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Pharmacological evaluation of the role of cytochrome P450 in intracellular calcium signalling in rat pancreatic acinar cells

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1 We have investigated whether the cytochrome P450 system is involved in Ca^{2+} signalling in rat pancreatic acinar cells. Intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured in collagenase-isolated cells using fura-2 microspectrofluorimetry and imaging.

2 The imidazole P450 inhibitor ketoconazole (5–50 μM) inhibited $[Ca^{2+}]_i$ oscillations induced by cholecystokinin octapeptide (CCK). However, ketoconazole also raised baseline $[Ca^{2+}]_i$ when applied in the absence of CCK. These effects were mimicked by 5–50 μM SKF96365, an imidazole widely used as an inhibitor of Ca^{2+} entry.

3 The non-imidazole P450 inhibitor proadifen (SKF525A) inhibited CCK-induced $[Ca^{2+}]_i$ oscillations at a concentration of 10–50 μM. Proadifen alone caused intracellular Ca^{2+} release at 25 or 50 μM, but not at 10 μM.

4 Octadecynoic acid and 1-aminobenzotriazole, structurally-unrelated non-imidazole P450 inhibitors, did not alter baseline $[Ca^{2+}]_i$ or CCK-evoked oscillations.

5 We compared cumulative CCK dose-response relationship in control cells and in cells where P450 had been induced by prior injection of animals with β-naphthoflavone. Only minor differences were apparent, with induced cells showing some decrease in responsiveness at moderate and higher concentration of CCK (30 pM–3 nM).

6 Direct assessment of depletion-activated Ca^{2+} entry showed no clear differences between control and induced cells.

7 In conclusion, we could find no compelling evidence for a role of P450 in controlling Ca^{2+} signalling generally, or Ca^{2+} entry in particular, in pancreatic acinar cells. Induction of P450 is therefore probably toxic to acinar cells *via* a Ca^{2+}-independent mechanism.
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Abbreviations: 1-ABT, 1-Aminobenzotriazole; ACh, Acetylcholine; cyclic AMP, cyclic adenosine monophosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; CCK, cholecystokinin octapeptide; DMSO, dimethylsulphoxide; EDTA, ethylenediamine-N,N,N′N′-tetraacetic acid; EET, epoxyeicosatrieneoic acid; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid; β-NF, β-naphthoflavone; 17-ODYA, 17-octadecynoic acid; P450, cytochrome P450; SERCA Ca^{2+}-ATPase, sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase; SKF96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1-*H*-imidazole hydrochloride

Introduction

Studies in several laboratories have suggested a role for P450 mono-oxygenases in intracellular Ca^{2+} signalling. In particular, the studies of Garcia-Sancho and co-workers have implicated P450 in linking Ca^{2+} release from intracellular stores to store-operated or capacitative Ca^{2+} entry in a variety of cell types (Alonso *et al*., 1991; Alvarez *et al*., 1991; 1992a; Montero *et al*., 1992; 1993a; Alonso-Torre *et al*., 1993). However, the evidence supporting this hypothesis relies heavily on the ability of imidazole antimycotics to inhibit store-operated Ca^{2+} entry. Econazole, which has been reported to be the most potent imidazole P450 inhibitor in most studies on Ca^{2+} signalling, shows structural homology to the non-selective Ca^{2+} channel blocker SKF96365, which has also been shown to inhibit store operated Ca^{2+} entry in several systems (Merritt *et al*., 1990). The question then arises whether imidazole antimycotics act *via* inhibition of P450 or by directly blocking the Ca^{2+} influx channel. There is also much convincing evidence that imidazoles have many other non-specific effects, such as inhibition of agonist-induced protein tyrosine phosphorylation, of inducible nitric oxide synthase, cyclo-oxygenase and lipoxygenase, of microsomal Ca^{2+}-ATPase, and of voltage-gated Ca channels, K^+ channels, and cyclic AMP regulated Cl^- channels (for references see Clementi & Meldolesi, 1996). High concentrations of SKF96365 have also been shown to stimulate intracellular Ca^{2+} release (Merritt *et al*., 1990; Mason *et al*., 1993).

Despite all the evidence that disputes a role for P450 in store operated Ca^{2+} entry, data supporting the 'P450 hypothesis' also continues to accumulate. Most of this work centres on the role of P450 mono-oxygenases in converting arachidonic acid to epoxyeicosatrieneoic acids (EETs). In particular, two recent papers from the same laboratory tested the hypothesis that agonist-stimulated Ca^{2+} entry in vascular endothelial cells is under the control of cytochrome P450 and the conversion of arachidonic acid to 5,6-epoxyeicosatrienoic acid (5,6-EET). Thus store depletion (using agonists, inositol-1,4,5-trisphosphate, thapsigargin or oxalate) evoked Ca^{2+} entry and activated P450, both of which responses were inhibited by a variety of structurally and mechanistically unrelated P450

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inhibitors (Graier *et al*., 1995; Hoebel *et al*., 1997). In addition, the P450 inducers β-naphthoflavone, dexamethasone and clofibrate all potentiated both agonist- and thapsigargin-induced Ca^{2+} entry (Graier *et al*., 1995; Hoebel *et al*., 1997). Finally, 5,6,-EET can activate Ca^{2+} entry in both endothelial and glial cells without depletion of intracellular stores or agonist stimulation (Graier *et al*., 1995; Rzigalinski *et al*., 1999).

We have been prompted to re-examine this issue in the pancreatic acinar cell by the possible association between P450 induction and pancreatic disease. Pancreatitis is a serious and sometimes fatal inflammatory disease of pancreatic acinar cells. Although the underlying pathology of pancreatitis remains poorly understood, one hypothesis centres around cytochrome P450 activity (Braganza, 1983). Epidemiological evidence shows a good correlation between the recurring or chronic form of pancreatitis and increased P450 activity resulting from occupational exposure to inducing agents (McNamee *et al*., 1994).

In the present study we used a pharmacological approach to examine critically whether P450 mono-oxygenases play any role in regulating $[Ca^{2+}]_i$ in pancreatic acinar cells.

A preliminary account of part of these results has been reported to the Physiological Society (Bruce & Elliott, 1997).

Methods

Cell isolation

Male Sprague-Dawley rats weighing between 100 and 300 g were killed by cervical dislocation and the pancreas removed. Small clusters of rat pancreatic acinar cells were isolated from the pancreas by collagenase digestion as previously described (Speake & Elliott, 1998). Following isolation the cells were resuspended in a HEPES- (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffered physiological saline (pH 7.4) containing (mM): NaCl 104, KCl 5, $MgSO_4$ 1.2, $KHPO_4$ 1.2, $CaCl_2$ 2, HEPES 25, glucose 15, L-glutamine 2. This solution was supplemented with 2% (v v^{-1}) Eagle's minimal essential medium amino acids, 0.12 mg ml^{-1} trypsin inhibitor (type II-S, Sigma) and 1% (w v^{-1}) bovine serum albumin (Fraction V, Sigma). Cells were then maintained on ice until use. All experimental solutions were made fresh each day.

Fura-2 microspectrofluorimetry and imaging

Cells were loaded with 4 μM fura-2 acetoxymethyl ester for 40 min at room temperature, transferred to a small superfusion chamber (volume 150 μl; Warner Instruments, Hamden, CT, U.S.A.) on the stage of a Nikon Diaphot inverted microscope, and allowed to adhere to the glass coverslip forming the base of the chamber. The cells were then continuously superfused with HEPES-buffered, 100% O_2-bubbled physiological saline (composition as above but omitting amino acids, trypsin inhibitor, bovine serum albumin and glutamine) from a gravity-fed perfusion system at a rate of 3 ml min^{-1}. Fluorescence was excited and observed through a 40$\times$ oil immersion objective (numerical aperture 1.3; Nikon).

Two different microscope-based systems were used to measure fura-2 fluorescence in this study. The first was a microspectrofluorimetric system in which excitation light at 350 and 380 nm was supplied *via* a filter wheel spinning at 40 Hz (Cairn Research Ltd). A diaphragm in the emitted light path was used to limit light collection to a single cell within a cell cluster and the emitted fluorescence from the cell was measured by a photomultiplier tube attached to a Cairn Research spectrophotometer (for details see Berrie & Elliott, 1994; Speake & Elliott, 1998). Experiments in which responses from large numbers of cells were analysed simultaneously employed an imaging system in which emitted fluorescence was captured and digitised at 12-bit resolution by a slow scan CCD camera (Digital Pixel Ltd, Brighton) under the control of the Kalcium-PC computer software package (Kinetic Imaging Ltd., Liverpool). The imaging system is described in detail in van de Put & Elliott (1996). Background fluorescence was determined from a region of the coverslip without cells and subtracted from the 340 and 380 nm images off-line before calculation of the 340 : 380 nm ratio image. During P450 inhibition experiments, a 2.5$\times$ relay lens was placed in the emission light-path, giving an image field typically containing 20 – 30 cells. The 340 : 380 ratio could then be plotted for each individual cell within this field. For P450 induction experiments, where particularly large numbers of cells were required for viable statistical analysis, a 1.0$\times$ relay lens was substituted, which gave a field containing 30 – 100 cells. All experiments were carried out at room temperature.

We did not routinely calibrate fura-2 signals in terms of absolute values of $[Ca^{2+}]_i$, since the accuracy of such estimates is debatable (Williams & Fay, 1990) and since it was not necessary for the analysis of the data. However, calibration was carried out on a limited number of cells chosen at random throughout the study. The calibration method was as previously described (Speake & Elliott, 1998), based on the original formulation of Grynkiewicz *et al*. (1985). The precise calibration values obtained depended on the optical components in the system. When the 1.0$\times$ relay lens was used calibration parameters were: R_{min}, 0.48 ± 0.02; R_{max}, 1.58 ± 0.03; F_{380f}/F_{380b} 1.40 ± 0.03; $n = 164$ cells (five individual experiments) from three rats. The mean resting ratio obtained was 0.75 ± 0.02 which corresponds to an $[Ca^{2+}]_i$ of 67 ± 8 nM. Typical values of the ratio at the peak of a CCK-induced $[Ca^{2+}]_i$ oscillation were between 1.2 and 1.4, corresponding to $[Ca^{2+}]_i$ between approximately 400 nM and 1 μM in different cells.

Experiments with P450 inhibitors

A number of structurally- and mechanistically-unrelated cytochrome P450 inhibitors were employed in this study. These were the imidazole antimycotic ketoconazole, proadifen (previously known as SKF525A), the older inhibitor 1-aminobenzotriazole (1-ABT), and 17-octadecynoic acid (17-ODYA), a suicide inhibitor of P450 subtypes which convert arachidonic acid to EETs. The imidazole non-selective Ca^{2+} channel blocker SKF96365 (1-{β-[3-(4-methoxyphenyl)propoxy]4-methoxyphenethyl}-1-H-imidazole hydrochloride) was also tested for comparison, since it has been widely used to inhibit Ca^{2+} entry in non-excitable cells and, like ketoconazole, contains an imidazole group. The effects of the inhibitors on CCK-evoked $[Ca^{2+}]_i$ oscillations were examined using a paired experimental design, whereby the effect of the inhibitor on CCK-evoked $[Ca^{2+}]_i$ oscillations was compared to a control period (CCK alone) in the same cell. All these experiments were carried out at a CCK concentration of 50 pM, since this concentration reliably produced $[Ca^{2+}]_i$ oscillations in 80 – 100% of the acinar cells. Normalization of the data was achieved by expressing each parameter (oscillation amplitude, oscillation frequency) as a percentage of that during the initial control period of CCK-evoked oscillations. Tests for differences in the presence of the inhibitor were made using a Wilcoxon test for paired samples. We have reported exclusively

effects on oscillation frequency in this study, since we have previously shown that this parameter remains constant for up to an hour during a CCK-evoked oscillation train, while oscillation amplitude declines with time (Elliott & Bruce, 1997).

In vivo *induction of cytochrome P450*

The effect of induction of cytochrome P450 on $[Ca^{2+}]_i$ signalling in rat pancreatic acinar cells was also investigated, by inducing P450 *in vivo* using β-naphthoflavone (β-NF). The major advantage of the induction protocol is that it removes the non-specific effects which are associated with P450 inhibitors.

Male Sprague Dawley rats (100–200 g) received daily intraperitoneal injections (2 ml kg^{-1}) of 100 mg kg^{-1} of β-NF (a known inducer of cytochrome oxidases, and widely used as an inducer of P4501A; Oakey, 1990) in corn oil on three consecutive days. Weight-matched control rats received injections of corn oil vehicle alone. Due to the low solubility of β-NF in corn oil the mixture was briefly probe sonicated to aid solubilization prior to injection. On the fourth day, both experimental and control animals were sacrificed and the liver and pancreas were rapidly removed. Small portions of liver and pancreas tissue were either prepared for Western analysis or snap frozen in liquid nitrogen and stored at $-80^{\circ}C$ until ready for use (see below). The remainder of the pancreas was digested using the standard collagenase digestion procedure described above to isolate acinar cells.

Experimental design: P450 induction

With the *in vivo* P450 induction protocol, Ca^{2+} signalling in cells from the test group of rats was compared to cells from a control group of rats. In this situation an unpaired experimental design must be applied. In order to maintain statistical viability large numbers of cells were simultaneously analysed (up to 100 cells) by use of the imaging system (see above). In addition, to account for possible variation due to cell isolation, each experiment was carried out on the same day as a parallel experiment on cells from a vehicle-injected rat, which therefore acted as a 'day-matched' control.

In one series of experiments, control or 'induced' acinar cells were treated sequentially with physiological saline containing increasing doses of CCK (3 pM, 30 pM, 300 pM and 3 nM). These data were then analysed by categorizing the $[Ca^{2+}]_i$ response in each cell in the microscope field into one of four typical $[Ca^{2+}]_i$ response types. These were: (i) no response; (ii) large $[Ca^{2+}]_i$ oscillations, with $[Ca^{2+}]_i$ returning to baseline between oscillations, often termed 'baseline spiking'; (iii) $[Ca^{2+}]_i$ oscillations over a raised baseline; and (iv) a sustained increase in $[Ca^{2+}]_i$ (no oscillations). An earlier example of a similar type of data analysis, although at only a single concentration of CCK, can be found in the work of Ward *et al.* (1996) on cells isolated from rats with experimental (hyperstimulation) pancreatitis. Cells which were completely unresponsive to CCK even at the highest CCK concentration of 3 nM were excluded from the analysis in the present study. For statistical analysis, the numbers of cells in each category from all the individual experiments on control or induced animals were summed to give a distribution of response types for the entire 'population' of analysed (control or induced) cells. The response distributions obtained in this way for induced and control cells were then compared to one another statistically by multiple χ^2 analysis.

We also computed the mean frequency of oscillations in each population at each CCK dose. In order to simplify the analysis, in these experiments the frequency of oscillations was calculated for each cell as the number of spikes during the 15 min of CCK treatment. Thus this includes cells showing only a single spike (e.g. at 3 pM CCK), as well as cells showing either baseline $[Ca^{2+}]_i$ spiking or $[Ca^{2+}]_i$ oscillations over a raised baseline. Cells which showed a transient increase followed by a lower sustained plateau (a typical response at high doses of CCK) were taken as showing a single spike. Comparisons of mean frequency between control and induced cells were made using an unpaired Mann-Whitney test.

In a separate set of experiments, control or 'induced' acinar cells were treated with a cocktail of the Ca^{2+}-ATPase inhibitors thapsigargin (1 μM) and cyclopiazonic acid (10 μM), to evoke depletion of intracellular stores and subsequent activation of capacitative Ca^{2+} entry (Thastrup *et al.*, 1990; Speake & Elliott, 1998). During the resulting plateau phase of high $[Ca^{2+}]_i$, cells were treated with the P450 inhibitor proadifen (50 μM). These experiments were analysed by comparing the mean response from all cells using an unpaired Student's *t*-test.

Western analysis of P450 protein

Expression of P4501A1 protein in liver and pancreas of β-NF-treated and control rats was detected by Western blotting. Briefly, a small segment of pancreas or liver (less than 0.5 cm^3) was homogenized in lysis buffer (Tris-HCl 50 mM, NaCl 250 mM, EDTA 5 mM, NaF 50 mM, 0.1% Triton X-100, 1 μM orthovanadate, 10 μg ml^{-1} phenylmethylsulphonylfluoride, 0.2 μg ml^{-1} leupeptin, 2 μg ml^{-1} trypsin inhibitor and 0.2 μg ml^{-1} aprotinin). These tissue homogenates were centrifuged at $2700 \times g$ for 10 min, and the supernatants then centrifuged at $18{,}800 \times g$ for 20 min. The supernatants from the latter centrifugation were further centrifuged at $43{,}500 \times g$ for 20 min to recover all membrane-bound protein as a pellet. This membrane pellet was then solubilised in fresh lysis buffer for determination of sample protein concentration using a protein assay kit (BioRad).

Levels of P4501A1 protein in the samples were determined with a commercially-available Western blotting kit (Amersham), which was used according to the maker's instructions. Five μg of molecular weight markers and of liver microsomes from β-NF treated rats (the positive control sample provided with the kit), and 20 μg per lane of tissue homogenate samples were separated on a sodium dodecyl sulphate-polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane (PVDF; BioRad). The membranes were incubated with a rabbit polyclonal anti-rat cytochrome P4501A1 primary antibody, followed by incubation with anti-rabbit Immunoglobulin biotinylated secondary antibody (both from Amersham). Finally the membranes were incubated with streptavidin-horseradish peroxidase conjugate and bands were detected using the Amersham enhanced chemiluminescence (ECL) system.

Chemicals and drugs

Fura2/AM was obtained from Molecular Probes and kept as a 2 mM stock solution in dry dimethylsulphoxide (DMSO). Ketoconazole and proadifen were from Research Biochemicals International; SKF96365 was from Calbiochem; 1-aminobenzotriazole from Aldrich; and 17-octadecynoic acid, β-naphthoflavone, thapsigargin and cyclopiazonic acid from Sigma. The P450 inhibitors were kept frozen as 50 mM stock solutions in

distilled water (SKF 96365 and proadifen), ethanol (ketoconazole) or DMSO (17-octadecynoic acid and 1-aminobenzotriazole). Thapsigargin and cyclopiazonic acid were kept frozen as 10 mM stock solutions in DMSO. The vehicle concentration added to experimental solutions never exceeded 0.1%. Cholecystokinin octapeptide (Bachem) was kept frozen in small aliquots as a 1 μM stock solution in distilled water. A single aliquot was defrosted for each day's experiments and then discarded.

Results

Effect of ketoconazole and SKF96365 on $[Ca^{2+}]_i$ signalling

Ketoconazole and the closely-related econazole are perhaps the most widely used P450 inhibitors in studies relating P450 to Ca^{2+} signalling. We tested the effects of ketoconazole on $[Ca^{2+}]_i$ signalling in acinar cells in parallel with the non-selective Ca^{2+} channel blocker, SKF96365. The two compounds had almost identical effects on both CCK-induced $[Ca^{2+}]_i$ oscillations and on baseline $[Ca^{2+}]_i$. At a concentration of 50 μM, ketoconazole caused a substantial decrease in oscillation frequency to $11 \pm 5\%$ of control ($n=4$). However, every experiment also showed a large increase in baseline $[Ca^{2+}]_i$. A lower dose, 25 μM ketoconazole (see e.g. Figure 1B) also significantly reduced frequency of oscillations (to $44 \pm 10\%$ of control, $n=6$; see Figure 1B), while 5 μM ketoconazole had no significant effect ($n=3$, data not shown, but see Figure 4). In experiments with 25 or 50 μM ketoconazole we never observed any recovery of oscillations after removal of the drug (see e.g. Figures 1B and 2). In addition, application of a maximal dose of ACh (10 μM) after removal of ketoconazole and CCK typically produced little or no Ca^{2+} release, indicating that the function of Ca^{2+} stores was severely impaired (not shown, but see Figures 1C and 2B).

Similarly to ketoconazole, 50 μM SKF96365 inhibited $[Ca^{2+}]_i$ oscillations (oscillation frequency decreased to $12 \pm 10\%$ of control, $n=4$) and caused an increase in baseline $[Ca^{2+}]_i$. (Figure 1C). Lower concentrations (10 and 5 μM) of SKF96365 also decreased oscillation frequency significantly, to 55 ± 12 and $71 \pm 6\%$ of control, respectively (both $n=6$; Figure 1A). However, in contrast to experiments with ketoconazole, there was almost complete recovery of $[Ca^{2+}]_i$ oscillations after removal of 5 or 10 μM SKF96365 (see Figure 1A). The effect of ketoconazole and SKF96365 on baseline $[Ca^{2+}]_i$ was also examined to determine whether either drug had any non-specific effects on $[Ca^{2+}]_i$ homeostasis (i.e. effects not attributable to blockade of Ca^{2+} entry). Figure 2A shows the effect of 50 μM ketoconazole on unstimulated cells. The cells were first stimulated with 50 pM CCK to test for cell viability/responsiveness. Both ketoconazole and SKF96365 produced a large and rapid rise in $[Ca^{2+}]_i$ when applied to cells at a concentration of 50 μM, in some cases with superimposed oscillations in $[Ca^{2+}]_i$, followed by a sustained and irreversible increase in $[Ca^{2+}]_i$. Subsequent application of CCK failed to produce any oscillations, and the $[Ca^{2+}]_i$ remained elevated long after removal of the drug (20 min), typically a good indication of irreversible cell damage. Lower doses (20–25 μM) of both drugs also produced a partial oscillatory change in $[Ca^{2+}]_i$ (see Figure 2B), suggesting that both compounds induced increases in $[Ca^{2+}]_i$ due, at least in part, to Ca^{2+} release from intracellular stores.

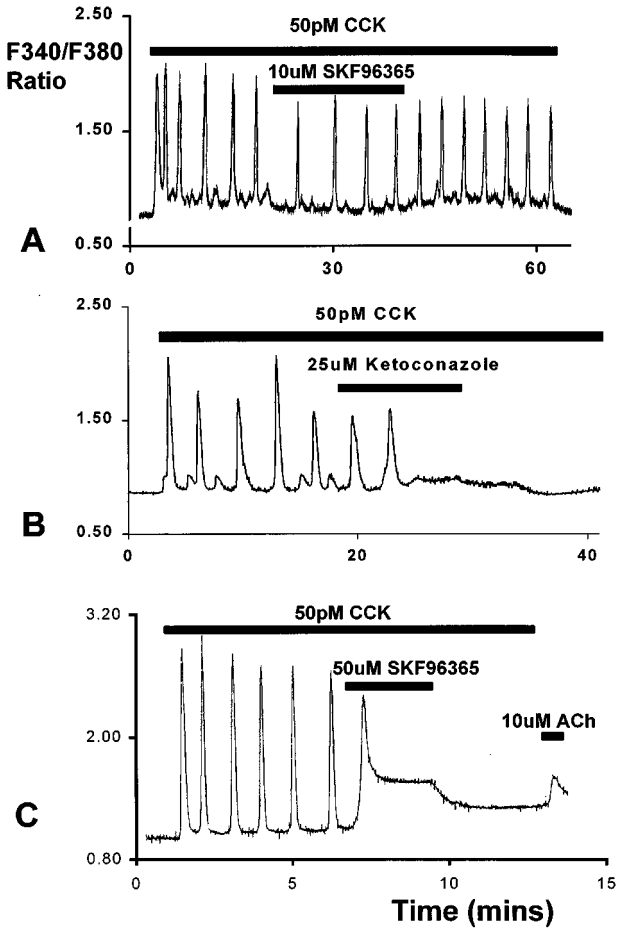

Figure 1 The effect of ketoconazole and SKF96365 on CCK-evoked oscillations of $[Ca^{2+}]_i$ in fura-2 loaded pancreatic acinar cells. (A–C) Traces of the fura-2 340:380 nm fluorescence ratio showing representative examples of the effects of the drugs applied at concentrations of 10, 25 and 50 μM respectively to cells which were oscillating in response to 50 pM CCK. The two drugs had essentially identical effects. Agents were applied for the times indicated by the bars. The traces are representative of six (A), six (B) and four (C) experiments respectively.

Effect of Proadifen (SKF525A) on $[Ca^{2+}]_i$ signalling

Proadifen (formerly known as SKF525A) is a P450 inhibitor which does not contain an imidazole group. Application of proadifen (10–50 μM) caused a broadly dose dependent inhibition of CCK-evoked $[Ca^{2+}]_i$ oscillations with only minor effects on baseline $[Ca^{2+}]_i$ (Figure 3). At 50 μM, proadifen decreased oscillation frequency to $38 \pm 9\%$ of control ($n=4$). A small increase in baseline $[Ca^{2+}]_i$ was observed on applying proadifen in three out of four experiments. However, the increase in baseline was much less marked than with ketoconazole or SKF96365. At 20 μM, proadifen decreased oscillation frequency to $77 \pm 8\%$ of control ($n=5$) and caused a small gradual rise in baseline $[Ca^{2+}]_i$ in two out of five of the experiments. At the lowest concentration tested, 10 μM, proadifen also caused a significant decrease in oscillation frequency to $73 \pm 7\%$ of control ($n=6$) with little or no increase in baseline $[Ca^{2+}]_i$. There was no recovery of $[Ca^{2+}]_i$ oscillations after removal of 50 μM proadifen, and a diminished ACh response was also observed after drug treatment, suggesting impaired function of Ca^{2+} stores. However, at the two lower doses of proadifen, partial recovery of CCK-evoked oscillations was observed in over half of the experiments (three out of five cells treated with 20 μM

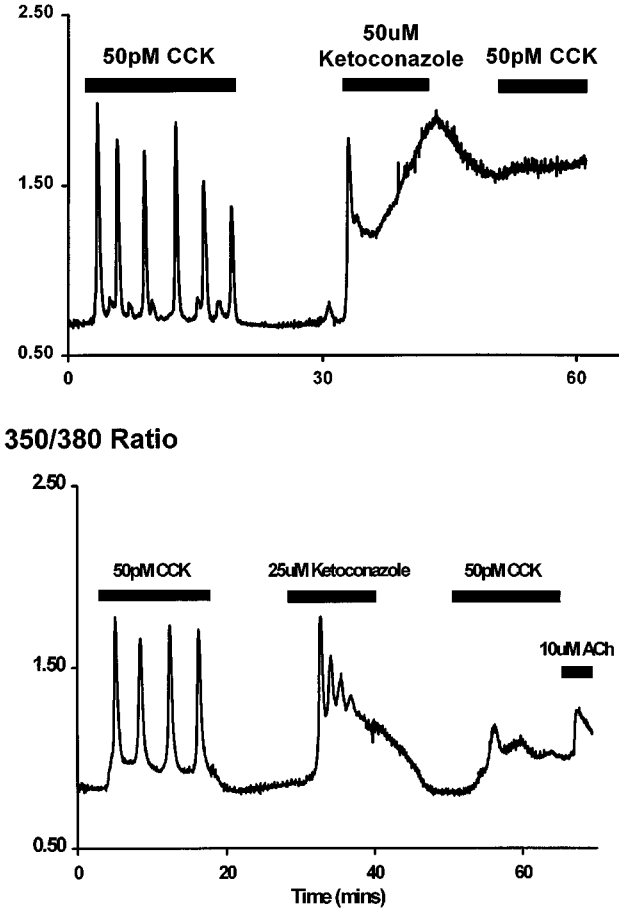

Figure 2 The effects of ketoconazole and SKF96365 on baseline$[Ca^{2+}]_i$ in fura-2 loaded pancreatic acinar cells. Upper and lower panels are traces of the fura-2 350 : 380 nm fluorescence ratio and show representative examples of the effects of the drugs applied at concentrations of 50 and 25 μM respectively to unstimulated cells. The two drugs had essentially identical effects. Agents were applied for the times indicated by the bars. The traces are representative of four and six experiments for ketoconazole at 25 and 50 μM respectively, and three and six for SKF96365.

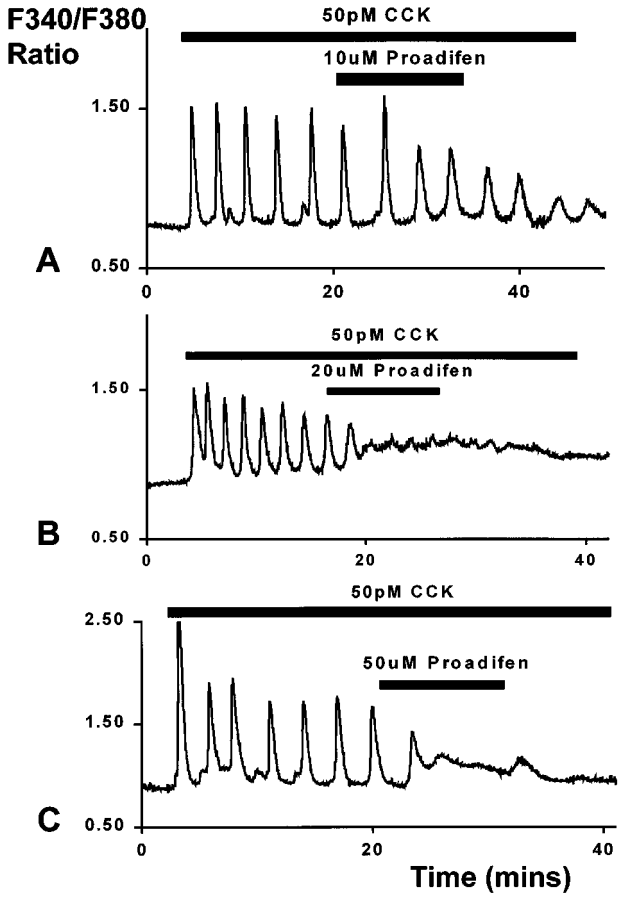

Figure 3 The effect of proadifen (SKF525A) on CCK-evoked oscillations of $[Ca^{2+}]_i$ in fura-2 loaded pancreatic acinar cells. Format smilar to Figure 1. (A–C) Traces of the fura-2 340 : 380 nm fluorescence ratio showing representative examples of the effects of proadifen applied at concentrations of 10, 20 and 50 μM respectively to cells which were oscillating in response to 50 pM CCK. The traces are representative of six, five, and four experiments for 10, 20, and 50 μM proadifen respectively.

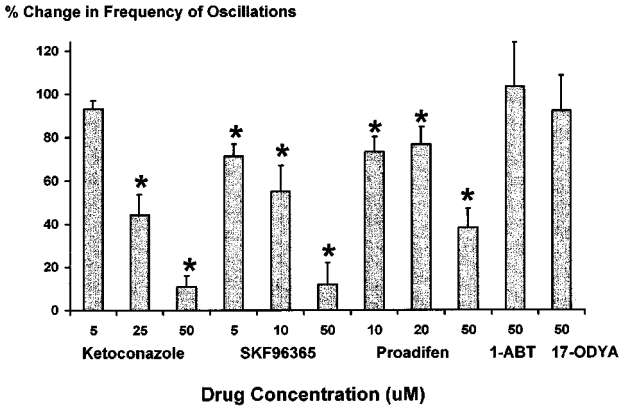

Figure 4 Summary of the effects of P450 inhibitors on the frequency of CCK-evoked oscillations in $[Ca^{2+}]_i$ in rat pancreatic acinar cells. Experiments such as those in Figures 1 and 3 were analysed in terms of changes in the frequency of CCK-evoked $[Ca^{2+}]_i$ oscillations as described in the text (see Methods). The number of experiments was between four and six for all experiments involving ketoconazole, SKF 96365 and proadifen. For 1-ABT and 17-ODYA the number of cells analysed was 86 cells (four rats) and 61 (four rats) respectively.

proadifen; four out of six cells treated with 10 μM). Furthermore, cells treated with 20 or 10 μM proadifen showed a 'normal' ACh response (defined as an increase in $[Ca^{2+}]_i$ equal to or larger than the initial spike of the CCK-evoked $[Ca^{2+}]_i$ oscillation train) after drug washout (not shown). This suggests that at these lower concentrations the effects of proadifen are reasonably reversible

We also tested whether proadifen altered resting $[Ca^{2+}]_i$ when applied in the absence of CCK. At 50 μM, the drug caused a small rise in $[Ca^{2+}]_i$ which slowly returned to baseline. Subsequent application of CCK produced $[Ca^{2+}]_i$ oscillations, although at a reduced rate and amplitude compared to control cells. At 10 μM, proadifen produced no obvious change in resting $[Ca^{2+}]_i$, although subsequent application of CCK again induced slightly impaired oscillations.

Effect of 1-ABT and 17-ODYA on $[Ca^{2+}]_i$ signalling

In order to attribute effects of P450 inhibitors to actions on the P450 system rather than to non-specific effects, it is customary to test several structurally and mechanistically unrelated inhibitors. We therefore tested two additional P450 inhibitors, 1-aminobenzotriazole (1-ABT) and 17-octadecynoic acid (17-ODYA). Aminobenzotriazole is an older broad spectrum P450 inhibitor (Micro *et al*., 1988), while 17-ODYA specifically inhibits the P450 epoxygenases which mediate conversion of arachidonate to EETs, including 5,6-EET (Zou *et al*., 1994).

For these two drugs the imaging system was used to examine large numbers of oscillating cells. However, neither drug significantly altered CCK-evoked $[Ca^{2+}]_i$ oscillations (1-ABT: $n=86$ cells from six rats; 17-ODYA: $n=61$ cells from four rats) or baseline $[Ca^{2+}]_i$ (1-ABT: $n=64$ cells from four rats; 17-ODYA: $n=78$ cells from four rats).

Figure 4 summarizes the effects of the various P450 inhibitors on the frequency of CCK-evoked $[Ca^{2+}]_i$ oscillations. The most dramatic effects were observed with high (50 μM) doses of ketoconazole and SKF96365. However, the evidence discussed above suggests strongly that these effects are related to inhibition of Ca^{2+} store function rather than to reduced Ca^{2+} entry. Of the non-imidazole drugs, proadifen was the only one to have inhibitory effects on $[Ca^{2+}]_i$ oscillations. Proadifen had much less pronounced non-specific effects than the imidazole compounds, and was thus used in subsequent studies involving P450 induction.

P450 induction protocol

Given the problems associated with the use of the P450 inhibitors, P450 induction provides an attractive alternative, as it potentially allows P450-dependent mechanisms to be identified *via* their selective enhancement in 'induced' cells. In particular, if a P450-mediated process produces an intermediate involved in stimulating Ca^{2+} entry, then inducing P450 would be expected to enhance the rate of Ca^{2+} influx, as experimentally observed following induction of several P450 isoenzymes, including P4501A1, in endothelial cells (Graier *et al*., 1995). We induced P450 by treating rats *in vivo* with β-NF. This compound is classically viewed as an inducer of the 1A1 subtype of P450, but it also appears to induce other P450 mono-oxygenases, notably the 2C8/34 subtype which has been reported to produce EETs in endothelial cells (Fisslthaler *et al*., 1999). Induction of P450 was confirmed by Western blot analysis of P450 1A1 protein levels. Figure 5 shows a representative blot where bands are visible in the lanes corresponding to liver and pancreas from β-NF-treated rats. These bands matched in molecular weight the P450 1A1 band seen in a positive control sample (induced liver microsomes). No P450 1A1 protein was detected in tissues from vehicle-treated rats. This is consistent with the negligible constitutive expression of P450 1A1 in uninduced tissues reported in many studies (Guengerich, 1987; Ioannides, 1990). In the induced tissues, the liver produced a stronger signal than the pancreas, as expected since the liver is known to express much larger amounts of P450 than any other tissue (Guengerich, 1987; Ioannides, 1990).

Comparison of CCK dose-response characteristics in cells isolated from induced and control rats

We isolated acini from the pancreas of β-NF treated rats by the same cell isolation protocol used for normal rat pancreas. However, there was a dramatic reduction in the number of viable cells obtained from the pancreas of β-NF treated rats compared to weight-matched control rats (typically around 10–20% of the cell yield of vehicle-treated control rats). In addition, the 'induced' cells tended to clump together and were very difficult to isolate as single cells or small acini. This suggests that treatment with β-NF and thus induction of P450 is generally toxic to the pancreas, although we did not carry out detailed histopathological examination of the tissue.

We examined the effects of P450 induction on CCK-evoked $[Ca^{2+}]_i$ oscillations by characterizing the CCK dose-response

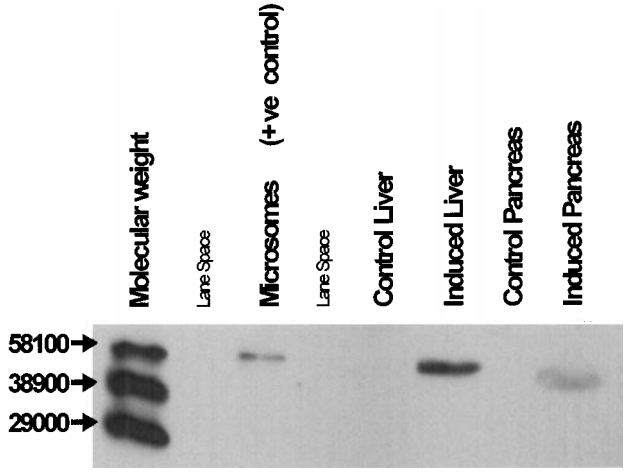

Figure 5 Western analysis of P4501A1 protein expression in pancreas and liver from control rats and from rats treated with β-NF to induce P450. Lanes were loaded with 20 μg of membrane protein from each tissue sample, except for the positive control lane which contained 5 μg of positive control sample (liver microsomes from β-NF treated rats). The position of molecular wt. markers is indicated.

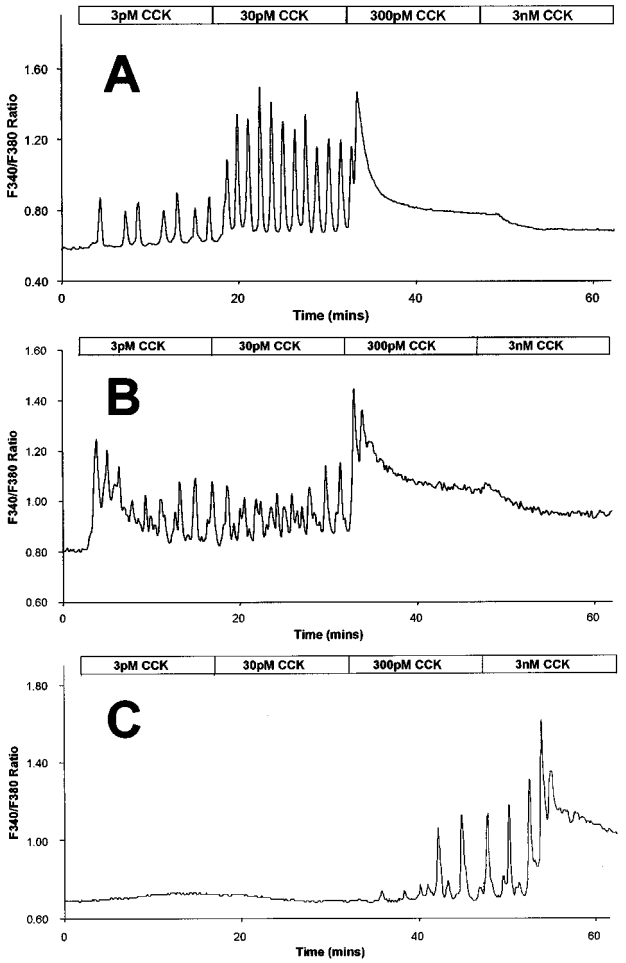

Figure 6 Typical $[Ca^{2+}]_i$ responses indicating the cell-to-cell variability observed in pancreatic acinar cells treated with increasing concentrations of CCK-octapeptide. (A) Shows a response from a 'typical' cell, where 3 pM CCK evoked small low frequency oscillations, 30 pM evoked large baseline spiking oscillations, and 300 pM evoked a sustained response. (B) Shows a cell which was very sensitive to CCK, since high frequency oscillations were evoked even at 3 pM CCK. (C) Shows a cell which was very insensitive to CCK, since oscillations were only evoked at very high concentrations of the hormone.

curve in the two cell populations. As described in other studies, individual acinar cells vary widely in their sensitivity to CCK (Willems *et al*., 1993). In addition, as the dose of CCK is increased the response of the cell changes from so-called 'baseline spiking' $[Ca^{2+}]_i$ oscillations, to oscillations over a raised baseline, and finally to a sustained increase in $[Ca^{2+}]_i$ (Willems *et al*., 1993). Figure 6 shows typical examples of the responses to CCK seen in different cells within the population during a cumulative dose-response experiment.

Cumulative CCK dose-response protocols of the type shown in Figure 6 were applied to large fields of cells typically containing around 60 cells. For analysis, the response to a given dose of CCK in each cell was allocated into one of four categories; no response; baseline spiking; oscillations above a raised baseline; and a sustained response. The distributions obtained from individual imaging runs were then summed to give 'total' distributions for control and induced cells, which were compared statistically as described in Methods.

The data obtained in the CCK dose-response experiments are summarized in Figure 7. A total of 441 cells from control rats (eight animals), and 279 cells from induced rats (eight animals) were analysed. Panels A–D show representative examples of the four response types described above and in Methods, while the lower panel shows histograms showing the distribution of responses (expressed as a percentage of the total number of cells analysed) obtained at the different concentrations of CCK in control cells (open bars) and induced cells (shaded bars). It is clear that the pattern of CCK-evoked response types was little different for induced and control cells at the low physiological CCK concentration of 3 pM. At higher CCK concentrations, however, differences were apparent. This was most obvious at 300 pM CCK, where many less induced cells than control cells produced a sustained response, while correspondingly more induced cells than control cells produced $[Ca^{2+}]_i$ oscillations. This implies that induction of P450 slightly decreased overall responsiveness to CCK. Multiple χ^2 analysis indicated that the response distributions differed significantly between control and induced cells for all CCK concentrations tested except 3 pM. The upper right panel shows the percentage of cells showing either baseline spiking or a sustained $[Ca^{2+}]_i$ response as a function of CCK concentration. Again, the decrease in CCK responsiveness in the induced cells (broken line) is clear.

We also compared the mean frequency of $[Ca^{2+}]_i$ oscillations between induced and control cells. This was only done for the two lowest CCK concentrations, 3 and 30 pM, where the responding cells produce primarily spike-like oscillations that lend themselves to this kind of analysis (see Methods, and Elliott & Bruce, 1997). This comparison revealed, somewhat surprisingly, that the mean frequency was not significantly different between induced and control cells at 3 pM CCK (mean number of oscillations was 5.9 ± 1.0 in control cells, $n = 141$, and 5.7 ± 0.3 in induced cells, $n = 92$). A slight decrease in mean oscillation frequency was observed at 30 pM CCK (7.8 ± 0.2 in control cells, $n = 428$; 6.3 ± 0.2 in induced cells, $n = 251$; values significantly different by Mann-Whitney test, $P < 0.01$).

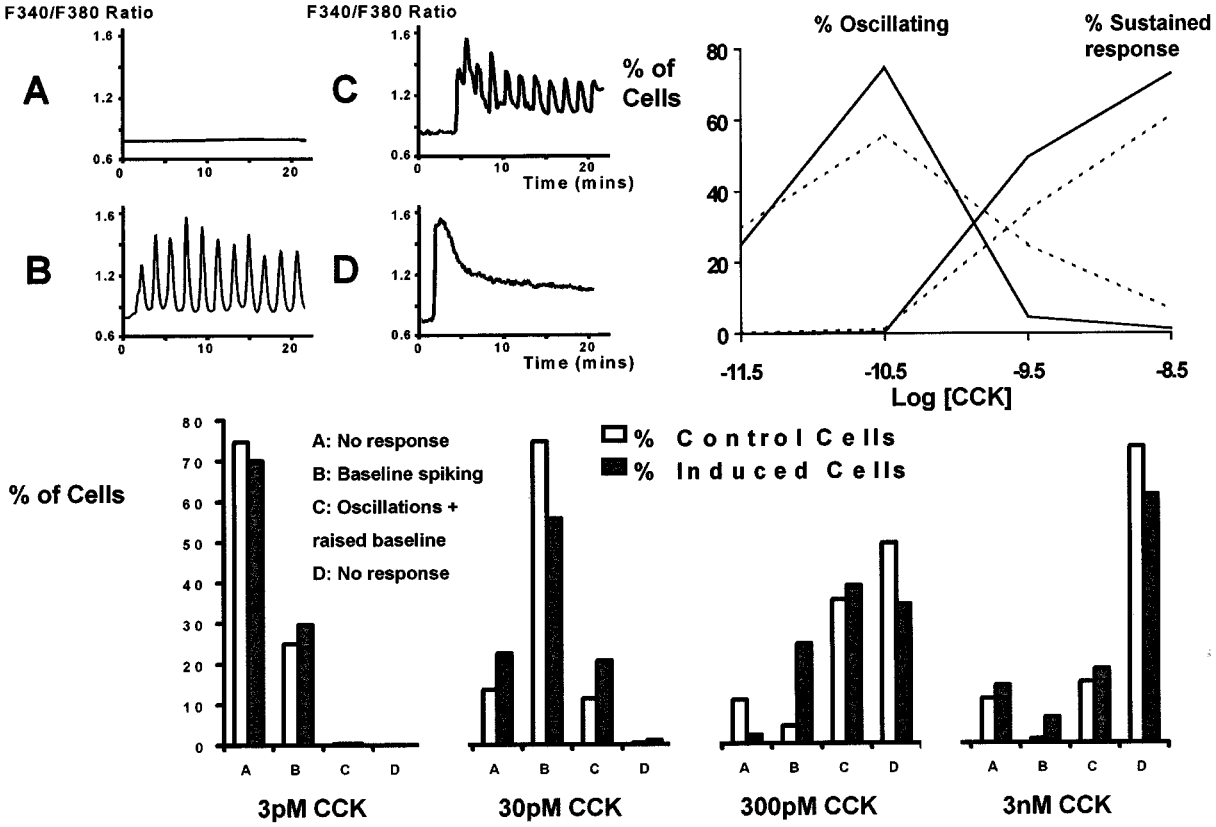

Figure 7 Analysis of CCK cumulative dose-response experiments in control and induced pancreatic acinar cells. The upper left-hand panel shows the four categories into which $[Ca^{2+}]_i$ responses to CCK were grouped for analysis, namely A (no response); B (baseline spiking); C (oscillations over raised baseline); and D (sustained response). The lower panel shows the responses to CCK categorized in this fashion for the four concentrations of CCK successively applied in cumulative dose-response experiments (see Figure 6). Open bars indicate control cells; shaded bars indicate induced cells. The upper right-hand panel shows the percentage of cells in the population showing baseline spiking $[Ca^{2+}]_i$ oscillations or a sustained $[Ca^{2+}]_i$ response. Solid lines indicate control cells; broken lines indicate induced cells.

Inhibition of SERCA Ca^{2+}-ATPases with thapsigargin and cyclopiazonic acid

We also directly compared depletion-activated Ca^{2+} entry in cells isolated from control and induced rats. Thapsigargin and cyclopiazonic acid both deplete intracellular Ca^{2+} stores by inhibition of microsomal SERCA Ca^{2+}-ATPase pumps, and thus activate capacitative Ca^{2+} entry (see e.g. Thastrup *et al*., 1990). The cocktail of the two drugs was used to ensure complete inhibition of SERCA pumps (Speake & Elliott, 1998). Somewhat surprisingly, the mean resting $[Ca^{2+}]_i$ was significantly higher in cells from β-NF treated rats (induced cells) than in control cells (Figure 8). However, this increased basal $[Ca^{2+}]_i$ was not observed in CCK-dose response experiments on the same cell populations (data not shown), where sample numbers were larger, and may therefore be artifactual. The mean peak thapsigargin- and cyclopiazonic acid-induced increase in $[Ca^{2+}]_i$ was significantly reduced in cells from β-NF treated rats (induced cells) compared to control cells (Figure 8), possibly indicating a decrease in the total releasable Ca^{2+} within intracellular stores.

The plateau (sustained) phase of the thapsigargin plus cyclopiazonic acid-induced $[Ca^{2+}]_i$ increase results from capacitative or store-operated Ca^{2+} entry, caused by depletion of intracellular stores (Putney, 1990). More accurately, assuming that all SERCA pumps are completely blocked, the 'plateau phase' represents the balance between capacitative Ca^{2+} entry and plasma membrane Ca^{2+} extrusion, mediated in this cell type by the plasma membrane (PMCA) Ca^{2+}-ATPase. In many other studies this plateau phase remains relatively constant but elevated, indicating that Ca^{2+} entry and extrusion are balanced. However, in the present study the plateau phase gradually declined almost to baseline, suggesting that Ca^{2+} extrusion actually exceeded Ca^{2+} entry during this period. In control cells, addition of 50 μM proadifen during this 'plateau phase' caused little or no change in $[Ca^{2+}]_i$ In contrast, in 'induced' cells proadifen caused a small but noticeable increase in $[Ca^{2+}]_i$.

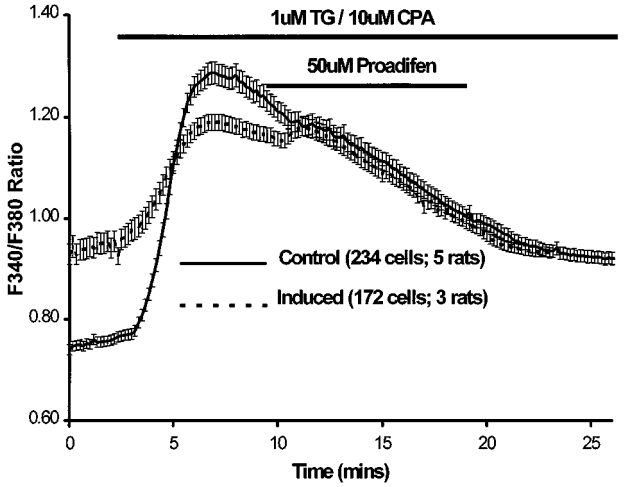

Figure 8 Mean data showing the effect of thapsigargin and cyclopiazonic acid, and of subsequent addition of proadifen, on $[Ca^{2+}]_i$ in control and induced pancreatic acinar cells.

Discussion

Ketoconazole and SKF96365

It is clear that the P450 inhibitors ketoconazole and proadifen dose-dependently inhibit $[Ca^{2+}]_i$ oscillations in pancreatic acinar cells. However, these results must be viewed with some caution, since high doses (50 μM) of ketoconazole in particular consistently raised baseline $[Ca^{2+}]_i$. This suggests that ketoconazole acts directly on $[Ca^{2+}]_i$-regulating mechanisms, most probably intracellular Ca^{2+} stores, as reported by several other authors (see Introduction). This was confirmed when both ketoconazole and the imidazole Ca^{2+} entry inhibitor SKF96365 caused a large and irreversible rise in baseline $[Ca^{2+}]_i$ when tested in the absence of CCK. The initial response to the two imidazoles was similar to that observed with supramaximal doses of ACh and CCK, i.e. a large and rapid transient increase in $[Ca^{2+}]_i$ which slowly declined. This pattern of rapid $[Ca^{2+}]_i$ increase is usually attributed to stimulation of Ca^{2+} release from intracellular stores. The second, sustained phase of the response to proadifen and SKF96365 was more reminiscent of inhibition of SERCA Ca^{2+}-ATPase, as observed with the combined application of thapsigargin and cyclopiazonic acid. Both ketoconazole and SKF96365 have previously been shown to stimulate intracellular Ca^{2+} release (Merritt *et al*., 1990) and inhibit microsomal (i.e. SERCA) Ca^{2+}-ATPase (Mason *et al*., 1993), in other cell types. This provides a plausible explanation for the rise in baseline $[Ca^{2+}]_i$ observed during CCK-induced $[Ca^{2+}]_i$ oscillations with 50 μM ketoconazole or SKF96365, and suggests that even the effects observed at lower doses may be due to non-P450-mediated effects.

The similarity in the actions of ketoconazole and SKF 96365 is not unexpected, given that the two compounds show a very high degree of structural homology. They share both an imidazole ring (the key functional group for inhibition of P450 by ketoconazole and related antimycotics) and also a substantial part of their carbon skeleton. Given this structural homology, one might predict that SKF96365 should also inhibit P450. Indeed, papers investigating the link between P450 and $[Ca^{2+}]_i$ signalling sometimes describe SKF 96365 as a 'P450 inhibitor' (see e.g. Mason *et al*., 1993; Franzius *et al*., 1994), although the only direct experimental confirmation of this is the unpublished observations of Garcia-Sancho and co-workers (Villalobos *et al*., 1992).

Given the actions of SKF96365 and the various imidazole antimycotics against Ca^{2+} entry, it has sometimes been suggested that it is the imidazole group which is the critical determinant of Ca^{2+} entry-blocking activity. It should be noted that this does not apply to any compound containing an imidazole moiety, since we have found that the simple imidazole-containing amino-acid histidine does not inhibit $[Ca^{2+}]_i$ oscillations in pancreatic acinar cells (unpublished observations).

1-Aminobenzotriazole and 17-octadecynoic acid

Given the non-specific effects of the imidazole P450 inhibitors, experiments were also carried out using structurally- and mechanistically-distinct P450 inhibitors. However, neither 1-ABT, a potent inhibitor of a range of P450 subtypes (Micro *et al*., 1988), nor 17-ODYA, a suicide substrate inhibitor of the P450 isoenzymes responsible for the synthesis of EETs (Zou *et al*., 1994), produced any effect on CCK-evoked $[Ca^{2+}]_i$ oscillations or baseline $[Ca^{2+}]_i$. This argues against a role of P450 in capacitative Ca^{2+} entry in pancreatic acinar cells. This is particularly important since 5,6-EET has been proposed to be the arachidonic acid metabolite responsible for activating Ca^{2+} entry in endothelial cells (Graier *et al*., 1995).

Proadifen

Like 1-ABT and 17-ODYA, proadifen (formerly SKF525A) is structurally and mechanistically unrelated to the imidazole antifungals such as ketoconazole. Many studies therefore interpret similar inhibitory effects of ketoconazole and proadifen on Ca^{2+} entry as indicative of a P450-dependent mechanism (see discussion in Graier *et al*., 1995). Results with microspectrofluorimetry showed that proadifen produced a dose-dependent inhibition of CCK-induced $[Ca^{2+}]_i$ oscillations. However, an increase in baseline $[Ca^{2+}]_i$ was also observed, although this was much less pronounced than with ketoconazole or SKF96365. In addition, there was recovery of $[Ca^{2+}]_i$ oscillations after the removal of proadifen, unlike with ketoconazole, and a fully functional ACh response could be observed after drug treatment, suggesting that Ca^{2+} store function was not modified irreversibly by proadifen. Furthermore, the lowest dose of proadifen used (10 μM), which produced a significant inhibition of $[Ca^{2+}]_i$ oscillations, had no effect on baseline $[Ca^{2+}]_i$ either during CCK-evoked oscillations or when tested alone. Nonetheless, when tested in the absence of CCK-induced Ca^{2+} oscillations, high doses (50 μM) of proadifen did produce an increase in $[Ca^{2+}]_i$ which slowly returned to baseline, and was sometimes accompanied by $[Ca^{2+}]_i$ oscillations. It is thus clear that proadifen itself does have some direct effects on Ca^{2+} store function. In fact, the only evidence supporting a role of P450 in controlling Ca^{2+} entry in this study was the effect of 10 μM proadifen on CCK-induced $[Ca^{2+}]_i$ oscillations. This is because 10 μM proadifen was the only dose of any drug tested which significantly inhibited oscillations but did not by itself cause changes in baseline $[Ca^{2+}]_i$ indicative of non-specific effects. Although the reported non-specific effects of proadifen are minimal compared to those observed with ketoconazole and the other imidazoles, non-specific effects of the drug have been observed in other systems. These include inhibition of voltage-operated Ca^{2+} channels (Villalobos *et al*., 1992), of nitric oxide synthase (Hecker *et al*., 1994), of a variety of K^+ channels (Alvarez *et al*., 1992b; Sakuta & Yoneda, 1994) and of Ca^{2+}-induced Ca^{2+} release in cardiac sarcoplasmic reticulum (e.g. Volpe *et al*., 1983).

Can P450 inhibitor data be used to demonstrate a role of P450 in controlling Ca^{2+} entry?

The present study highlights once again the difficulty of showing definitively whether cytochrome P450 is involved in controlling Ca^{2+} entry, particularly when the experimental design is heavily reliant on the use of P450 inhibitors (see the review by Clementi & Meldolesi, 1996). In general, it seems clear that a reasonable case for P450 playing a role in Ca^{2+} entry in any given cell type can only be made if several structurally and mechanistically distinct P450 inhibitors have a similar inhibitory effect on Ca^{2+} entry without showing any 'non-specific' effects. Similar results with an imidazole inhibitor and with proadifen should be viewed with caution, since proadifen clearly has non-specific effects, although not as marked as those of the imidazole antimycotics. Thus the inhibitor selection in a study on P450 in Ca^{2+} entry should clearly include a broad spectrum inhibitor, such as 1-ABT or carbon monoxide, and/or an inhibitor of epoxygenases such as 17-ODYA or arachidonyl-trifluoromethylketone. Examples of recent studies meeting these criteria are those by Hoebel *et al*., (1997) on endothelial cells and Rzigalinski *et al*., (1999) on astrocytes. However, since even these studies are not free from worries about the specificities of the drugs, additional evidence should also be sought, such as stimulatory effects of P450 induction (Graier *et al*., 1995) or of exogenously added EETs (Graier *et al*., 1995; Rzigalinski *et al*., 1999) on Ca^{2+} entry.

P450 induction

In view of the equivocal results obtained with P450 inhibitors, we turned to an *in vivo* P450 induction protocol in an attempt to bypass the need for using the drugs. Western blotting confirmed that injection of β-NF induced substantial expression of P450 in the pancreas as well as in the liver. By analogy with the work of Graier *et al*., (1995), this would be expected to enhance Ca^{2+} entry if a P450-mediated process is important in mediating activation of Ca^{2+} influx. In fact, induction of P450 in pancreatic acinar cells appeared to have small, though clear, inhibitory effects on $[Ca^{2+}]_i$ signalling.

CCK dose-response curves

The inhibitory effects of P450 induction on CCK-evoked $[Ca^{2+}]_i$ signalling could be observed in a number of parameters. At low doses of CCK there was a decrease in the frequency of $[Ca^{2+}]_i$ oscillations and/or a decrease in the number of cells that produced $[Ca^{2+}]_i$ oscillations compared to control cells. Similarly, at higher doses of CCK, there was a decrease in the number of cells producing a sustained elevation in $[Ca^{2+}]_i$. Induction of P450 thus effectively shifted the CCK dose response curve to the right, suggesting a general loss of sensitivity to CCK with P450 induction. This could be explained by a desensitization of CCK receptors. However, it was noticeable that P450 induction did not significantly affect the response to 3 pM CCK, the lowest dose applied. These observations are the opposite to what would be expected based on the current hypothesis for the involvement of P450 in capacitative Ca^{2+} entry. Induction of P450 would theoretically be expected to enhance capacitative Ca^{2+} entry and thus generally increase CCK sensitivity (i.e. cause a shift in the CCK dose response curve to the left). Since this was not observed, the CCK dose-response data tend to indicate that P450 is probably not involved in store-operated Ca^{2+} entry in pancreatic acinar cells.

Stimulation of Ca^{2+} entry via *inhibition of SERCA pumps*

The experiments activating Ca^{2+} entry with thapsigargin and cyclopiazonic acid also showed some differences between control and induced cells. Firstly, P450 induction appeared to decrease the total releasable Ca^{2+}, since thapsigargin plus cyclopiazonic acid evoked a smaller change in $[Ca^{2+}]_i$ in induced than in control cells. Secondly, proadifen caused a small additional increase in $[Ca^{2+}]_i$ in induced, but not in control, cells. It was clear, however, that proadifen did not inhibit capacitative Ca^{2+} entry in either control or induced cells. This reinforces the conclusion that P450 is not involved in Ca^{2+} entry. In fact, proadifen appeared to produce the opposite effect, namely a slight increase in the plateau phase of the response in induced cells.

Taken together, the induction data do not support a role for P450 in controlling Ca^{2+} entry in pancreatic acinar cells. It should be noted, however, that β-naphthoflavone does not necessarily induce all subtypes of P450, and that cytochrome P450 enzymes of several families (1A, 2B, 2C, 2E and 2J) can catalyse the formation of EETs (Capdevila *et al*., 1999). In the present study, we showed that induction of P450 1A1 (and possibly also 2C8/34, since this subtype has been shown to be

induced in endothelial cells by β-NF treatment, Fisslthaler *et al*., 1999) in the pancreas is not associated with alterations in Ca^{2+} entry. It remains conceivable that other P450 isoenzymes which are not induced by β-NF might still have a physiological role in intracellular Ca^{2+} signalling in this cell type, although the lack of clear-cut effects with P450 inhibitors speaks against this. Apart from P450 1A1, the P450 3A and P450 2E isoenzymes have also been shown to be induced in the pancreas during pancreatitis (Foster *et al*., 1993), and are therefore candidates for further investigation. The P450 2E subfamily, which can produce EETs, is induced by alcohols, acetone and carbon tetrachloride (for review see Gonzalez, 1990) which may be important when considering the role of ethanol and diabetes (i.e. ketoacidosis) in predisposing to pancreatitis. In contrast to the P450 1A1 and 2C8/34 subtypes, the induction of P450 2E occurs primarily by post-transcriptional mechanisms (Guengerich, 1987; Ryan & Levin, 1989).

Pancreatic toxicity during P450 induction

As discussed above, P450 induction had a general, though small, inhibitory effect on $[Ca^{2+}]_i$ signalling in pancreatic acinar cells. In addition, the reduced cell yields from the pancreas of β-NF-treated rats implies that P450 induction caused cellular damage in the pancreas. All these effects could conceivably be consequences of an increase in cellular oxidative stress, which is known to occur during P450 induction (Sandlands *et al*., 1990). This was, in fact, the original basis of the hypothesis that P450 induction might play a role in the pathogenesis of pancreatitis (Braganza & Chaloner, 1995). In addition, those cells which survived digestion, and thus were used experimentally, presumably represent a sub-population of those cells which were most resistant to toxicity, perhaps due to increased antioxidant capacity. Therefore, P450-mediated cellular toxicity (due to increased oxidative stress) and impaired signal transduction may well account for the observed differences in $[Ca^{2+}]_i$ signalling between induced and control cells.

Summary

In summary, we have used a pharmacological approach to determine whether a cytochrome P450-mediated process might be involved in controlling Ca^{2+} entry in rat pancreatic acinar cells. Results with P450 inhibitors were mixed. Ketoconazole, SKF96365 and proadifen all inhibited CCK-evoked $[Ca^{2+}]_i$ oscillations. However, these results are probably attributable to direct effects of the drugs on Ca^{2+} stores, or directly on the Ca^{2+} channels mediating Ca^{2+} entry, rather than on cytochrome P450. Two other P450 inhibitors, 1-aminobenzotriazole and 17-octadecynoic acid, failed to produce any effect on $[Ca^{2+}]_i$. Experiments in which P450 was induced *in vivo* showed only minor differences in $[Ca^{2+}]_i$ signalling in induced cell compared to control cells, and these differences were probably attributable to pancreatic toxicity during P450 induction. We therefore conclude that it is unlikely that P450 has a physiological role in CCK-evoked $[Ca^{2+}]_i$ signalling in rat pancreatic acinar cells.

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