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Involvement of the cGMP signalling pathway in the regulation of viability in insulin-secreting BRIN-BD11 cells

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Abstract We have evaluated the hypothesis that cGMP may serve as an intracellular messenger regulating the viability of pancreatic β-cells. A direct activator of soluble guanylyl cyclase, YC-1, caused a time- and dose-dependent loss of viability in clonal BRIN-BD11 β-cells. This was accompanied by a rise in cGMP and was antagonised by Rp-8-pCPT-cGMPS, a selective inhibitor of protein kinase G (PKG). Reverse transcription polymerase chain reaction analysis confirmed that BRIN-BD11 cells (and human islets) express all three known isoforms of PKG (PKG-Iα, -Iβ and II). Cell death induced by YC-1 was not sensitive to cell-permeable caspase inhibitors and was not accompanied by oligonucleosomal DNA fragmentation. The response was, however, inhibited by actinomycin D, suggesting that a transcription-dependent pathway of programmed cell death is involved in the actions of cGMP.

Key words: Diabetes; Apoptosis; cGMP-dependent kinase; Islets of Langerhans; YC-1; Guanylyl cyclase

1. Introduction

The viability of pancreatic β-cells is regulated by a variety of hormones, growth factors, metabolites and cytokines [1–3]. Some of these (e.g. IGF-1 [4] and mono-unsaturated fatty acids [5,6]) actively protect β-cells from cytotoxicity, while others (e.g. interleukin-Iβ and interferon-γ [1,2,7], saturated fatty acids [5,6,8], Fas [7,9], hyperglycaemia [10,11]) directly promote the loss of viability. Thus, under (patho)physiological conditions, the long-term fate of the β-cells is determined by the relative balance of these various factors and by the activation state of the signalling systems they regulate.

Considerable effort has been invested in understanding the signalling pathways controlling β-cell viability and this work has revealed that transcriptional regulation of multiple target genes underlies the pro-apoptotic effects of cytotoxic agents [12–16]. However, the 'upstream' signalling events that control β-cell apoptosis have proved more difficult to define. In this context, we previously reported that prolonged elevation of cGMP is associated with entry of both rat [17] and human [7] islet cells into apoptosis and proposed that cGMP may be a hitherto unrecognised intracellular messenger involved in the control of β-cell viability. This hypothesis is consistent with data obtained in cardiomyocytes [18–21], retinal photoreceptors [22,23], lung [24], neutrophils [25] and arterial smooth muscle [26] and was supported by the findings of others [27,28] in β-cells. However, it was also reported that elevation of cGMP can result in a mode of β-cell death that does not always display the classical hallmarks of apoptosis [27].

In the majority of earlier work, either chemical NO donors or cytokines acting to increase the expression of inducible NO synthase were used to elevate cGMP in islet cells [17,27,28]. This is because a rise in NO leads to a secondary increase in cGMP by promoting the activation of a soluble isoform of guanylyl cyclase [29,30]. Such studies confirmed that elevation of cGMP might be involved in mediating some of the cytotoxic effects of NO donors and cytokines, and this conclusion was supported by the observation that cell-permeable cGMP analogues can also reduce viability [7,17,27]. However, the results of experiments with cytokines or chemical NO donors can be difficult to interpret since NO is, itself, a highly reactive radical that can inhibit metabolic enzymes and damage cellular DNA, thereby leading to cell death by pathways that do not involve a rise in cGMP [31–33]. Moreover, treatment of cells with chemical NO donors can often lead to the production of other radical species (e.g. superoxide, peroxynitrite) which may contribute to the cytotoxic effects [34]. Thus, in order to substantiate the involvement of cGMP in control of β-cell viability, it is important that further studies are undertaken with agents that lack these additional effects.

In addition, a further important issue concerns the possible involvement of cGMP-dependent protein kinase (PKG) in mediating β-cell apoptosis. Earlier work implicated this enzyme in mediating the pro-apoptotic actions of cGMP but was based largely on the use of a purportedly selective inhibitor, KT5823 [17,27,28]. This reagent has been employed widely to study the activation of PKG in mammalian cells but recent work has questioned the specificity and mode of action of KT5823 [35,36]. Therefore, it is important that these conclusions are re-evaluated by the use of a more specific PKG inhibitor.

In the present work, we have undertaken studies designed to re-examine the involvement of cGMP and PKG in the regulation of β-cell apoptosis. To achieve this, 1-benzyl-3-(5′-hydroxymethyl-2′-furyl) indazole, YC-1 [37,38], has been employed to directly activate soluble guanylyl cyclase in a clonal pancreatic β-cell line, BRIN-BD11, without the need to generate intracellular NO. In addition, we have also investigated the expression of PKG isoforms in these cells and have

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employed a highly selective substrate analogue, Rp-8-pCPT-cGMPS, as an inhibitor of PKG. The aim was to establish whether activation of PKG underlies the pro-apoptotic actions of a rise in cGMP in β-cells.

2. Materials and methods

2.1. Materials

RPMI 1640, trypsin/EDTA, phosphate-buffered saline (PBS), penicillin and streptomycin were purchased from Invitrogen, UK. YC-1, annexin-V-Cy3 apoptosis detection kit and propidium iodide were from Sigma-Aldrich, UK. Rp-8-pCPT-cGMPS, Z-DEVD-fmk and Z-VAD-fmk were purchased from Calbiochem, UK and RNase (DNase-free) was from Roche, UK. cGMP Direct enzyme-linked immunosorbent assay (ELISA) kit was purchased from Amersham Biosciences, UK. TACS DNA Laddering kit was purchased from R&D Systems.

2.2. Growth and incubation of BRIN-BD11 cells

BRIN-BD11 β-cells [39] were seeded at a concentration of 10^5 cells per well, in 6-well cell culture plates containing RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, penicillin (400 IU/ml) and streptomycin sulphate (200 μg/ml). The cells were cultured at 37°C (5% CO_2) in the absence or presence of test reagents. Stock solutions of all the reagents were prepared in dimethyl sulphoxide (DMSO) and the solvent was added to all control cultures.

2.3. Analysis of BRIN cell death

Cell death was analysed using light microscopy and flow cytometry. Following incubation with test reagents, the culture medium containing detached cells was harvested. Adherent cells were incubated with trypsin/EDTA (0.5 ml per well) for 5 min at 37°C and 2 ml of complete culture medium was then added. The cells were collected by centrifugation (300×*g*; 5 min) and the pellet resuspended in 150 μl of culture medium. 150 μl of 0.2% trypan blue (in PBS) was added and the number of dead cells counted using a haemocytometer. Experiments were performed in duplicate and repeated on at least three occasions.

For analysis of DNA fragmentation by flow cytometry, cells were harvested as above and resuspended in 200 μl of PBS. The cells were then fixed with ice-cold ethanol:PBS (70:30 v:v) for 30 min on ice. After fixation, the cells were centrifuged for 5 min at 300×*g* and resuspended in 820 μl of PBS. 80 μl of propidium iodide (0.5 mg/ml) and 5 μl of DNase-free ribonuclease were then added and incubation continued for 30 min at 37°C. After this time, cellular DNA was analysed using a flow cytometer (FACScan, Becton Dickinson).

Flow cytometric analysis of β-cell apoptosis was performed by annexin-V-Cy3 staining. Following incubation with test reagents, cells were harvested and washed twice with PBS before resuspension at 10^6 cells/ml. 500 μl of this suspension was removed and 5 μl of annexin-V-Cy3 added. The cells were incubated for 10 min at room temperature and then analysed by flow cytometry.

2.4. DNA laddering

For the detection of DNA laddering, a TACS DNA Laddering kit (R&D Systems) was used according to the manufacturer's protocol. DNA was isolated from both adherent and non-adherent cells after incubation with test reagents and was electrophoresed on 1.5% agarose gels. Gels were post-stained for 15 min in 0.5 mg/ml ethidium bromide and visualised using a UV transilluminator.

2.5. Measurement of cGMP levels

BRIN cell cGMP levels were measured using a cGMP ELISA kit (Amersham Biosciences, UK) according to the manufacturer's instructions. Cells were cultured in 96-well plates then lysed by addition of 5% dodecyltrimethylammonium bromide. Samples were stored at −20°C prior to measurement of cGMP by reference to appropriate standards run in parallel.

2.6. RT-PCR analysis of PKG expression in BRIN-BD11 cells

Total RNA was extracted from BRIN cells with Trizol reagent and was amplified by reverse transcription polymerase chain reaction (RT-PCR) in a single tube reaction (Abgene, UK) using primers designed against sequences of the human PKG isoforms. The primer sequences were: PKG-Iα: F-tgccaagattctcatgctca, R-ggaaggacctgtacgtctgc; PKG-Iβ: F-caccttgcgggatttacagt, R-gatcaccgagcggtacttgt, PKG II: F-aagcacccagatggacactc, R-tggatgcacttgttctggag. Cycling conditions (30 cycles) were 94°C for 60 s, 56°C for 60 s, 72°C for 60 s. Products were separated on agarose gels then extracted and subcloned into Topo-2 vectors (Promega, UK) for DNA sequencing.

2.7. Statistical analysis

Statistical analysis was performed by Student's *t*-test or by analysis of variance, and results were considered significant when $P < 0.05$.

3. Results

3.1. Effects of YC-1 on the viability of BRIN-BD11 cells

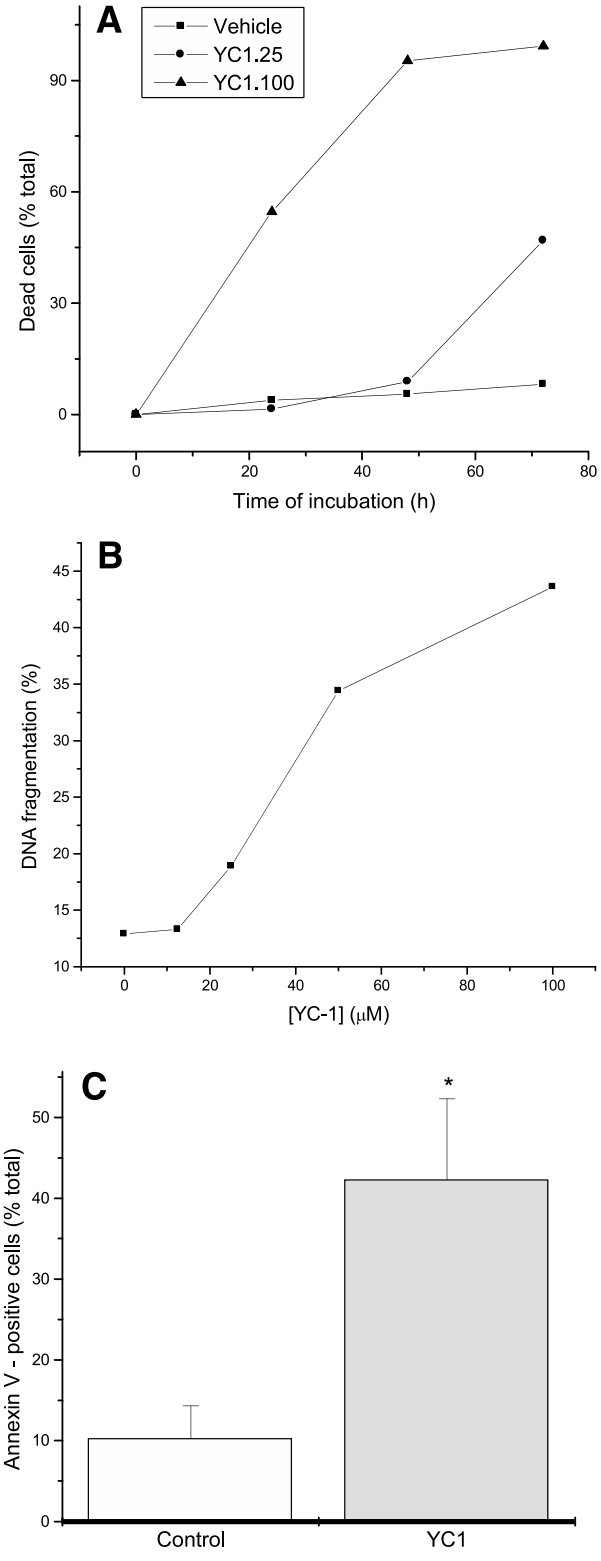
Initially, it was important to establish whether exposure of BRIN-BD11 (BRIN) cells to the guanylyl cyclase activator, YC-1, resulted in loss of viability. Cells were treated with either YC-1 (25 and 100 μM) or vehicle (DMSO) for up to 72 h and the extent of cell death monitored by vital dye staining (Fig. 1A). Control cells remained viable throughout the experiment whereas cells exposed to YC-1 progressively lost viability. This effect was most pronounced with the higher concentration of YC-1 where exposure for as little as 24 h resulted in the death of more than 50% of the cells. A similar loss of viability was also achieved with 25 μM YC-1, but this response required a longer period of incubation (72 h). Examination of the dose–response curve for induction of cell death (Fig. 1B) revealed an EC_{50} of approximately 40 μM for YC-1. Confirmation that the mode of cell death involved induction of apoptosis was provided by an examination of the surface labeling of phosphatidylserine with annexin-V (Fig. 1C). Cells incubated under control conditions were largely negative for annexin-V, whereas exposure to YC-1 for 24 h resulted in a large increase in annexin-V-positive cells, consistent with the entry of these cells into the apoptotic pathway.

3.2. Involvement of cGMP and the PKG pathway in mediating the effects of YC-1 in BRIN-BD11 cells

In order to confirm that YC-1 induces a rise in cGMP in BRIN cells, direct measurements were made of cGMP levels following exposure of cells to increasing concentrations of the compound (Fig. 2). A detectable rise in cGMP was elicited by as little as 6 μM YC-1 and the level increased progressively as the concentration was raised, such that, at 100 μM, cGMP was increased more than eight-fold above control.

There is currently no information on the expression of PKG isoforms in β-cells and we considered it important to establish this before attempting to assess the involvement of PKG in mediating the pro-apoptotic effects of YC-1. Accordingly, RNA was extracted and amplified by RT-PCR to investigate the expression of the three known isoforms of PKG, Iα, Iβ and II. As the full sequences of all three enzymes were not available for rat, we used primers directed to regions of the appropriate human sequence. These amplified products of the correct size for all three isoforms in BRIN cells (Fig. 3A) and direct sequence analysis confirmed that these were more than 90% identical with the equivalent human PKG sequence, in each case. Thus, we conclude that BRIN-BD11 cells express transcripts for all three isoforms of PKG. In order to confirm that this reflects the situation in primary islet cells, RNA was also extracted from two separate batches of isolated human islets and amplified by RT-PCR. Again, all three PKG isoforms were successfully amplified (not presented).

Having established that BRIN cells express PKG, the effects of a highly selective PKG inhibitor (Rp-8-pCPT-cGMPS [40]) on YC-1-induced cell death, were investigated (Fig. 3B). Rp-8-pCPT-cGMPS significantly attenuated the loss of viability caused by YC-1, which was reduced by more than 50% in the presence of the inhibitor (Fig. 3B).



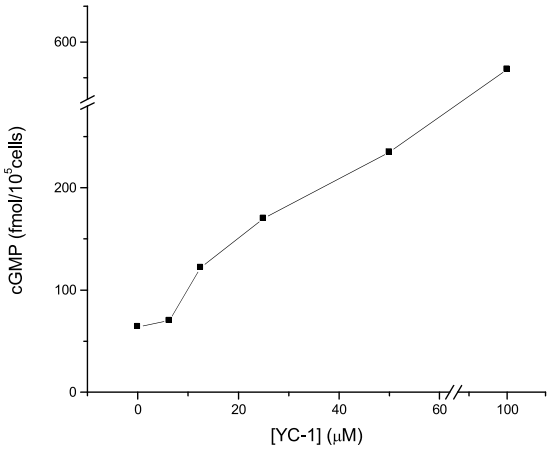


Fig. 2. Effect of YC-1 on cGMP levels in BRIN cells. BRIN cells were treated with increasing concentrations of YC-1 for 6 h then extracted for measurement of cGMP by ELISA. Each point is the mean of duplicate cell incubations. The experiment was repeated twice with similar results.

3.3. Mode of cell death induced by YC-1 in BRIN-BD11 cells

Measurement of annexin-V staining revealed that treatment of BRIN cells with YC-1 was associated with phosphatidylserine translocation (Fig. 1C) consistent with induction of apoptosis. However, pre-incubation of the cells with the cell-permeable caspase 3 inhibitor, Z-DEVD-fmk, failed to improve the viability of cells exposed to YC-1 (Fig. 3C). Similar results were also obtained when a lower concentration of YC-1 was used (25 μM) or when a different caspase inhibitor having less restricted specificity (Z-VAD-fmk) was employed (not shown).

In view of these findings, the effects of YC-1 on DNA fragmentation in BRIN cells were studied by flow cytometry and gel electrophoresis. Flow cytometric analysis confirmed that cells exposed to YC-1 contained fragmented DNA. In four separate experiments, the sub-diploid DNA peak was increased from a mean of $9.7 \pm 1.6\%$ in control cells to $33.5 \pm 3.3\%$ after 18 h exposure to 100 μM YC-1 ($P < 0.001$). Despite this, analysis of DNA fragmentation in YC-1-treated cells by gel electrophoresis failed to reveal evidence of the pattern of oligonucleosomal DNA fragmentation characteristic of caspase-dependent apoptosis (Fig. 4). For comparison, cells were also exposed to 5 mM NaF and, as expected from previous studies [41], this did result in oligonucleosomal DNA fragmentation (Fig. 4).

To explore further the implications of these phenomena,

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Fig. 1. Induction of cell death in BRIN-BD11 cells by YC-1. A: BRIN cells were cultured in the absence (squares) or presence of either 25 μM YC-1 (circles) or 100 μM YC-1 (triangles) for the time periods shown. After incubation, the cells were harvested and stained with trypan blue to monitor viability. Each point represents the mean of duplicate determinations from a single experiment that was repeated twice with similar results. B: BRIN cells were exposed to increasing concentrations of YC-1 for 48 h then analysed by flow cytometry. Each point is the mean of duplicate determinations from a single experiment that was repeated twice with similar results. C: BRIN cells were treated with 100 μM YC-1 overnight then stained with annexin-V-Cy3 and analysed by flow cytometry. Results represent mean values (±S.E.M.) from four separate experiments. *$P < 0.001$ relative to control cells.

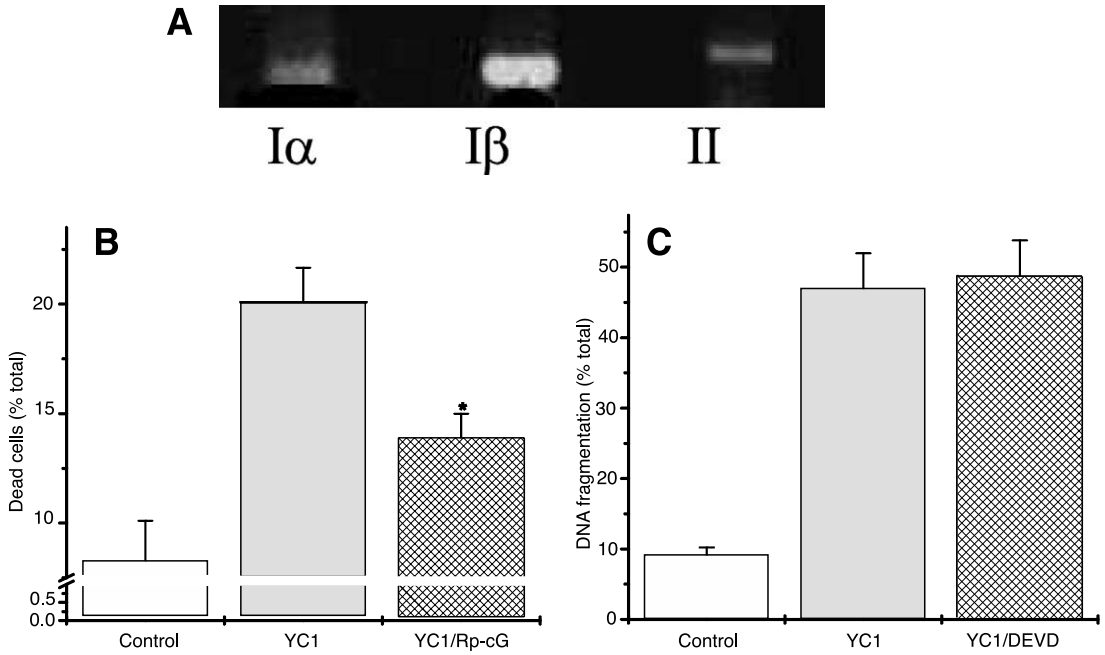

Fig. 3. Involvement of PKG and caspases in YC-1-induced cell death. A: PKG isoform expression in BRIN cells was determined by RT-PCR after extraction of total RNA. Amplified bands corresponding to PKG-Iα, -Iβ and II were separated on agarose gels and visualised with ethidium bromide under UV illumination. The identity of each band was confirmed by DNA sequencing. B: BRIN cells were incubated under control conditions or with 25 μM YC-I in the absence (grey bar) or presence (hatched bar) of 0.5 μM Rp-8-pCTP-cGMPS. 24 h later the cells were harvested and stained with trypan blue to monitor viability. Each point is the mean (±S.E.M.) from four separate experiments. C: BRIN cells were incubated under control conditions or with 100 μM YC-1 in the absence (grey bar) or presence (hatched bar) of 50 μM Z-DEVD-fmk. Cells were treated with the caspase inhibitor 2 h prior to addition of YC-1. Following incubation for a further 24 h, cells were harvested and analysed by flow cytometry. Each point is the mean (±S.E.M.) from three separate experiments. *$P < 0.01$ relative to YC-1 alone.

additional experiments were performed in which BRIN cells were treated with the protein synthesis inhibitor, actinomycin D. This agent promotes the loss of viability in many cell types [42–44] but, despite this, it has also been reported to attenuate the cytotoxic effects of compounds acting via 'atypical' pathways of programmed cell death [45].

Treatment of BRIN-BD11 cells with actinomycin D resulted in a dose-dependent reduction in viability at concentrations up to 1 μM (Fig. 5). Vital dye staining revealed that this response was sensitive to inhibition by Z-DEVD-fmk (0.5 μg/ml actinomycin-23.0 ± 1.7% dead cells; actinomycin+10 μM Z-DEVD-fmk-16.8 ± 0.8%; $P < 0.01$). Strikingly, it was observed that simultaneous exposure of BRIN cells to both actinomycin D and YC-1 was associated with a mutually antagonistic inhibition of cell death (Fig. 5). Thus, when the drugs were used in combination, the viability of the cells was maintained at much higher levels than when either compound was applied alone.

4. Discussion

Considerable progress has been made in defining the 'downstream' effectors involved in regulating β-cell apoptosis, whereas the more proximal, 'upstream', signalling events are much less well characterised. However, these are likely to be critically important to β-cell viability and an understanding of their regulation may offer the opportunity for therapeutic intervention to minimise β-cell loss in diabetes.

In the present work we have evaluated the previous suggestion [17,27,28] that changes in cGMP may be important for determining the viability of pancreatic β-cells. This conclusion was originally derived from studies performed with chemical NO donors which promote an increase in cGMP indirectly, by raising the concentration of intracellular NO [17,27]. Some of this NO then interacts with the haem group of soluble guanylyl cyclase, leading to activation of the enzyme and a rise in cGMP [27,28]. However, although NO donors are effective in raising cGMP [46], the time course of the response can be very short-lived and the interpretation of results is complicated by the fact that NO can exert a range of other detrimental effects that are independent of cGMP [31–33]. Moreover, in one report, it was suggested that a rise in cGMP (which was assumed to derive from exposure of islets to carbon monoxide) might be protective to β-cells [47]. Therefore, in the current study, we have exploited the potential of a structurally different compound, YC-1, that binds directly to guanylyl cyclase and elevates cGMP without requiring the intermediacy of a rise in NO [37,38].

Treatment of BRIN-BD11 cells with YC-1 caused a dose-dependent increase in cGMP (Fig. 2) confirming that the soluble form of guanylyl cyclase expressed in BRIN cells is sensitive to this reagent. In addition, YC-1 also caused a time- and dose-dependent loss of BRIN cell viability, which is consistent with the proposition that cGMP may play a role in regulating the viability of these cells independently of NO formation. It should be noted, however, that YC-1 may also cause cell death by cGMP-independent mechanisms in some cell types [48]. Firm evidence that this was not the case in the present work was provided by the results of experiments with Rp-8-pCPT-cGMPS. This compound is a highly selective inhibitor of PKG [40] and the finding that a low concentration (0.5 μM) markedly attenuated the response to YC-1 is consistent

with the conclusion that at least part of the induction of cell death was secondary to activation of PKG by cGMP.

In drawing this conclusion it is important to note that the expression of PKG has been investigated to only a very limited extent in β-cells and that, although PKG activity has been measured [27], there are no data on the pattern of isoform expression in these cells. Accordingly, we examined PKG expression by RT-PCR and observed that all three known isoforms, PKG-Iα, -Iβ and -II, are present. Similar results were obtained both in clonal BRIN cells and in primary human islets, suggesting that all three isoforms are likely to be expressed in the β-cells in vivo. The respective roles of these enzymes remain to be established but it seems probable that the signals arising from a rise in cGMP will be transduced differentially according to the subcellular localisation and substrate availability of each isoform. Nevertheless, the results suggest that at least one isoform is involved in controlling β-cell viability.

The proposal that PKG activation may mediate the effects of cGMP on β-cell viability was originally advanced on the basis of data obtained with the inhibitor, KT5823 [17,27,28]. However, the efficacy and specificity of this compound have recently been questioned [35] and it has even been suggested that its functional activity might be attributable to the presence of an unidentified contaminant [36]. Thus, the present results employing a more potent and highly selective substrate analogue as an inhibitor of PKG provide important confirmation that the enzyme is involved in mediating the pro-apoptotic effects of cGMP in β-cells.

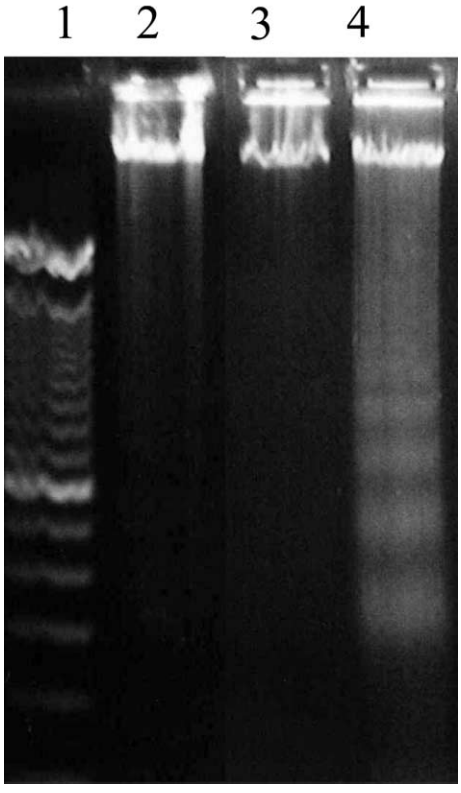

Fig. 4. Gel electrophoresis of DNA extracted from BRIN cells. BRIN cells were incubated under control conditions (lane 2) or in the presence of either 100 μM YC-1 (lane 3) or 5 mM NaF (lane 4) for 24 h. DNA was then extracted from the cells, separated by electrophoresis on agarose gels and visualised by staining with ethidium bromide. Marker DNA was run in parallel in lane 1.

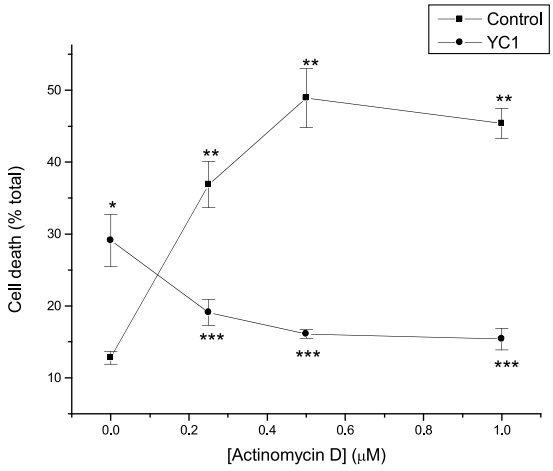

Fig. 5. Effects of actinomycin D on YC-I-induced cell death in BRIN cells. BRIN cells were incubated in the absence (squares) or presence (circles) of 100 μM YC-1 and increasing concentrations of actinomycin D, as shown. Following incubation for 24 h, cells were harvested and their viability monitored. Each point represents the mean ± S.E.M. from triplicate experiments. $*P < 0.001$ relative to control cells incubated in the absence of YC-1. $**P < 0.001$ relative to control cells incubated in the absence of actinomycin D. $***P < 0.01$ relative to cells incubated with YC-1 or actinomycin D alone.

Analysis of YC-1-treated BRIN cells by annexin-V staining revealed that the compound promotes a marked increase in the number of positively stained cells. Annexin-V labels phosphatidylserine, a lipid that is normally localised within the inner leaflet of the plasma membrane but which becomes translocated across the bilayer during the early stages of apoptosis [49]. Thus, these results imply that YC-1 elevates cGMP and thereby leads to the activation of PKG and the entry of BRIN cells into apoptosis. The time course and dose–response data (Figs. 1 and 2) suggest that a modest, acute elevation of cGMP may not be sufficient to elicit this response but that a more sustained elevation is required. Thus, it is probable that both temporal (time course of cGMP elevation) and spatial (subcellular distribution of PKG and its substrates) effects are important in determining the outcome of PKG activation. Indeed, it is even conceivable that the effects of cGMP may be differentially anti- [47] or pro-apoptotic [17] according to the time course and magnitude of the response.

Despite the evidence that YC-1 causes BRIN cells to enter apoptosis, the response was not sensitive to blockade by either a selective inhibitor of the effector caspase, caspase 3 (Z-DEVD), nor by the pan-caspase inhibitor, Z-VAD. Thus, it appears that PKG-mediated cell death may occur by a pathway that does not require caspase activation, in BRIN cells. In support of this, it was also discovered that the induction of cell death by YC-1 occurred without any increase in oligonucleosomal DNA fragmentation (Fig. 4), a process that normally accompanies caspase-dependent activation of apoptosis [45]. We also examined caspase activation using fluorescent substrates in BRIN cells treated with YC-1 and, again, observed no marked increase in response to the agent (A. Kaminski and N.G. Morgan, unpublished).

Taken together, these results imply that PKG activation mediates a pathway of β-cell death that occurs independently

of caspase activation but which leads to phosphatidylserine translocation and loss of high molecular weight DNA (as revealed by flow cytometry). Thus, these findings raise the possibility that YC-1 (and, hence, cGMP and PKG activation) elicits a mode of programmed cell death that differs from 'classical' apoptosis in β-cells [45]. As such, the results are in accord with the studies of Tejedo et al. [27] who reported that cGMP-induced cell death in RINm5F β-cells is not mediated by a typical apoptotic mechanism even though it is accompanied by certain features that accompany programmed cell death.

This proposition is supported most clearly by the outcome of experiments performed with actinomycin D. This reagent is unusual in that it has the ability to induce caspase activation and cell death in some cells (including BRIN cells, as shown here) while also inhibiting certain alternative, transcription-dependent, pathways of programmed cell death [45]. Strikingly, despite causing extensive cell death itself, actinomycin D dramatically attenuated the extent of cell death in cells exposed to YC-1 (Fig. 5). Thus, the two drugs exerted mutually antagonistic effects, consistent with the induction of a pathway of programmed cell death in each case.

Further studies will be required to understand the molecular mechanisms involved in the pathway of programmed cell death induced by cGMP and PKG, but there is evidence that down-regulation of Bcl-2 expression occurs as a consequence of PKG activation in β-cells [27]. Whether this is a primary event mediating the loss of cell viability or whether it follows as a secondary consequence of more fundamental changes remains to be established. Nevertheless, the present findings are consistent with the proposal that sustained PKG activation, mediated by a rise in cGMP, provides a signal to activate programmed cell death in β-cells.

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