Stock solutions of glibenclamide, meglitinide, and repaglinide were prepared in DMSO and mitiglinide in ethanol. All stock solutions were stored at −20°C and diluted in intracellular solution to achieve final concentrations immediately before use. Because repaglinide is light-sensitive, stock solutions of this drug were kept in the dark at all times. There was no change in the potency of repaglinide during the course of experiments. The vehicles, DMSO and ethanol, did not affect the activity of CFTR Cl^- channels ($n = 4$ in each case).

Statistics

Results are expressed as means ± s.e.mean of n observations. To compare sets of data, we used either Student's t-test or the Wilcoxon signed rank test. Differences were considered statistically significant when $P < 0.05$. Tests were performed using SigmaStat^{TM} (version 1.03, Jandel Scientific GmbH, Erkrath, Germany).

Results

Non-sulphonylurea hypoglycaemic agents inhibit CFTR Cl^- currents

To examine the effects of the non-sulphonylurea hypoglycaemic agents, meglitinide, repaglinide, and mitiglinide on wild-type human CFTR, we studied CFTR Cl^- currents in excised inside-out membrane patches from C127 cells stably expressing wild-type human CFTR. Following the activation of CFTR Cl^- currents by cAMP-dependent phosphorylation, drugs were added to the solution bathing the intracellular side of the membrane in the continued presence of PKA and ATP. Figure 2 demonstrates that meglitinide inhibited CFTR Cl^- currents. Addition of meglitinide (50 – 1000 μM) to the intracellular solution caused a concentration-dependent decrease in CFTR Cl^- current. The relationship between meglitinide concentration and current inhibition was well fitted by the Hill equation with $K_i = 164 \pm 19$ μM ($n = 6$) and $n = -1.0 \pm 0.1$ ($n = 6$) at −50 mV (Figure 2b). Inhibition of CFTR Cl^- currents by meglitinide was reversible (Figure 2a).

Repaglinide is a new hypoglycaemic agent that inhibits K_{ATP} channels in pancreatic β-cells with a potency similar to that of glibenclamide (Gromada *et al.*, 1995). In contrast to its

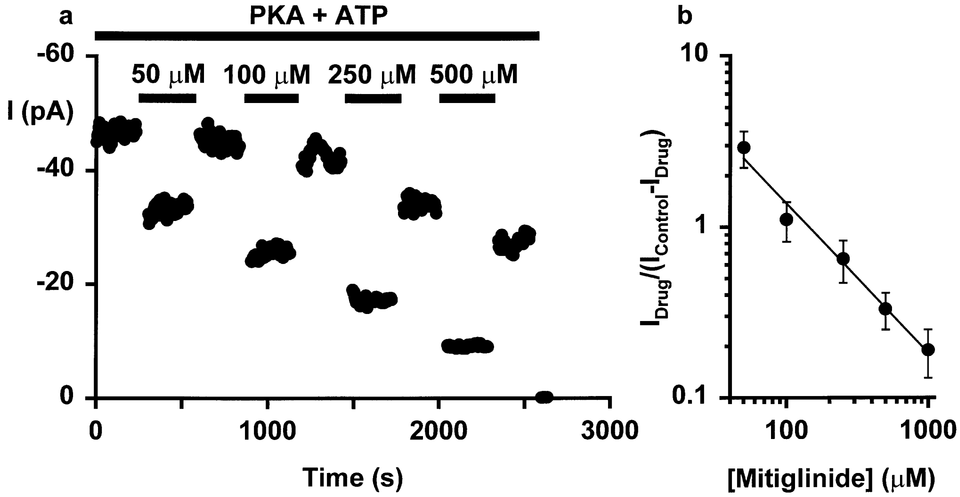

Figure 4 Mitiglinide inhibits CFTR Cl^- currents. (a) Time-course of CFTR Cl^- current in an excised inside-out membrane patch. ATP (0.3 mM), PKA (75 nM), and mitiglinide (50 – 500 μM) were present in the intracellular solution during the times indicated by the bars; other details as in Figure 2a. (b) Hill plot of the effect of mitiglinide concentration on CFTR Cl^- currents. Data are means ± s.e.mean; $n = 4 – 5$ observations at each concentration. The continuous line is the fit of a first-order regression to the data. Other details as in (a).

effect on K_{ATP} channels, repaglinide was a much less potent inhibitor of CFTR Cl^- currents than glibenclamide. Figure 3 shows that repaglinide (50 μM) caused only a small reduction in CFTR Cl^- current, whereas glibenclamide (50 μM) produced a much larger decrease. Addition of higher concentrations of repaglinide (100 – 200 μM) to the intracellular solution caused only a modest increase in channel blockade (see below) and repaglinide failed to potentiate glibenclamide inhibition of CFTR (see Figure 3 legend). Inhibition of CFTR Cl^- currents by repaglinide was weakly reversible (not shown).

Like meglitinide, mitiglinide caused a concentration-dependent decrease in CFTR Cl^- current (Figure 4). The relationship between mitiglinide concentration and current inhibition was well fitted by the Hill equation with $K_i = 148 \pm 36$ μM ($n = 5$) and $n = -0.90 \pm 0.1$ ($n = 5$) at -50 mV. Inhibition of CFTR Cl^- currents by mitiglinide was partially reversible (Figure 4a).

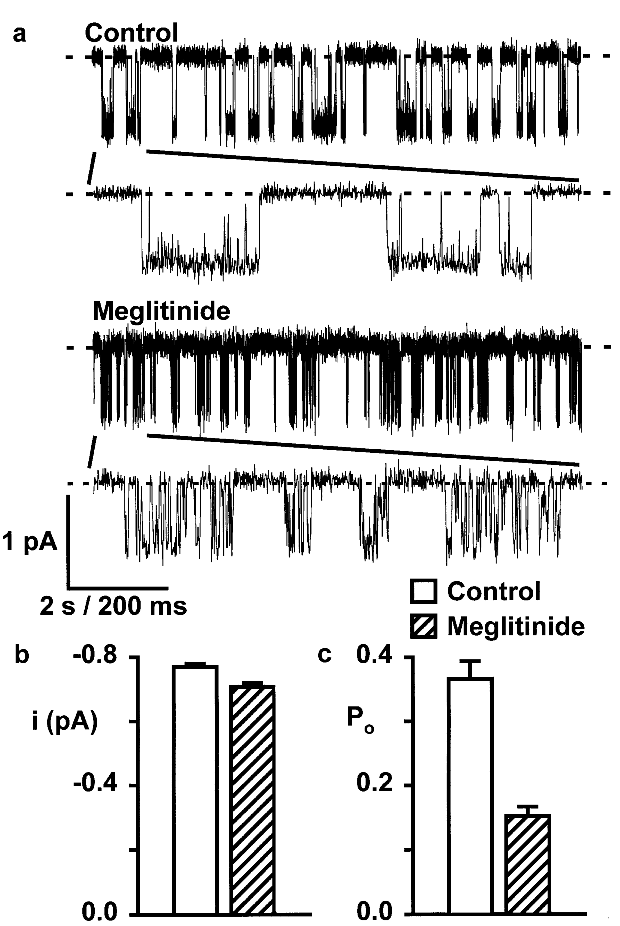

Figure 5 Effect of meglitinide on the single-channel activity of CFTR. (a) Representative recordings show the effect of meglitinide (250 μM) on the activity of a single CFTR Cl^- channel. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution. Voltage was -50 mV, and there was a large Cl^- concentration gradient across the membrane patch (internal $[Cl^-] = 147$ mM; external $[Cl^-] = 10$ mM). Scale bar, 2 s and 200 ms for prolonged and expanded recordings, respectively. Dashed lines indicate the closed channel state and downward deflections correspond to channel openings. For the purpose of illustration, records were filtered at 500 Hz and digitised at 1 kHz. (b) and (c) Effect of meglitinide (250 μM) on i and P_o, respectively. Columns and error bars indicate means ± s.e.mean ($n = 7$) for each concentration. Meglitinide (250 μM) decreased i to $92 \pm 1\%$ of the control value ($n = 7$; $P < 0.001$) and P_o to $42 \pm 3\%$ of the control value ($n = 7$; $P < 0.001$). Other details as in (a).

Effects of non-sulphonylurea hypoglycaemic agents on the single-channel properties of CFTR

To understand better how non-sulphonylurea hypoglycaemic agents inhibit CFTR, we studied single-channels. Figure 5a shows the effect of meglitinide (250 μM) on the activity of a single CFTR Cl^- channel following phosphorylation by PKA. These single-channel records demonstrate that meglitinide dramatically altered the gating behaviour of CFTR Cl^- channels. The pattern of gating of wild-type human CFTR is characterized by bursts of activity interrupted by brief flickery closures, separated by longer closures between bursts (Figure 5a, top trace). In the presence of meglitinide (250 μM), the gating behaviour of CFTR was characterized by a large increase in the frequency of flickery closures (Figure 5a). To quantify the effect of meglitinide on channel gating, we measured i and P_o. Figure 5b,c show that meglitinide (250 μM) caused a small reduction in i ($P < 0.05$), but a large decrease in P_o ($P < 0.001$).

In contrast to meglitinide, repaglinide (50 μM) did not alter the gating behaviour of CFTR Cl^- channels (Figure 6a). However, repaglinide (50 μM) caused a small decrease in i ($P < 0.01$; Figure 6b). This effect was not readily apparent by

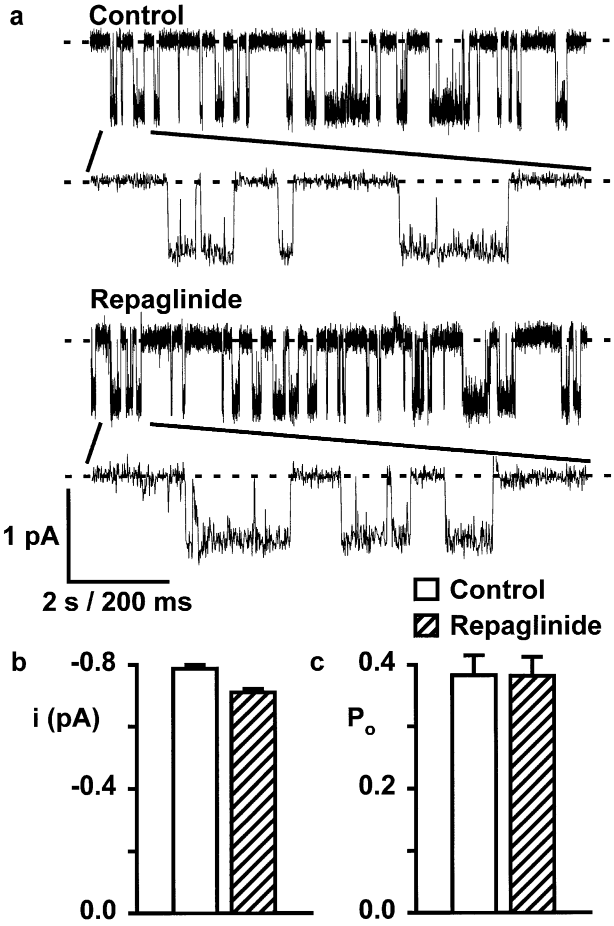

Figure 6 Effect of repaglinide on the single-channel activity of CFTR. (a) Representative recordings show the effect of repaglinide (50 μM) on the activity of a single CFTR Cl^- channel. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution; voltage was -50 mV. Scale bar, 2 s and 200 ms for prolonged and expanded recordings, respectively. (b) and (c) Effect of repaglinide (50 μM) on i and P_o, respectively. Columns and error bars indicate means ± s.e.mean ($n = 6$) for each concentration. Repaglinide (50 μM) decreased i to $91 \pm 2\%$ of the control value ($n = 6$; $P = 0.005$), but was without effect on P_o ($n = 6$; $P > 0.05$). Other details as in (a).

visual inspection of single-channel records (Figure 6a). Consistent with its lack of effect on channel gating, repaglinide (50 μM) did not alter P_o ($P>0.05$; Figure 6c). Because repaglinide (50 μM) only weakly inhibited CFTR Cl^- channels, we tested higher concentrations. Repaglinide (200 μM) decreased i to $80\pm3\%$ of the control value ($n=3$; $P<0.05$), but was without effect on P_o ($n=3$; $P>0.05$). Higher concentrations of the drug were not soluble in the intracellular solution.

Figure 7a shows the effect of mitiglinide (250 μM) on the activity of a single CFTR Cl^- channel following phosphorylation by PKA. In contrast to meglitinide and repaglinide, mitiglinide (250 μM) was without effect on i ($P>0.05$; Figure 7a,b). However, mitiglinide did alter channel gating. In the presence of mitiglinide (250 μM), the gating behaviour of CFTR was characterized by flickery channel closures and P_o was significantly decreased ($P<0.001$; Figure 7a,c).

Meglitinide and mitiglinide alter the open and closed time distributions of CFTR

To determine how meglitinide and mitiglinide decreased P_o, we investigated the effect of these agents on the gating kinetics of phosphorylated CFTR Cl^- channels using membrane patches that contained only a single active channel. We analysed histograms of open and closed times in the absence and presence of either meglitinide or mitiglinide to determine whether we could detect new populations of channel closures that represented channels blocked by these drugs. Consistent with previous results, the open and closed time histograms of wild-type human CFTR were best fitted with one- and two-component functions, respectively (Figure 8 and Table 1; Winter *et al.*, 1994).

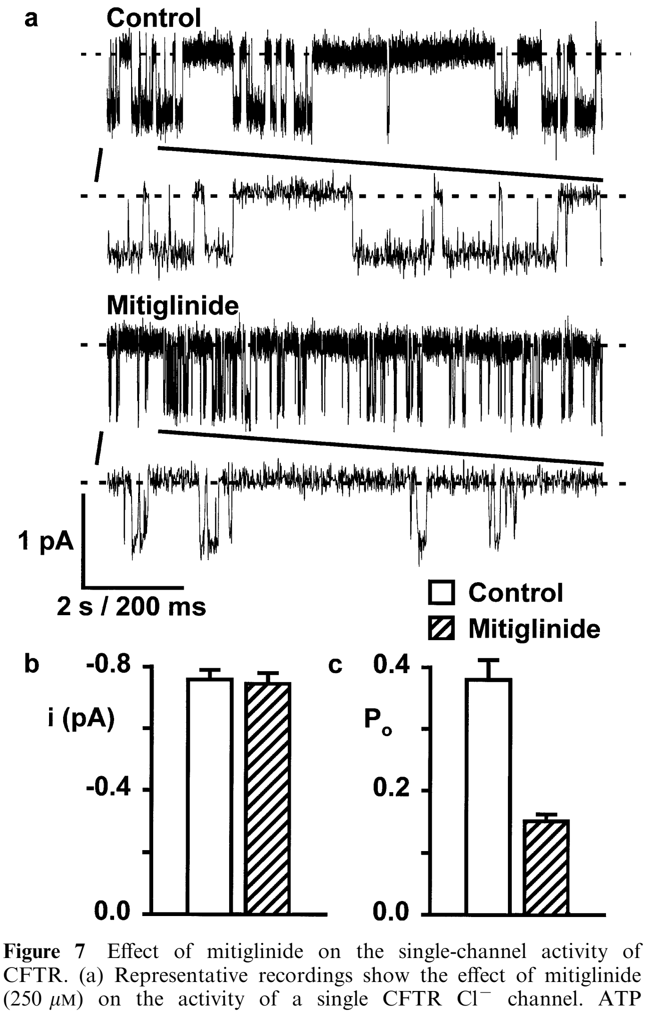

Figure 7 Effect of mitiglinide on the single-channel activity of CFTR. (a) Representative recordings show the effect of mitiglinide (250 μM) on the activity of a single CFTR Cl^- channel. ATP (0.3 mM) and PKA (75 nM) were continuously present in the intracellular solution; voltage was -50 mV. Scale bar, 2 s and 200 ms for prolonged and expanded recordings, respectively. (b) and (c) Effect of mitiglinide (250 μM) on i and P_o, respectively. Columns and error bars indicate means±s.e.mean ($n=6$) for each concentration. Mitiglinide (250 μM) was without effect on i ($n=6$; $P>0.05$), but decreased P_o to $41\pm5\%$ ($n=6$; $P<0.05$). Other details as in (a).

In the presence of meglitinide (250 μM), open and closed time histograms were still best fitted with one- and two-component functions, respectively (Figure 8a and Table 1). However, meglitinide altered the distribution of open and closed times in several ways. First, meglitinide decreased the open time constant (τ_{O1}) by 15-fold (Figure 8a and Table 1). Second, meglitinide increased the fast closed time constant (τ_{C1}) by 2-fold, while its share of the closed time distribution expanded from 67% to 92% (Figure 8a and Table 1). Third, meglitinide was without effect on the slow closed time constant (τ_{C3}), although its share of the closed time distribution decreased from 33% to 8% (Figure 8a and Table 1).

In the presence of mitiglinide (250 μM), open and closed time histograms were best fitted with two- and three-component functions, respectively (Figure 8b and Table 1). The new population of open times was described by an intermediate open time constant (τ_{O2}) and the new population of closed times was described by an intermediate closed time constant (τ_{C2}; Figure 8b and Table 1). In addition, mitiglinide decreased the open time constant (τ_{O1}) by 16-fold, but was without effect on the fast closed (τ_{C1}) and slow closed (τ_{C3}) time constants of CFTR. In the presence of mitiglinide, the closed time distribution was equally divided between τ_{C1}, τ_{C2}, and τ_{C3} (Figure 8b and Table 1). These data show that two different non-sulphonylurea hypoglycaemic agents, both with similar effects on CFTR Cl^- currents, have distinct effects on the gating kinetics of phosphorylated CFTR Cl^- channels.

The data presented in Figure 8 and Table 1 indicate that meglitinide and mitiglinide decrease the open time of CFTR Cl^- channels, suggesting that these drugs may be open-channel blockers of CFTR. Open-channel block of CFTR may be described by the simple kinetic model:

$$\mathrm{C} \underset{\alpha}{\overset{\beta}{\longleftrightarrow}} \mathrm{O} \underset{\mathrm{k_{off}}}{\overset{\mathrm{k_{on}[drug]}}{\longleftrightarrow}} \mathrm{B} \qquad (3)$$

where C, O, and B represent the closed, open, and blocked states of the channel, respectively; β and α are the transition rates for channel opening and closing; k_{on} is the second order binding constant for drug binding to CFTR; and k_{off} is the first-order rate constant for dissociation of drug from CFTR. The equilibrium dissociation constant for drug binding to CFTR, $K_D=k_{off}/k_{on}$; $k_{off}=1/\tau_C$; and $k_{on}=(1/\tau_O)\times[\mathrm{drug}]^{-1}$. For meglitinide, using values of τ_O and τ_{C1} from Table 1, we calculate values of $k_{on}=1.0\times10^6\ M^{-1}\ s^{-1}$, $k_{off}=152\ s^{-1}$, and $K_D=148\ \mu$M, at -50 mV. For mitiglinide, using values of τ_{O1} and τ_{C2} from Table 1, we calculate values of $k_{on}=1.0\times10^6\ M^{-1}\ s^{-1}$, $k_{off}=49\ s^{-1}$, and $K_D=47\ \mu$M, at -50 mV. These values suggest that both drugs bind rapidly to CFTR, but that mitiglinide remains bound to CFTR 3-fold longer than meglitinide. The data also suggest that meglitinide and mitiglinide are less potent inhibitors of CFTR than glibenclamide ($K_D=14-26\ \mu$M; Schultz *et al.*, 1996; Sheppard & Robinson, 1997), but more potent than tolbutamide ($K_D=430\ \mu$M; Venglarik *et al.*, 1996).

Voltage-dependence of meglitinide and mitiglinide inhibition of CFTR

Previous work has shown that the inhibition of CFTR by a number of agents, including arylaminobenzoates, disulphonic stilbenes, and sulphonylureas, is voltage-dependent (McCarty *et al*., 1993; Linsdell & Hanrahan, 1996a; Sheppard & Robinson, 1997; Walsh *et al*., 1999). Therefore, we speculated that meglitinide and mitiglinide may cause a voltage-dependent block of CFTR Cl^- channels. To test this possibility, we bathed membrane patches in symmetrical 147 mM Cl^- solutions and measured P_o in the absence and presence of either meglitinide or mitiglinide over the voltage range −80 mV to +60 mV. As previously observed (Shep-

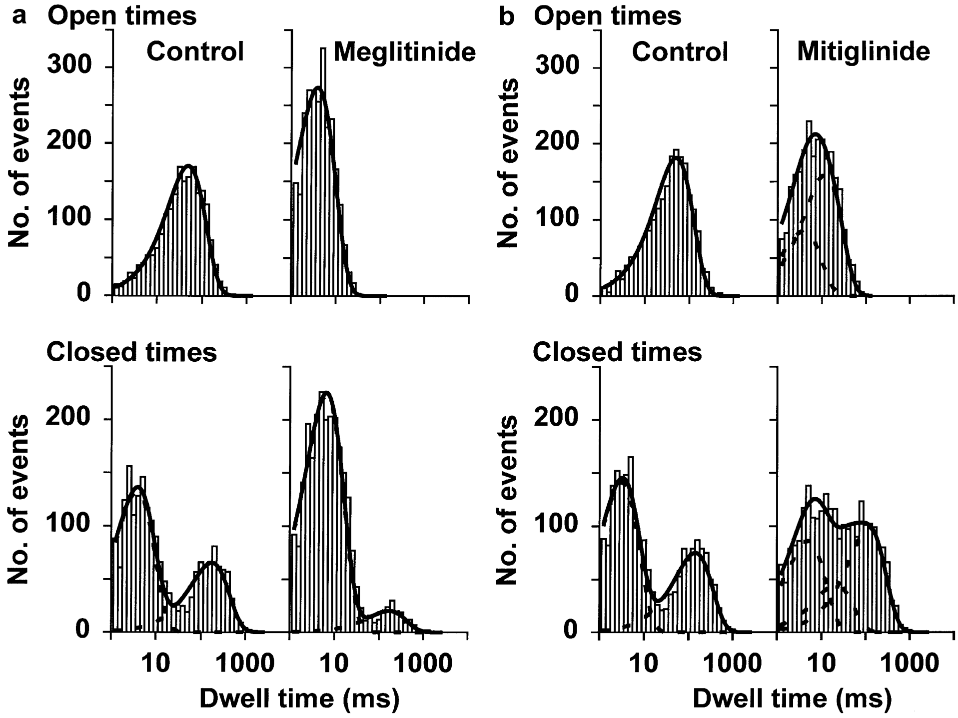

Figure 8 Open and closed time histograms of single CFTR Cl^- channels inhibited by meglitinide and mitiglinide. (a) and (b) Effect of meglitinide and mitiglinide on the open (top) and closed (bottom) time histograms of CFTR, respectively. Data are from experiments in which the membrane patch contained only one active channel, studied in the absence and presence of either meglitinide (250 μM) or mitiglinide (250 μM); ATP (0.3 mM) and PKA (75 nM) were present throughout and voltage was −50 mV. For open time histograms, the continuous line is the fit of either a one- (control, meglitinide) or a two- (mitiglinide) component exponential function. For closed time histograms, the continuous line is the fit of either a two- (control, meglitinide) or a three- (mitiglinide) component exponential function. Dashed lines show the individual components of the exponential functions. Logarithmic x-axes with 10 bins $decade^{-1}$ were used for both open and closed time histograms. Other details as in Table 1.

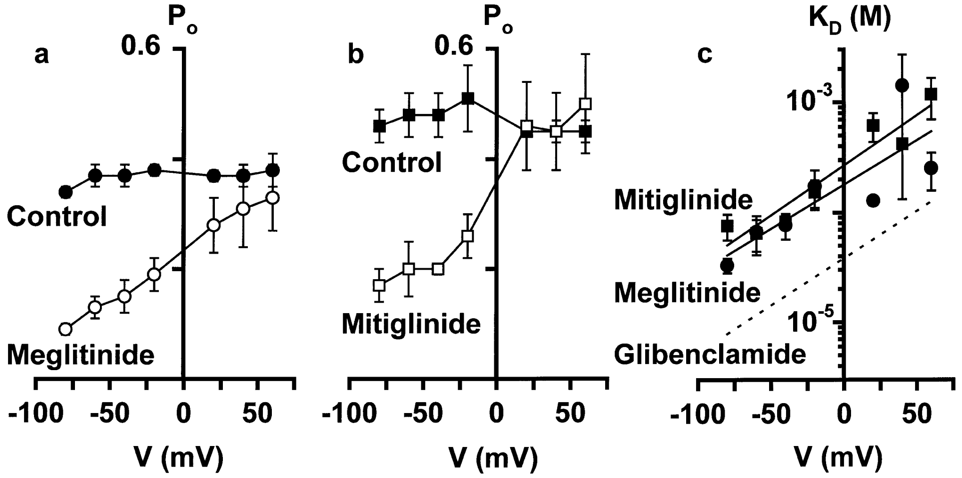

Figure 9 Voltage-dependent inhibition of CFTR Cl^- channels by meglitinide and mitiglinide. (a) and (b) Relationships between P_o and voltage in the absence (filled symbols) and presence (open symbols) of meglitinide (250 μM) and mitiglinide (250 μM) when the membrane patch was bathed in symmetrical 147 mM Cl^- solutions. Data points are means±s.e.mean (meglitinide: $n=3-5$; mitiglinide: $n=3-4$) at each voltage. (c) Relationship between K_D and voltage for the data shown in (a) and (b). K_D values were calculated as described in Results and the continuous lines are the fit of first-order regressions to the data. For comparison, the dotted line shows the relationship between K_D and voltage for glibenclamide under similar experimental conditions. Data for glibenclamide are from Sheppard & Robinson (1997).

pard & Robinson, 1997), the P_o of CFTR was voltage-independent (Figure 9a,b). In the presence of both meglitinide and mitiglinide, P_o decreased significantly at negative voltages, but at positive voltages inhibition was relieved (Figure 9a,b).

The voltage-dependent dissociation constant (K_D) for meglitinide and mitiglinide inhibition of CFTR can be calculated with data from multichannel patches using the relationship (Benham *et al*., 1985):

$$K_D(V) = ([\text{drug}] \bullet P_{\text{drug}} \bullet P)/(P - P_{\text{drug}}) \tag{4}$$

where K_D is the voltage-dependent dissociation constant at voltage V, and P_{drug} and P are the open probabilities in the presence and absence of drug, respectively. Figure 9c shows that K_D was strongly voltage-dependent for both meglitinide and mitiglinide. However, both drugs were less potent inhibitors of CFTR than glibenclamide (Figure 9c). At −50 mV, the K_D value for meglitinide was 68 ± 17 μM ($n=5$) and that of mitiglinide was 96 ± 12 μM ($n=4$), in reasonable agreement with the K_D values calculated using single-channel data.

The voltage-dependence of meglitinide and mitiglinide inhibition of CFTR suggests that these drugs bind within the electric field of the membrane. The electrical distance sensed by blocking ions (δ) can be calculated using the relationship (Woodhull, 1973):

$$K_D(V) = K_D(0\ \text{mV})\exp[(-z\delta FV)/(RT)] \tag{5}$$

where K_D (0 mV) is the voltage-dependent dissociation constant at 0 mV, z is the valency of the blocking ion (−1 for both meglitinide and mitiglinide), and F, R, and T are the Faraday constant, gas constant, and absolute temperature, respectively. Using the data in Figure 9c, for meglitinide, $\delta = 0.49 \pm 0.09$ ($n=5$) and for mitiglinide, $\delta = 0.61 \pm 0.07$ ($n=4$) measured from the inside of the membrane over the voltage range −80 to −40 mV.

Discussion

The goal of this study was to identify new modulators of CFTR. The data demonstrate that non-sulphonylurea hypoglycaemic agents inhibit CFTR Cl^- channels with a rank order of potency of mitiglinide ⩾ meglitinide > > repaglinide. Thus, the data indicate that these agents have a wider specificity of action than previously recognized.

Our study of the effects of non-sulphonylurea hypoglycaemic agents on CFTR was prompted by the finding that modulators of K_{ATP} channels inhibit CFTR Cl^- channels (Sheppard & Welsh, 1992; Schultz *et al*., 1996; Venglarik *et al*., 1996; Sheppard & Robinson, 1997). K_{ATP} channels are composed of at least two subunits: a pore-forming subunit, the inwardly rectifying K^+ channel (Kir6.2; Inagaki *et al*., 1995) and a regulatory subunit, the SUR that binds sulphonylurea drugs (Aguilar-Bryan *et al*., 1995). Like CFTR, SUR is a member of the ATP-binding cassette transporter family (Hyde *et al*., 1990). This suggests that CFTR and SUR likely share some features in common in their drug binding sites. Consistent with this idea, with the exception of repaglinide, the rank order of potency of inhibition of CFTR and K_{ATP} channels by hypoglycaemic drugs is similar. However, the affinity of hypoglycaemic drugs for K_{ATP} channels in pancreatic β-cells far exceeds their affinity for CFTR Cl^- channels. For example, mitiglinide (KAD-1229) inhibited K_{ATP} and CFTR channels with K_i of ~20 nM and 150 μM, respectively (Mogami *et al*., 1994; present study). These data suggest that some feature(s) of the binding site for hypoglycaemic drugs in SUR1, not shared with that of CFTR, strongly stabilise the interaction of hypoglycaemic drugs with K_{ATP} channels in pancreatic β-cells.

Repaglinide inhibits K_{ATP} channels in pancreatic β-cells with a potency similar to that of glibenclamide (Gromada *et al*., 1995). However, the present results demonstrate that repaglinide only weakly inhibits CFTR. Because K_{ATP} channels are built from pore-forming and regulatory subunits, one possible explanation for these differences between K_{ATP} and CFTR channels is that the high-affinity repaglinide binding site may be located on the pore-forming subunit, Kir6.2. Several lines of evidence support this idea. First, Fuhlendorff *et al*. (1998) identified two binding sites on βTC3 cells: a high-affinity repaglinide site ($K_D = 4$ nM) that has a lower affinity for glibenclamide (14 nM) and a high-affinity glibenclamide site ($K_D = 25$ nM) that has a lower affinity for repaglinide (550 nM). Second, repaglinide specifically binds to a 36 kDa protein in βTC3 cells consistent with the predicted molecular weight of Kir6.2 (42 kDa; Fuhlendorff, 1998). Third, in the absence of nucleotides, tolbutamide inhibits K_{ATP} channels expressed in *Xenopus* oocytes by binding to both high- and low-affinity sites located on SUR1 and Kir6.2, respectively (Gribble *et al*., 1997). These data indicate that hypoglycaemic agents inhibit K_{ATP} channels by binding to sites

Table 1 Effect of meglitinide and mitiglinide on open and closed time constants

	[Meglitinide] (μM)		*[Mitiglinide]* (μM)	
	0	250	0	250
n	5	5	3	3
τ_{O1} (ms)	58.29 ± 7.98	3.89 ± 0.18	62.62 ± 12.78	3.85 ± 0.48
τ_{O2} (ms)				12.31 ± 1.03
τ_{C1} (ms)	3.09 ± 0.23	6.59 ± 0.10	2.76 ± 0.23	4.07 ± 0.47
τ_{C2} (ms)				20.33 ± 0.31
τ_{C3} (ms)	228.4 ± 18.2	215.1 ± 33.7	162.6 ± 10.6	170.6 ± 44.8
Area under curve τ_{C1}	0.67 ± 0.01	0.92 ± 0.01	0.59 ± 0.06	0.35 ± 0.03
Area under curve τ_{C2}				0.32 ± 0.06
Area under curve τ_{C3}	0.33 ± 0.01	0.08 ± 0.01	0.41 ± 0.06	0.33 ± 0.06
Total time	1224	433	595	402

Open and closed time constants were measured at the indicated concentrations of meglitinide and mitiglinide by the fitting of one-, two, or three-component exponential functions to open and closed time histograms as described in Methods. Area under curve indicates the proportion of the total closed time distribution that corresponds to the different closed time constants. The total time analysed for each concentration of meglitinide and mitiglinide is shown, and in each patch, approximately 5000 events were analysed per intervention. Values are means ± s.e.mean of *n* observations. Measurements were made in the presence of PKA (75 nM) and ATP (0.3 mM) in the intracellular solution; voltage was −50 mV.

located on both the regulatory and pore-forming subunits. They also suggest that for sulphonylureas the high-affinity binding site is located on SUR, but for repaglinide it may be located on Kir6.2. This would explain the failure of repaglinide to potently inhibit CFTR.

In addition to affinity of block, the characteristics of CFTR inhibition by hypoglycaemic agents differ from their effects on K_{ATP} channels in pancreatic β-cells in one other important way: voltage-dependence of channel blockade. Inhibition of CFTR Cl^- channels by hypoglycaemic agents was strongly voltage-dependent (Sheppard & Robinson, 1997; present study). In contrast, the blockade of K_{ATP} channels by hypoglycaemic agents was voltage-independent (Gillis *et al*., 1989; Akiyoshi *et al*., 1995), suggesting that the binding sites for hypoglycaemic agents may be located outside the K_{ATP} channel pore. Consistent with this idea, sulphonylureas inhibit K_{ATP} channels by modulating the interaction of nucleotides with the nucleotide-binding domains (NBDs) of SUR1 (Gribble *et al*., 1997). However, site-directed mutations in the NBDs did not alter the effect of glibenclamide on CFTR (Sheppard & Welsh, 1992). Instead, the data suggest that glibenclamide binds within the CFTR pore (Sheppard & Robinson, 1997).

Previous work has shown that a number of agents inhibit CFTR by a common mechanism. The arylaminobenzoates, diphenylamine-2-carboxylate (DPC), flufenamic acid, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), the disulphonic stilbenes 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), and 4,4′-dinitrostilbene-2,2′-disulphonic acid (DNDS), glibenclamide, gluconate, and glutamate caused a voltage-dependent block of CFTR, and their binding sites were located 15–50% of the way through the transmembrane electric field from the intracellular side (McCarty *et al*., 1993; Linsdell & Hanrahan, 1996a, 1996b; Sheppard & Robinson, 1997; Walsh *et al*., 1999). All these agents are anions, and with the exception of DPC and flufenamic acid they are too large to pass through the CFTR pore. Moreover, the effects of intracellular DPC, glibenclamide, gluconate, and glutamate were enhanced when the external Cl^- concentration was decreased (McDonough *et al*., 1994; Linsdell & Hanrahan, 1996b; Sheppard & Robinson, 1997), and DPC, DIDS, DNDS, and NPPB interacted with residues that contribute to the formation of the CFTR pore (McDonough *et al*., 1994; Linsdell & Hanrahan, 1996a; Walsh *et al*., 1999). Thus, the data suggest that the CFTR pore contains a wide intracellular vestibule where large anions bind and prevent Cl^- permeation (Linsdell & Hanrahan, 1996a; Sheppard & Robinson, 1997).

Our observation that the anionic form of glibenclamide inhibits CFTR (Sheppard & Robinson, 1997) suggests that the anionic forms of non-sulphonylurea hypoglycaemic agents may inhibit CFTR Cl^- channels. Consistent with this idea, block of CFTR by meglitinide and mitiglinide was only observed at negative voltages which would propel anions from the intracellular solution into the CFTR pore. The voltage-dependence of meglinitide and mitiglinide inhibition of CFTR suggests that the binding sites for these agents are located about 50–60% of the electrical distance across the membrane from the intracellular side, in good agreement with the location of the glibenclamide binding site (Sheppard & Robinson, 1997; present study). Single-channel analysis of channel blockade indicated that both meglitinide and mitiglinide decreased open time and dramatically increased both the frequency and duration of flickery closures, suggesting that these agents are open-channel blockers of CFTR. In contrast, block of CFTR by repaglinide was not resolved as discrete closures. Instead, repaglinide decreased current amplitude, indicating that the kinetics of block were too fast to be resolved with our recording conditions. Other inhibitors of CFTR that decrease current amplitude include gluconate and glutamate, two agents that occlude the CFTR pore (Linsdell & Hanrahan, 1996b).

A defining characteristic of open-channel block is that in the presence of the blocker the mean open time per burst is unaltered; the burst is merely prolonged by numerous intervening visits to the blocked state (Neher & Steinbach, 1978). Our previous data suggested that glibenclamide increases the mean burst length of CFTR (Sheppard & Robinson, 1997). In the present study, we have not determined the effect of meglitinide and mitiglinide on mean burst length because of the difficulty of distinguishing closed and blocked times. Nevertheless, the most likely explanation of our data is that meglitinide and mitiglinide are open-channel blockers of CFTR. Other mechanisms of CFTR inhibition, such as changes in the rates of channel opening and closing, are not consistent with our data. Thus, the data suggest that meglitinide and mitiglinide may enter the CFTR pore from the intracellular side of the membrane and bind to a site within the pore. While they are bound, meglitinide and mitiglinide occlude the CFTR pore, preventing Cl^- permeation.

The present results have implications for the relationship between the chemical structure of hypoglycaemic agents and CFTR Cl^- channel inhibition. Structure-activity studies of hypoglycaemic sulphonylureas indicate that the potency of second-generation sulphonylurea drugs, such as glibenclamide, is conferred by binding at two distinct, but related sites on the SUR: one site interacts with the sulphonylurea moiety ($SO_2NHCONH$) and the second site interacts with the benzamide group (CONH) (Brown & Foubister, 1984). In contrast, first generation sulphonylurea drugs, such as tolbutamide, bind the SUR with reduced affinity because they lack the benzamide group (Brown & Foubister, 1984). Meglitinide, a benzoic acid derivative of the non-sulphonylurea moiety of glibenclamide has intermediate potency compared with glibenclamide and tolbutamide. It interacts with both binding sites on SUR, because the COOH group of meglitinide is able to bind at the same site as the sulphonylurea moiety (Brown & Foubister, 1984; see Figure 1). Other studies using a benzenesulphonic acid derivative of glibenclamide that is completely dissociated at pH 7.40 indicate that the anionic form of hypoglycaemic sulphonylureas interacts with SUR (Schwanstecher *et al*., 1994). Because, glibenclamide, tolbutamide, and meglitinide inhibit K_{ATP} and CFTR channels with a similar rank order of potency (Sturgess *et al*., 1988; Schultz *et al*., 1996; Venglarik *et al*., 1996; Sheppard & Robinson, 1997; present study), and because the anionic form of glibenclamide inhibits CFTR (Sheppard & Robinson, 1997), we speculate that glibenclamide interacts with CFTR at two sites. One site interacts with the acidic sulphonylurea moiety and a second site interacts with the benzamide group to stabilise the interaction of glibenclamide with CFTR (Figure 1).

In conclusion, our data demonstrate that non-sulphonylurea hypoglycaemic agents inhibit CFTR Cl^- channels. This suggests that these or related agents may be of value in the development of therapeutically active blockers of CFTR. Such drugs may be of value in the treatment of diseases that may involve increased activity of the CFTR Cl^- channel such as secretory diarrhoea and polycystic kidney disease (Gabriel *et al*., 1994; Sullivan *et al*., 1998).

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