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Effects of tyrosine kinase inhibitors on cell death induced by sodium fluoride and pertussis toxin in the pancreatic β-cell line, RINm5F

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1 Sodium fluoride causes apoptosis of pancreatic β-cells and this response is enhanced by pre-treatment with pertussis toxin. In the present study, tyrosine kinase inhibitors were used to investigate the mechanisms of action of NaF and pertussis toxin in the β-cell line, RINm5F.

2 Exposure of RINm5F cells to low concentrations of genistein or tyrphostin A25 resulted in significant inhibition of cell death induced by 5 mM NaF. Higher concentrations (>25 μM) were cytotoxic in the absence of NaF but, paradoxically, the combination of genistein and NaF induced less cell death than when each agent was used alone.

3 The increase in cell death induced by 100 μM genistein was markedly inhibited by ciprofloxacin, a drug which binds to topoisomerase II. Etoposide (which inhibits topoisomerase II but has no effect on tyrosine kinase activity) also caused an increase in RINm5F cell death. Neither etoposide nor ciprofloxacin altered the response to 5 mM NaF.

4 Pertussis toxin markedly enhanced the extent of RINm5F cell death induced by NaF and this effect was completely prevented by 25 μM genistein. The inhibition caused by genistein was not affected by ciprofloxacin but was reproduced by a structurally dissimilar tyrosine kinase inhibitor, herbimycin A.

5 The results demonstrate that RINm5F β-cells express a pertussis toxin sensitive pathway that is anti-apoptotic. The activity of this pathway is most evident in cells exposed to pro-apoptotic stimuli where the effects of pertussis toxin can be blocked by inhibitors of tyrosine kinase enzymes. A genistein-sensitive tyrosine kinase does not appear to be involved in RINm5F cell survival under basal conditions. *British Journal of Pharmacology* (2001) **132,** 119–126

Keywords: β-cell; endocrine pancreas; apoptosis; topoisomerase II; pertussis toxin; tyrosine kinase; genistein; diadzein; etoposide; ciprofloxacin

Abbreviations: EDTA, ethylenediamine tetra-acetic acid; IGF-1, insulin-like growth factor-1; NaF, sodium fluoride; PBS, phosphate buffered saline; PI-3-K, phosphatidylinositol-3-kinase; Ptx, pertussis toxin; RINm5F cells, cultured rat insulinoma cells

Introduction

The pathway of apoptosis in mammalian cells is subject to multiple levels of regulation, many of which are cell-specific (reviewed by Hale *et al*., 1996; Kiess & Gallaher, 1998; Mallat & Tedgui, 2000). This specificity is established principally at the level of the up-stream components of the pathway (i.e. *via* signalling molecules involved in initiation of inhibition of apoptosis) while the down-stream effectors of apoptosis are common to a variety of different cell types (Hale *et al*., 1996; McConkey & Orrenius, 1997; Schulze-Osthoff *et al*., 1998; Utz & Anderson, 2000).

In a number of cells, there is evidence that pertussis toxin (Ptx) sensitive G-proteins are involved in the regulation of apoptosis and, in many cases, treatment of such cells with the toxin is associated with inhibition of cell death (Yin *et al*., 1997; Farkas *et al*., 1998; Okazawa *et al*., 1998; Sharma & Srikant, 1998). This implies that a Ptx-sensitive G-protein(s) controls a pathway that is involved in the activation of cell death in these cells. As a consequence, blockade of G-protein dissociation by Ptx results in a reduction in the sensitivity to pro-apoptotic stimuli and cell viability is maintained. By contrast, the pancreatic β-cell displays the reverse specificity to Ptx (Loweth *et al*., 1996). In this cell type, treatment with pertussis toxin leads to an enhancement of cell death in response to certain inducers of apoptosis. This implies that, in β-cells, Ptx sensitive G-proteins are involved in the regulation of an inhibitory pathway that acts to restrain their entry into apoptosis (Loweth *et al*., 1996). Since induction of apoptosis is increasingly implicated in the early loss of β-cells associated with progression towards type 1 diabetes (reviewed by Mauricio & Mandrup-Poulsen, 1998; Kay *et al*., 2000), an understanding of this inhibitory mechanism may suggest a means of intervention to minimize the rate of cell death in the pre-diabetic phase. Moreover, since this mechanism appears to be differentially controlled compared to that found in many, though not all (Communal *et al*., 1999; 2000) other cell types, it offers the potential for relatively cell-specific targetting.

At present, very little is known about the mechanisms involved in the control of apoptosis by Ptx-sensitive G-proteins in pancreatic β-cells, although it is evident that cyclic AMP-dependent processes (which are subject to regulation by Ptx sensitive G-proteins) can influence viability in these cells

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(Loweth *et al*., 1996). However, previous studies have indicated that changes in cyclic AMP are unlikely to account for the actions of Ptx on β-cell apoptosis (Loweth *et al*., 1996). In this context, it is also known that, in some cell types, Ptx-sensitive G-proteins can interact with intracellular effector mechanisms involving tyrosine kinases (Alric *et al*., 1999; Luttrell *et al*., 1999; Chang & Wang, 2000; Germack & Dickenson, 2000) and there is evidence that pathways involving tyrosine kinase activation can modulate β-cell apoptosis (Mabley *et al*., 1997; Harrison *et al*., 1998; Hill *et al*., 1999). Thus, in the present work, we have sought to clarify whether activation of protein tyrosine kinases may be implicated in the enhancement of cell death mediated by Ptx in the β-cell line, RINm5F.

Methods

Culture of RINm5F cells

RINm5F cells were seeded at 3×10^4 cells per well in 24 well cell culture plates containing RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, penicillin (400 iu ml^{-1}) and streptomycin sulphate (200 μg ml^{-1}). The cells were cultured for 72 h at 37°C (95% O_2: 5% CO_2; 100% humidity) prior to treatment. The medium was then changed and test reagents added from concentrated stock solutions and incubation continued for between 4–24 h according to the experimental protocol. Control cells received vehicle alone. In the case of cells treated with pertussis toxin, the toxin was added for the final 24 h of culture, prior to addition of test reagents.

Analysis of cell death in RINm5F cells

Following the incubation period with test reagents, the culture medium was removed and any dead cells harvested by centrifugation of the supernatant at $1500 \times g$ for 3 min. The medium was removed and the cells resuspended in 20 μl of fresh medium. Ten μl of the suspension was removed and incubated with an equal volume of 0.2% Trypan blue (in phosphate buffered saline; PBS) and the number of dead cells counted using a haemocytometer. The total dead cell count (cells ml^{-1} in the original culture) was then calculated for each incubation condition. Experiments were normally performed in replicates of four and were repeated on a minimum of three occasions.

For estimation of apoptosis, cells were stained with either acridine orange or annexin-V-Cy3. Following the culture period, the cells were harvested from the supernatant and from the culture flask (adherent cells were released from the substrate with trypsin/EDTA) by centrifugation at $1500 \times g$ for 3 min. The supernatant was removed and the cells washed in 300 μl of PBS. They were centrifuged for 3 min ($1500 \times g$) after which the supernatant was removed and replaced with 20 μl of fresh PBS. The pellet was resuspended and 10 μl removed for dilution into 10 μl of PBS containing acridine orange (10 μg ml^{-1}). Apoptotic cells were scored by visual inspection under a fluorescence microscope. In the case of annexin-V-Cy3, cells were stained with an apoptosis detection kit according to the manufacturer's instructions and were viewed under a fluorescence microscope.

DNA fragmentation

For analysis of DNA fragmentation, batches of 10^6 RINm5F cells were harvested and centrifuged ($300 \times g$; 5 min) before resuspension in 20 μl of lysis bufer (50 mM Tris-HCl, 10 mM EDTA, 0.5% sodium lauryl sarcosine, pH 8) and incubation at 55°C for 1 h. RNAse A (0.5 mg ml^{-1}) was added and the incubation continued at 55°C for a further 1 h. The lysed cell suspension was then electrophoresed on a 2% agarose gel for 2 h at 40 V and the DNA visualized by post-staining with ethidium bromide and UV illumination.

Materials

RPMI-1640, NaF, pertussis toxin, genistein, wortmannin, herbimycin A, sodium penicillin G, streptomycin sulphate, etoposide, annexin-V-Cy3 apoptosis detection kit and acridine orange were purchased from Sigma Chemicals (Poole, Dorset, U.K.). Diadzein and tyrphostin A25 were from Calbiochem (Nottingham, U.K.) and ciprofloxacin was obtained from Miles Diagnostics, Kankakee, IL, U.S.A.

Statistical analysis

Statistical analysis was performed by analysis of variance and results were considered significant when $P < 0.05$.

Results

Effects of the tyrosine kinase inhibitor, genistein, on β-cell death

In confirmation of previous work (Loweth *et al*., 1996) 5 mM NaF caused a significant increase in RINm5F cell death (Figure 1) which was evident within 4 h of addition of the agent and was maximal within 24 h. Examination of the cells by acridine orange cytochemistry; after staining with an

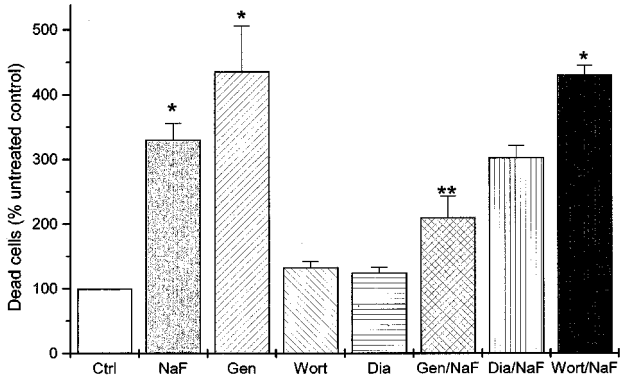

Figure 1 Effects of various kinase inhibitors on cell death in RINm5F cells. Cultured RINm5F cells were treated for 4 h with 5 mM NaF, 100 μM genistein (Gen), 300 nM wortmannin (Wort), 100 μM diadzein (Dia) or combinations of these agents as shown. After this time, the dead cells were harvested from the culture medium, stained and counted. The number of dead cells in each case was expressed relative to the untreated control (Ctrl) samples. Each value is the mean ± s.e.mean from three experiments. *$P < 0.001$ relative to cells incubated under control conditions. **$P < 0.01$ relative to genistein or NaF alone.

annexin V-Cy3 conjugate or by agarose gel electrophoresis of extracted DNA (Figure 2) confirmed that induction of cell death occurred, at least in part, by apoptosis.

Previous studies have revealed that genistein is a selective inhibitor of tyrosine kinases (Akiyama *et al*., 1987) that is effective in pancreatic β-cells (Persaud *et al*., 1999; Nedachi *et al*., 2000). Thus, we elected to use this agent in initial experiments to study the involvement of tyrosine kinase enzymes in the regulation of β-cell death by NaF and Ptx. However, it was immediately evident that treatment of RINm5F cells with genistein alone caused a consistent and marked increase in cell death over a period of 4–24 h (Figure 1). This response was similar in magnitude to that induced by NaF and was principally due to increased apoptosis as judged by acridine orange cytochemistry and DNA electrophoresis (Figure 2). The effects of genistein were dose-dependent over the range 5–100 μM (Figure 3) and were not reproduced by its inactive analogue diadzein (Figure 1).

Surprisingly, when RINm5F cells were exposed to 100 μM genistein in the presence of 5 mM NaF, the increase in cell death was reduced below that seen with either agent alone (Figure 1). This paradoxical effect was investigated in more detail in experiments in which RINm5F cells were exposed to increasing concentrations of genistein in the absence or presence of 5 mM NaF (Figure 3). Under these conditions, cell death in response to NaF was significantly reduced as the concentration of genistein was increased, despite the fact that genistein itself progressively increased apoptosis over the same dose range. Thus, the net effect of the combined stimulus was to produce less cell death (at all concentrations of genistein) than when either agent was used alone. Despite this, the extent of cell death was still elevated above control levels in the presence of NaF plus genistein and significant fragmentation of RINm5F cell DNA was observed in the presence of either drug alone and when they were used in combination (Figure 2).

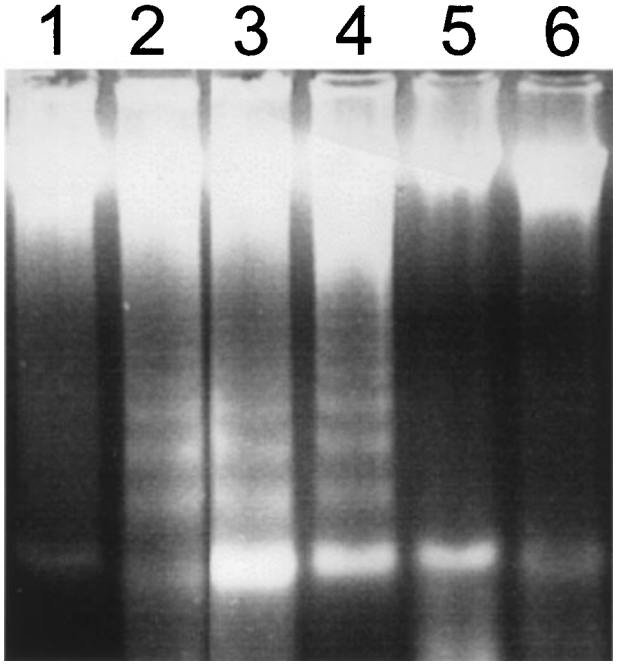

Figure 2 DNA fragmentation patterns in RINm5F cells treated with NaF, genistein and ciprofloxacin. RINm5F cells were cultured under control conditions (lane 1) or in the presence of 5 mM NaF (lane 2), 100 μM genistein (lane 3), 5 mM NaF plus 100 μM genistein (lane 4), 400 μM ciprofloxacin (lane 5) and 400 μM ciprofloxacin plus 100 μM genistein (lane 6). DNA was extracted from the cells and analysed after electrophoresis on a 2% agarose gel and post-staining with ethidium bromide.

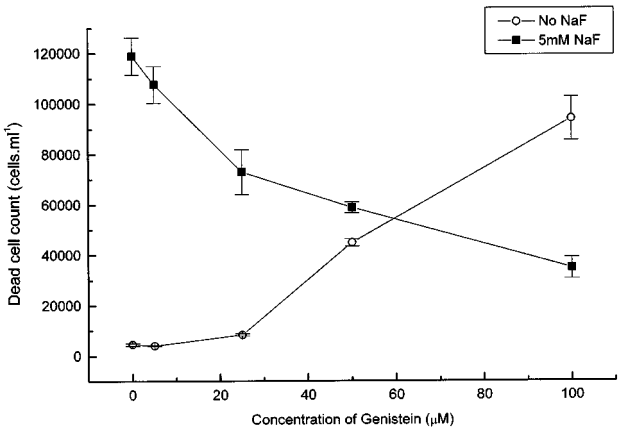

Figure 3 Effects of genistein and NaF on cell death in RINm5F cells. Cultured RINm5F cells were incubated for 24 h with either increasing concentrations of genistein alone (open circles) or increasing concentrations of genistein in the presence of 5 mM NaF (closed squares). Following incubation, the culture medium was harvested and the number of dead cells counted. Each point is the mean ± s.e.mean ($n=4$) from a representative experiment that was repeated three times with similar results.

Mechanisms involved in the control of β-cell viability by genistein

Analysis of the genistein dose-response data (Figure 3) revealed that 25 μM genistein exerted a significant inhibitory effect on the cell death response to NaF (reduction of ~40%) while causing only a marginal increase itself. This suggests that a major component of the apoptotic response to NaF in RINm5F cells may have been caused by activation of a tyrosine kinase that is sensitive to inhibition by genistein. In confirmation of this, it was observed that a second tyrosine kinase inhibitor, tyrphostin A25 (25 μM) also significantly reduced the NaF response (Figure 4). Interestingly, in common with genistein, higher concentrations of this inhibitor caused a marked loss of RINm5F cell viability although, in the case of tyrphostin A25, combination with NaF did not lead to a reduction in the level of cell death below that seen with each agent alone (Figure 4).

Effects of ciprofloxacin on cell death induced by genistein in RINm5F cells

In order to investigate further the unexplained increase in cell death induced by high concentrations of genistein in RINm5F cells, the cells were exposed to the drug in the presence of ciprofloxacin. This reagent does not interact with tyrosine kinases but, rather, it binds to DNA topoisomerase enzymes (Elsea *et al*., 1997; Hammonds *et al*., 2000). Over periods up to 24 h, ciprofloxacin did not cause any direct loss of RINm5F cell viability (Figure 5) nor did it alter the extent of cell death induced by NaF (not shown). By contrast, the

drug abolished DNA fragmentation (Figure 2) and markedly reduced the extent of cell death (Figure 5) in RINm5F cells treated with 100 μM genistein. To confirm that inhibition of topoisomerase II can increase cell death in RINm5F cells, the cells were exposed to a second topoisomerase II inhibitor, etoposide, which has no effects on tyrosine kinases. Etoposide induced a potent, dose-dependent, loss of viability in a manner that was independent of the presence of NaF (Figure 6).

Effects of genistein on the enhancement of RINm5F cell death caused by pertussis toxin

In view of the earlier finding that 25 μM genistein was able to significantly improve the viability of RINm5F cells exposed to NaF, while itself causing only a small increase in death (Figure 3) this concentration was selected for use in experiments with Ptx. As reported previously (Loweth *et al*., 1996), pre-treatment of RINm5F cells with Ptx did not induce a significant increase in cell death. By contrast, pre-treatment with Ptx routinely and significantly enhanced the extent of cell death caused by exposure to NaF (Figure 7). Annexin-V staining confirmed that this was due to an increase in apoptosis since the percentage of intact, annexin-V-positive cells increased from 1.3±0.3% in controls to 11.2±2.4% after NaF (5 mM) treatment and this was enhanced to 25.3±3.6% in the combined presence of NaF

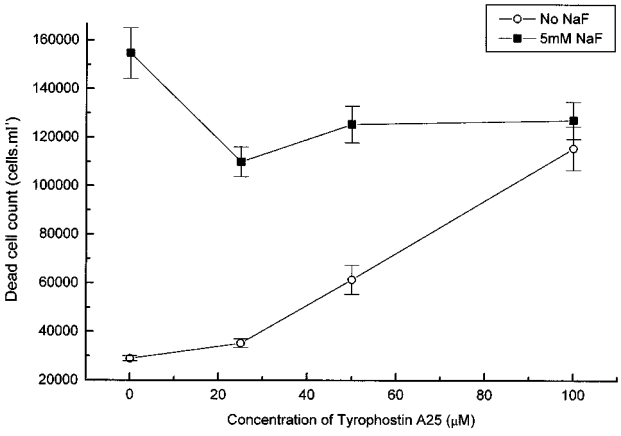

Figure 4 Effects of tyrphostin A25 and NaF on cell death in RINm5F cells. Cultured RINm5F cells were incubated for 24 h with either increasing concentrations of tyrphostin A25 alone (open circles) or increasing concentrations of tyrphostin A25 in the presence of 5 mM NaF (closed squares). Following incubation, the culture medium was harvested and the number of dead cells counted. Each point is the mean±s.e.mean ($n=4$) from a representative experiment that was repeated three times with similar results.

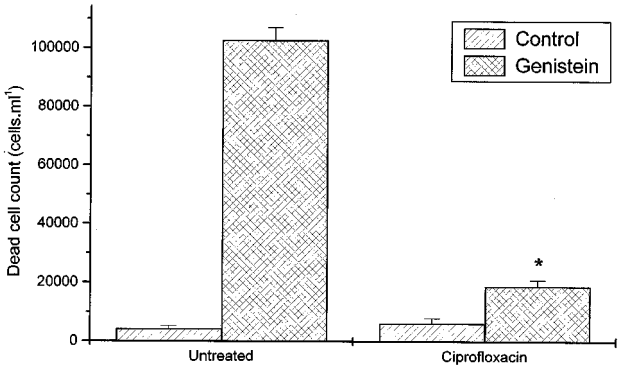

Figure 5 Effect of ciprofloxacin on genistein-induced cell death in RINm5F cells. Cultured RINm5F cells were exposed to 100 μM genistein in the presence of 400 μM ciprofloxacin for 24 h as shown. Following the culture period, the medium was collected and the number of dead cells counted after staining with Trypan blue. Each point represents the mean±s.e.mean ($n=4$) from a representative experiment that was repeated twice with similar results. *$P<0.001$ relative to genistein in the absence of ciprofloxacin.

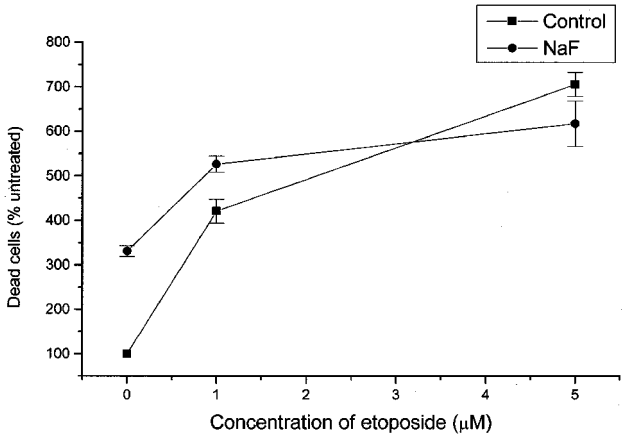

Figure 6 Effects of etoposide on cell death in RINm5F cells. Cultured RINm5F cells were treated with either etoposide alone (squares) or etoposide in the presence of 5 mM NaF (circles) for 4 h. The culture medium was then aspirated and the number of dead cells counted. Each point represents the mean number of dead cells (relative to the untreated control)±s.e.mean from three experiments.

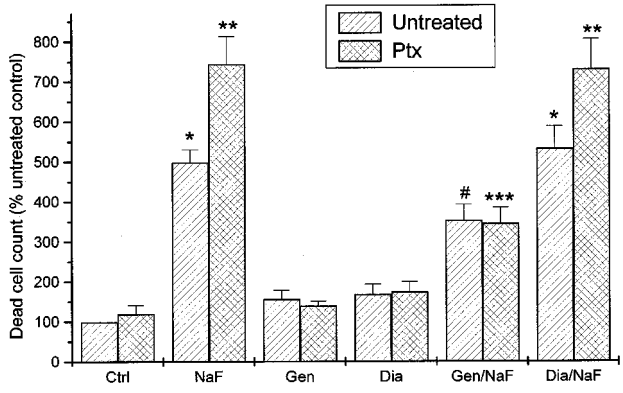

Figure 7 Effects of genistein and diadzein on RINm5F cell death in response to NaF and pertussis toxin. RINm5F cells were cultured for 24 h in either the absence (right hatches) or presence (cross hatches) of 0.2 μg ml^{-1} pertussis toxin. During the final 4 h of incubation, the culture medium was supplemented with 5 mM NaF, 25 μM genistein (Gen) or 100 μM diadzein (Dia) as shown. Following incubation, the medium was aspirated and the number of dead cells counted. Each point is the mean number of dead cells (relative to untreated controls)±s.e.mean from three experiments. *$P<0.001$ relative to cells incubated under control conditions. **$P<0.01$ relative to NaF in the absence of pertussis toxin. ***$P<0.001$ relative to NaF plus pertussis toxin in the absence of genistein. #$P<0.01$ relative to NaF in the absence of genistein.

and Ptx. Ptx alone did not increase the number of annexin-V positive cells above the control level.

The increase in cell death in response to Ptx was entirely absent when 25 μM genistein was also included in the culture medium (Figure 7) whereas the inactive analogue of genistein, diadzein, failed to block the response (Figure 7). A second tyrosine kinase inhibitor that is structurally dissimilar to genistein, herbimycin A (1 μg/ml) also completely prevented the increase in NaF-induced apoptosis caused by Ptx (Figure 8).

In a final series of experiments, the effects of ciprofloxacin were investigated on the ability of genistein to block the response to Ptx in RINm5F cells (Figure 9). In the presence of ciprofloxacin, Ptx was still able to enhance NaF-induced cell death and the ability of genistein to inhibit this effect was not impaired.

Discussion

The control of cell death in pancreatic β-cells involves a number of interacting signalling pathways which converge to regulate the final effectors involved in the execution phase of apoptosis (Loweth *et al*., 1996; 1997; 1998; 2000; Harrison *et al*., 1998; Mauricio & Mandrup-Poulsen, 1998; Paraskevas *et al*., 1998; Ishizuka *et al*., 1999; Kay *et al*., 2000). We have provided previous evidence that one such pathway involves a Ptx sensitive component (presumably a G-protein) which acts to restrain the entry of the cells into apoptosis (Loweth *et al*., 1996). In the present work this observation has been confirmed and extended to investigate the mechanisms involved.

The study was focused on the possibility that regulation of a tyrosine kinase may underlie the capacity of Ptx to enhance NaF-induced cell death in the pancreatic β-cell line, RINm5F. This hypothesis was based on evidence from other cell types where it is known that cross-talk from Ptx-regulated pathways can involve tyrosine kinases (Alric *et al*., 1999; Luttrell *et al*., 1999; Chang & Wang, 2000; Germack & Dickenson, 2000) and on the recent demonstration that regulation of tyrosine kinases may be important for the control of survival in pancreatic islet cells (Mabley *et al*., 1997; Harrison *et al*., 1998; Hill *et al*., 1999). On this basis, agents which inhibit tyrosine kinase enzymes might be expected to alter the viability of RINm5F cells. In order to test this hypothesis, we selected the tyrosine kinase inhibitor, genistein, for initial experiments since this agent has been employed in a number of previous studies of β-cell function (Sorenson *et al*., 1994; Corbett *et al*., 1994; 1996; Jonas & Henquin, 1996; Zhang *et al*. 1998; Persaud *et al*., 1999)

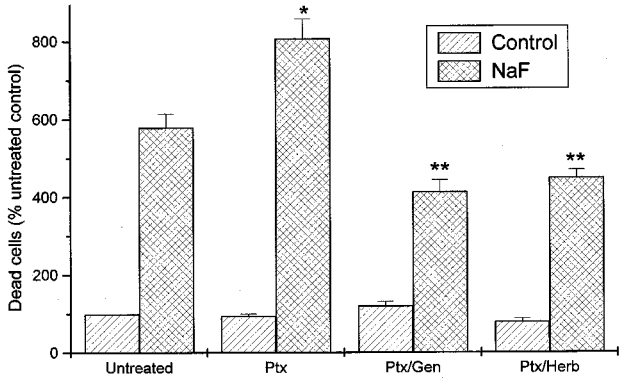

Figure 8 Effect of herbimycin A on the ability of pertussis toxin to enhance NaF-induced cell death in RINm5F cells. RINm5F cells were cultured in the absence (right hatches) or presence (cross hatches) of 5 mM NaF. Cells were also exposed to pertussis toxin (0.2 μg ml^{-1}; Ptx) as shown. Either genistein (25 μM; Gen) or Herbimycin A (1 μg ml^{-1}; Herb) was included during the period of exposure to NaF and the number of dead cells counted at the end of the incubation period. Each point represents the mean ± s.e.mean (relative to untreated control cells) from three experiments. *$P<0.01$ relative to NaF alone. **$P<0.001$ relative to NaF plus pertussis toxin.

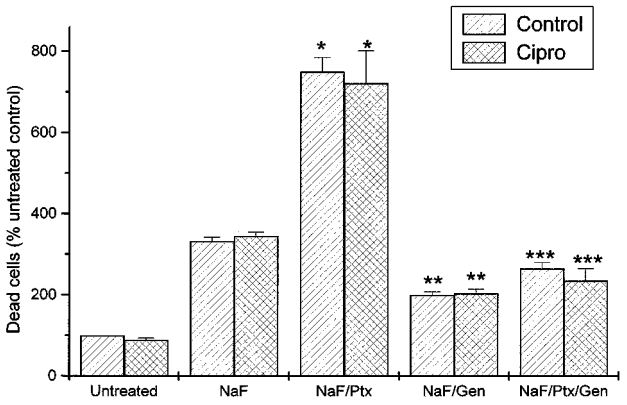

Figure 9 Effect of ciprofloxacin on the ability of genistein to inhibit the actions of pertussis toxin on cell death in RINm5F cells. RINm5F cells were cultured in the absence (right hatches) or presence (cross hatches) of 400 μM ciprofloxacin. NaF (5 mM) genistein (25 μM; Gen) and pertussis toxin (0.2 μg ml^{-1}) were included as shown and the number of dead cells counted at the end of the incubation period. Each point represents the mean ± s.e.mean (relative to untreated control cells) from three experiments. *$P<0.001$ relative to NaF alone. **$P<0.01$ relative to NaF alone. ***$P<0.001$ relative to NaF plus pertussis toxin.

Effects of high concentrations of genistein on the viability of RINm5F cells

Initially, the effects of genistein alone were investigated and it was discovered that addition of 100 μM genistein to the culture medium was detrimental to the survival of RINm5F cells. This concentration of genistein has been used in previous work with β-cells (Sorenson *et al*., 1994; Jonas & Henquin, 1996) but we observed that, within 4 h of exposure to 100 μM genistein, the cells began to exhibit the characteristic features of apoptosis, including extensive oligonucleosomal fragmentation of DNA (Figure 2). A similar loss of viability was also observed with increasing concentrations of tyrphostin A25 (Figure 4). By contrast, this effect was not seen with the inactive analogue of genistein, diadzein, suggesting that the toxicity was not the result of a non-specific action of the drugs.

One obvious explanation for these effects is that the cells express a critical tyrosine kinase enzyme whose activity is required for the maintenance of cell viability under resting conditions. Inhibition of this enzyme by genistein or tyrphostin A25 would then result in entry of the cells into apoptosis. However, it should be emphasized that these direct

effects of genistein and tyrphostin A25 were only seen at elevated concentrations of the drugs (50 μM and above) and that lower concentrations exerted minimal effects. Moreover, a low concentration of a third tyrosine kinase inhibitor, herbimycin A, also failed to alter cell viability in RINm5F cells cultured under control conditions (Figure 8). Thus, it cannot be excluded that additional mechanisms might account for the loss of viability observed with these inhibitors.

In an attempt to investigate this issue more directly, the extent of tyrosine phosphorylation of RINm5F cell proteins was studied by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies. In these experiments, a number of tyrosine phosphorylated proteins were evident in extracts prepared from RINm5F cells incubated under control conditions (not shown) but, in common with previous findings in normal islets (Jonas & Henquin, 1996) and in other cultured cells (Peterson, 1995) no specific changes could be detected in RINm5F cells treated with genistein. These results do not necessarily exclude the possibility that a genistein-sensitive tyrosine kinase is involved in maintenance of β-cell viability, since it is possible that the critical substrate protein(s) for the kinase may be a minor β-cell component. However, they support the view that further consideration should be given to the possibility that high concentrations of genistein may exert effects on β-cell viability by mechanisms that are independent of changes in protein phosphorylation state. Indeed, the results of experiments with ciprofloxacin suggest that this was the case.

Interactions between genistein and ciprofloxacin in RINm5F cells

Ciprofloxacin is a quinolone antibiotic that binds to mammalian DNA topoisomerase II at a site which overlaps the binding site for genistein (Elsea *et al*., 1997; Hammonds *et al*., 2000). Binding of ciprofloxacin does not cause any loss of enzyme activity (Elsea *et al*., 1997; Hammonds *et al*., 2000) whereas the interaction of genistein with topoisomerase II leads to loss of the DNA ligase activity of the enzyme. Hence, treatment of cells with ciprofloxacin does not lead to the appearance of DNA strand breaks (Figure 2) or to loss of viability (Figure 9) whereas genistein induces DNA strand breaks (Figure 2) and causes cell death by apoptosis (Figures 1–3; McCabe & Orrenius, 1993; Kulling & Metzler, 1997; Salti *et al*., 2000). Importantly, because the two drugs bind to topoisomerase II at overlapping sites but exert differential effects on enzyme activity, the pro-apoptotic effects of genistein can be overcome by the presence of ciprofloxacin (Elsea *et al*., 1997).

Since, in the present study, ciprofloxacin dramatically improved the viability of RINm5F cells treated with 100 μM genistein (Figure 5) and prevented the appearance of oligonucleosomal DNA fragmentation (Figure 2) this suggests very strongly, that the direct cytotoxic actions of genistein were mediated by interaction with topoisomerase II. Furthermore, the increase in cell death following exposure of RINm5F cells to tyrphostin A25 could be accounted for by a similar mechanism since tyrphostins have also been reported to inhibit topoisomerase II (Markovits *et al*., 1994). In support of this, it was observed that a more specific topoisomerase II inhibitor, etoposide, which does not inhibit tyrosine kinases, caused apoptosis of RINm5F cells. The observation that diadzein failed to reproduce the cytotoxic effects of genistein is also consistent with the involvement of topoisomerase II since, unlike genistein, diadzein does not interact with this enzyme to cause DNA strand breaks in mammalian cells (Kulling & Metzler, 1997).

By contrast with genistein, neither etoposide nor ciprofloxacin altered the extent of RINm5F cell death in cells exposed to NaF. This suggests that the ability of low concentrations of genistein to block the increase in cell death caused by NaF does not derive from inhibition of topoisomerase II but results from inhibition of a tyrosine kinase. This is supported by the finding that a low concentration of tyrphostin A25 also reduced the level of cell death in cells treated with NaF. Thus, whereas high concentrations of genistein and tyrphostin may exert effects on cell viability that are independent of tyrosine kinases, the effects of lower concentrations are likely to be due to inhibition of these enzymes. This conclusion is consistent with the findings of others that high μM concentrations of genistein are required for the cytotoxic (Salti *et al*., 2000) and genotoxic effects mediated by inhibition of topoisomerase II (Boos & Stopper, 2000) whereas lower concentrations can inhibit tyrosine kinases (Akiyama *et al*., 1987). Even under these latter conditions, however, neither genistein nor tyrphostin A25 completely blocked the ability of NaF to promote cell death. Thus, it is likely that mechanisms in addition to tyrosine kinase activation also account for part of the response to NaF.

One further feature of the effects of genistein and NaF that merits comment was the observation that, when used in combination, the overall level of cell death was reduced below that seen with either agent alone (Figures 1 and 3). This is unlikely to have resulted from a direct chemical interaction between the two agents but suggests the existence of a mutually antagonistic response within the cells. The molecular basis of this effect has not been disclosed but it is noteworthy that a similar mutual antagonism of cell death has been observed in macrophages treated with lipopolysaccharide and genistein (Sadowska-Krowicka *et al*., 1998).

In a variety of cells, including β-cells, an important signal that promotes cell survival is derived from the activity of the insulin-like growth factor-1 (IGF-1) receptor tyrosine kinase (Mabley *et al*., 1997; Harrison *et al*., 1998; Grimberg & Cohen, 2000). Since the IGF-1 receptor kinase is known to be inhibited by genistein in RINm5F cells (Zhang *et al*., 1998) this could account for the increase in apoptosis seen in response to the drug. Signals arising from the IGF-1 receptor in RINm5F cells are propagated *via* the substrate protein IRS-2 which, in turn, recruits and activates phosphatidylinositol-3-kinase (PI-3-K; Zhang *et al*., 1998). Thus, if this signalling pathway is important for β-cell survival, then blockade of PI-3-K might also be expected to lead to RINm5F cell death. However, by contrast with the effects of genistein, treatment of RINm5F cells with the selective PI-3-K inhibitor wortmannin, failed to decrease cell viability and did not inhibit the response to NaF (Figure 1).

Effects of low concentrations of genistein on cell death in RINm5F cells

Since 25 μM genistein did not, itself, cause a marked increase in RINm5F cell death but significantly lowered the response

to NaF (consistent with inhibition of tyrosine kinase activity) this concentration was chosen for use in experiments with Ptx.

In confirmation of previous work (Loweth *et al*., 1996) culture of RINm5F cells in the presence of Ptx did not cause any direct loss of viability whereas Ptx treatment promoted a significant increase in death when cells were exposed to NaF. This result confirms that RINm5F cells express a Ptx-sensitive pathway involved in the maintenance of cell viability. Strikingly, incubation of RINm5F cells with a low concentration of genistein (25 μM) completely blocked the ability of Ptx to promote cell death (Figure 7) providing strong evidence that activation of a tyrosine kinase is involved in the response to Ptx. In support of this, it was observed that this effect of genistein was unaltered by ciprofloxacin, confirming that it did not result from the interaction of the drug with topoisomerase II (Figure 9). Moreover, another tyrosine kinase inhibitor, herbimycin A, which does not inhibit topoisomerase II (Azuma *et al*., 1995) also abrogated the Ptx-induced increase in cell death observed in cells exposed to NaF (Figure 8).

Taken together, these results strongly implicate the involvement of a genistein-sensitive tyrosine kinase enzyme in the enhancement of cell death mediated by Ptx in RINm5F cells. Since Ptx did not increase cell death in unstimulated cells, it may be concluded that this kinase does not play a critical role in the mechanism(s) that promotes cell survival under basal conditions. However, the results suggest that control of this enzyme becomes important when cells are exposed to apoptotic stimuli. In support of this, we have observed that the pro-apoptotic effects of both interleukin-1β and chemical nitric oxide donors are also markedly enhanced in RINm5F cells treated with Ptx (manuscript in preparation). Thus, the effects of Ptx are not restricted to cells exposed to NaF but are also seen with other inducers of apoptosis.

Overall, therefore, we conclude that RINm5F β-cells express a Ptx-sensitive pathway that is anti-apoptotic. The activity of this pathway is most evident in cells exposed to pro-apoptotic stimuli where it acts to restrain the loss of cell viability. The presence of Ptx releases this restraint, with the result that there is a marked increase in the pro-apoptotic stimulus and in cell death. Blockade of tyrosine kinase activity counteracts the response to Ptx, suggesting that the pro-apoptotic pathway may involve the activation of a tyrosine kinase and that this mechanism is subject to regulation by a Ptx-sensitive G-protein. On this basis, we propose that regulation of tyrosine phosphorylation by a Ptx-sensitive G-protein may be crucial to the maintenance of β-cell viability. The present data do not, however, provide support for the view that a genistein-sensitive tyrosine kinase is involved in RINm5F cell survival during culture under basal conditions.

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