Role of Ca^{2+} in apoptosis evoked by human amylin in pancreatic islet β-cells

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The objective of these studies was to clarify the role of Ca^{2+} in the mechanism of death evoked by human amylin (hA) in islet β-cells. hA forms fibrils *in vitro* and islet amyloid *in vivo*. Here we show that pure synthetic hA aggregated in solution, formed fibrils and evoked death in cultured RINm5F islet β-cells in a time-dependent (0–24 h) and concentration-dependent (0–20 μM) manner. Dying cells underwent shrinkage of the nucleus, with clumping and segregation of chromatin into masses that lay against the nuclear envelope, and internucleosomal DNA fragmentation. These cells therefore show many features of apoptosis, although aspects of the morphology might be characteristic of this particular cell type rather than of a general apoptotic nature. Aurintricarboxylic acid, an inhibitor of both Ca^{2+}-dependent and Ca^{2+}-independent nucleases, suppressed this DNA fragmentation and inhibited apoptosis at concentrations between 25 and 200 μM. Direct measurements of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2 acetoxymethyl ester (AM)-loaded β-cells showed that neither hA nor its non-cytotoxic homologue, rat amylin were effective in raising $[Ca^{2+}]_i$. Modulators of Ca^{2+} regulation were tested for their effects on hA-induced β-cell apoptosis. Ca^{2+} ionophore (A23187) and thapsigargin (an inhibitor of endoplasmic reticular Ca^{2+}-ATPase activity) by themselves evoked apoptosis accompanied by increased $[Ca^{2+}]_i$. Neither the Ca^{2+} channel blocker verapamil, the extracellular Ca^{2+} chelator EGTA nor the cytosolic Ca^{2+} buffer bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid ('BAPTA')/AM protected β-cells from hA-evoked apoptosis. Prolonged incubation of β-cells with a lethal dose of hA altered neither the basal $[Ca^{2+}]_i$ nor the thapsigargin-induced release of Ca^{2+} from intracellular stores. Furthermore, $^{45}CaCl_2$ uptake by RINm5F cells did not differ in the presence or absence of hA. These results suggest that, whereas alterations in cytosolic Ca^{2+} homoeostasis do have a significant role in certain forms of β-cell death, they do not contribute to the pathway of apoptosis evoked by hA in islet β-cells.

Key words: fibril, islet amyloid, Type 2 diabetes mellitus.

INTRODUCTION

Amylin [1], also known as islet amyloid polypeptide [2], is a 37-residue peptide usually secreted together with insulin from pancreatic islet β-cells [3]. Since its discovery in diabetes-associated islet amyloid [4], human amylin (hA) has been implicated in the regulation of carbohydrate metabolism [3,5], tissue insulin sensitivity [6], Ca^{2+} and bone metabolism [7] and other cellular processes [3].

The deposition of amyloid formed by hA and a progressive decline in β-cell numbers are characteristics of non-insulin-dependent diabetes mellitus (NIDDM) [8,9]. Recently, transgenic mice overexpressing hA in their islet β-cells have been found to develop islet amyloid, β-cell depletion and NIDDM [10,11]. Furthermore, fibril-forming amylin evokes apoptosis in cultured primary islet β-cells [12]. However, the pathway by which hA elicits apoptosis is currently unknown. Accumulating evidence suggests that altered regulation of intracellular $[Ca^{2+}]$ and the generation of reactive oxygen species (ROS) could be significant in the cytotoxicity evoked by different fibril-forming peptides, including hA, the Alzheimer disease-associated peptide (βA) and β_2-microglobulin [13–16].

It is well established that both Ca^{2+} [17] and oxidative stress [18] are important mediators of apoptosis. Significant elevations in resting cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the production of ROS have been detected in apoptotic neurons exposed to hA [13,14] and βA [14,19]. Cell death induced by βA can be attenuated by blockers of voltage-gated Ca^{2+} channels [20]. βA itself was reported to form Ca^{2+} channels in a bilayer membrane system *in vitro* [20], to impair membrane ion-mediated ATPase activity [19] and to evoke the generation of free radicals [20]. Similarly, specific Ca^{2+} channel blockers can abrogate apoptosis in pancreatic β-cells treated with serum from patients with type I diabetes [21]. hA has also been reported to form ion-permeable channels in a lipid bilayer system [22]. Furthermore, the induction of increased $[Ca^{2+}]_i$ [14,19] and the cytotoxicity of either βA or hA [14,15,19] can be attenuated by antioxidants in some systems.

That $[Ca^{2+}]_i$ is central to the mechanism of apoptosis is supported by the observation that the oxidative disruption of $[Ca^{2+}]_i$ homoeostasis has frequently been implicated in models of apoptosis [17,23] and that diverse types of apoptosis can be inhibited by the oncoprotein bcl-2 [23]. bcl-2 can itself regulate intracellular $[Ca^{2+}]_i$ [17], acts as an inhibitor of oxidation and is located together with major intracellular Ca^{2+} transport and ROS generation sites, including mitochondrial, endoplasmic reticular and nuclear membranes [17,23].

Taken together, these observations suggest that altered $[Ca^{2+}]_i$ homoeostasis could be central to fibril-evoked apoptosis. However, contradictory findings also exist. For example, in one study

Abbreviations used: AM, acetoxymethyl ester; ATA, aurintricarboxylic acid; βA, Alzheimer disease-associated peptide; BAPTA, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; hA, human amylin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NIDDM, non-insulin-dependent diabetes mellitus; ROS, reactive oxygen species; Tg, thapsigargin; TEM, transmission electron microscopy.

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of βA-induced neural apoptosis, examination with fura-2 imaging failed to detect any significant changes in $[Ca^{2+}]_i$ during lethal exposure for 24 h [24]. Furthermore, inhibition of Ca^{2+} influx either by the channel blocker verapamil or by the Ca^{2+}-chelator EGTA failed to prevent apoptotic β-cell death induced by hA amyloid [12].

In the present study we examined the role of Ca^{2+} in hA-activated apoptosis in a continuous pancreatic islet β-cell line, RINm5F. Apoptosis was induced in RINm5F cells by synthetic hA. A number of well-defined Ca^{2+} modulators, such as ionophore A23187 [25], thapsigargin (Tg) (an endoplasmic-reticulum-specific Ca^{2+}-ATPase inhibitor) [26], verapamil (a Ca^{2+} channel blocker), EGTA (an extracellular Ca^{2+} chelator) and bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA)/acetoxymethyl ester (AM) (a cytosolic Ca^{2+} buffer) [27], were tested for their effects on cell viability during the development of hA-evoked cell death. The direct effect of hA on intracellular Ca^{2+} mobilization and uptake of extracellular Ca^{2+} were also determined, after either immediate administration or prolonged lethal exposure, with the fluorescent Ca^{2+} indicator fura-2/AM and ^{45}Ca uptake.

MATERIALS AND METHODS

Materials

Synthetic human (lot ZL934) and rat amylin (lot ZM275) were HPLC-purified products from Bachem California (Torrance, CA, U.S.A.). Peptides were freshly dissolved in sterile MilliQ water at a concentration of 500 μM, then diluted in medium before addition to cultures. Fura 2/AM, Tg, A23187, EGTA, verapamil, RPMI 1640 tissue culture medium and supplements, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St Louis, MO, U.S.A.). BAPTA/AM (B-1205) was purchased from Molecular Probes (Eugene, OR, U.S.A.). $^{45}CaCl_2$ was from ICN (cat. no. 62005; Irvine, CA, U.S.A.), and fetal calf serum was from Gibco-BRL-Life Technologies (Auckland, New Zealand). Tissue culture plasticware was supplied by Falcon (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). All other chemicals used were of analytical grade or better.

Cell culture

The insulin-secreting RINm5F cell line, which was derived from a rat islet cell tumour [28], was kindly provided by Dr. H. K. Oie (National Institutes of Health, Bethesda, MD, U.S.A.). Cells were cultured at 37 °C in air/CO_2 (19:1) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 290 μg/ml L-glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin, as described previously [29]. In brief, cells were maintained in T75 tissue culture flasks and the medium was changed every 3 days. After reaching confluence, cells were harvested by dissociation with 0.25% trypsin/0.02% EDTA, then replated at the required density. All experiments were performed with cells between passages 30 and 50.

Transmission electron microscopy (TEM) of amylin fibrils

Aqueous solutions of synthetic hA (25 μM) were prepared in deionized water. Aliquots of peptide solutions were loaded on glow-discharged carbon-coated collodion film on 400 mesh/inch copper grids (Probing and Structure, Queensland, Australia), then adsorbed and blotted. Grids were stained with a droplet of 5% (w/v) aqueous uranyl acetate (BDH, Lutterworth, Leics., U.K.), blotted and dried in air. Specimens were viewed and photographed with a Hitachi (Tokyo, Japan) H-8000 transmission electron microscope operated at 100 kV.

TEM of cultured β-cells

RINm5F cells (passage 35) were cultured to 80% confluence in 24-well plates (LINBRO®; Flow Laboratories), otherwise as above. Cell monolayers were washed with PBS; 390 μl of fresh medium was then added. Freeze-dried synthetic hA was dissolved in 10 μl of sterile water, preincubated for 1 h at room temperature and added to each well to a final concentration of 10 μM, then mixed by trituration and incubated for 22 h; control wells received equal quantities of water. Medium was then removed and cells were fixed with 3% (w/v) glutaraldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.2, for 3 h at room temperature. Cells were washed three times with 0.1 M Sorenson's buffer (10 min each), then post-fixed with 0.1% osmium tetroxide/0.1 M Sorenson's phosphate buffer (pH 7.2) for 1 h and serially dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90% and twice with 100%, all v/v). Cells were infiltrated with 812 epoxy resin/ethanol (1:1, v/v) for 1 h, then with 100% epoxy resin overnight; they were then embedded *in situ* with fresh 100% epoxy resin and cured for 48 h at 60 °C. Sections (70 nm) were cut with a Diatome M5624 diamond knife. Sections were picked up on acetone-cleaned 200-mesh copper grids, and stained with uranyl acetate for 15 min by floating the grid specimen-side down on a uranyl acetate drop placed on a strip of dental wax under moist conditions, followed by thorough washes with distilled water. Excess water was blotted from the the grid edge. Specimens were then stained with lead citrate for 3 min by flotation, as described for uranyl acetate, and washed with distilled water; excess water was then blotted and the specimens were dried in air. They were viewed with either Phillips 301 or Phillips CM-12 transmission electron microscopes.

^{125}I-hA precipitation assay *in vitro*

Tracer amounts of ^{125}I-hA (Peninsula Laboratories, Belmont, CA, U.S.A.) were incubated in the absence or presence of 10 μM hA for various durations. Incubation mixtures were then centrifuged (15000 ***g***, 20 min) and ^{125}I-hA in both supernatants and pellets was determined (1480 Wizard TM 3-inch Gamma Counter; Wallac Oy, Turku, Finland). Results are expressed as percentages of the radioactivity (c.p.m.) in supernatants relative to totals.

Cell viability assay

The reduction of MTT was used to assess cell viability, as described previously [30]. RINm5F cells, seeded at 8×10^5/ml, were cultured overnight in 96-well plates, treated with various agents as described, then incubated with 100 μl of MTT (final concentration 0.5 mg/ml) at 37 °C for 4 h. After the overnight addition of 100 μl of cell lysis buffer [20% (w/v) SDS/50% (v/v) *N*,*N*-dimethylformamide (pH 4.7)], colorimetric measurements were made at 562 nm with an ELISA plate reader (Spectramax 340; Molecular Devices, Sunnyvale, CA, U.S.A.). Results are expressed as percentages of MTT reduction (means ± S.E.M.) relative to culture medium controls (defined as 100%) after the removal of non-cell-derived background absorbances by subtracting relevant blank readings.

DNA fragmentation assay

Total genomic DNA was isolated from cell pellets as described [31], with minor modifications. In brief, cells were suspended in

lysis buffer [10 mM Tris/HCl (pH 8.0)/10 mM NaCl/10 mM EDTA/100 μg/ml proteinase K/1 % SDS], then incubated overnight at 55 °C. DNA was extracted with phenol/chloroform (1:1, v/v), precipitated on solid CO_2 with 0.3 M sodium acetate in ethanol, then washed with 70 % (v/v) aqueous ethanol. The DNA pellet was then resuspended in 10 mM Tris/HCl (pH 8.0)/1 mM EDTA and incubated for 2 h at 37 °C with 0.5 mg/ml DNase-free RNase (lot 1,119,915; Boehringer Mannheim, Mannheim, Germany). After re-extraction by the same method, DNA was analysed with 1.1 % (w/v) agarose gels and detected with ethidium bromide.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by a modified method with the Ca^{2+}-sensitive fluorescent indicator fura-2/AM [32]. RINm5F cells were dissociated with 0.25 % trypsin/5 mM EDTA in PBS and washed once with fresh culture medium. They were resuspended in culture medium containing 2 μM fura-2/AM at 10^6 cells/ml and incubated in the dark for 30 min at 37 °C. After centrifugation (100 ***g***, 10 min) and two washes with Hepes-buffered saline [120 mM NaCl/5 mM KCl/1 mM magnesium acetate/1 mM $CaCl_2$/20 mM Hepes/5.6 mM glucose (pH 7.4)], cells were resuspended in the same buffer at approx. 10^6 cells/ml for fluorescence measurement within the next hour. A Hitachi F-4500 spectrofluorometer was used to measure $[Ca^{2+}]_i$ with excitation at 340 nm and 380 nm (bandpass 5 nm) and emission at 510 nm (bandpass 20 nm). The intensity of fluorescence (F_{340} and F_{380}) at 510 nm was measured in cells suspended in a quartz cuvette (0.5 ml volume) with constant stirring at 37 °C. Ca^{2+} concentrations were calculated from the ratio of the fluorescence intensity ($R = F_{340}/F_{380}$) by the equation $[Ca^{2+}]_i = K_d\,[(R-R_{min})/(R_{max}-R)](F_{min}/F_{max})$. K_d, the dissociation constant for fura-2/Ca^{2+}, was taken as 224 [32]. F_{max} and F_{min} are values of F_{380} after treatment with 0.1 % (v/v) aqueous Triton X-100 and 10 mM EGTA respectively (maximum and minimum binding of Ca^{2+} with fura-2). R_{max} and R_{min} were obtained after treatment of cells with Triton X-100 and EGTA respectively.

Cytosolic Ca^{2+} buffering studies

A 2 mM stock solution of BAPTA/AM (B-1205; Molecular Probes) was prepared in DMSO. For use in experiments, this was diluted with culture medium and added to RINm5F cells in 96-well plates to give final concentrations of 0 (DMSO controls), 1, 5, 10, 20, 40, 50 and 100 μM. Cells were preincubated for 1 h, then incubated for a further 24 h in the presence or absence of 10 μM hA. Cell viability was determined by using MTT reduction.

^{45}Ca uptake assay

RINm5F cells, seeded in 12-well plates at 8×10^6 cells/ml, were cultured overnight then treated with test agent or vehicle in the presence of 2 μCi/ml $^{45}Ca^{2+}$, as described. At appropriate time points the medium was removed and cells were washed three times with PBS. Amounts of radioactivity in both the culture medium and cells after lysis with 0.1 % (v/v) Triton X-100 were determined with a scintillation counter (LS3801; Beckman, Fullerton, CA, U.S.A.). Results are expressed as percentages of radioactivity taken up by cells divided by total amounts.

Statistical analysis

Except for the representative traces for Ca^{2+} determination, all results are presented as means ± S.E.M. for at least three independent experiments. Statistical analysis was performed with STATISTICA (StatSoft, Tulsa, OK, U.S.A.). Comparisons were made by analysis of variance (ANOVA) with post hoc analysis by the Tukey test.

RESULTS

Fibril formation by hA

hA forms islet amyloid in more than 90 % of humans with NIDDM [3]. hA fibrils, which are found adjacent to β-cell surface membranes *in vivo* [4,11], were previously suggested to directly kill β-cells in primary culture by evoking apoptosis [12]. To test the validity of our *in vitro* cellular model of fibril formation and β-cell death evoked by hA, we initially verified that synthetic hA forms fibrils under the experimental conditions employed. As shown in Figure 1(A), TEM studies showed the

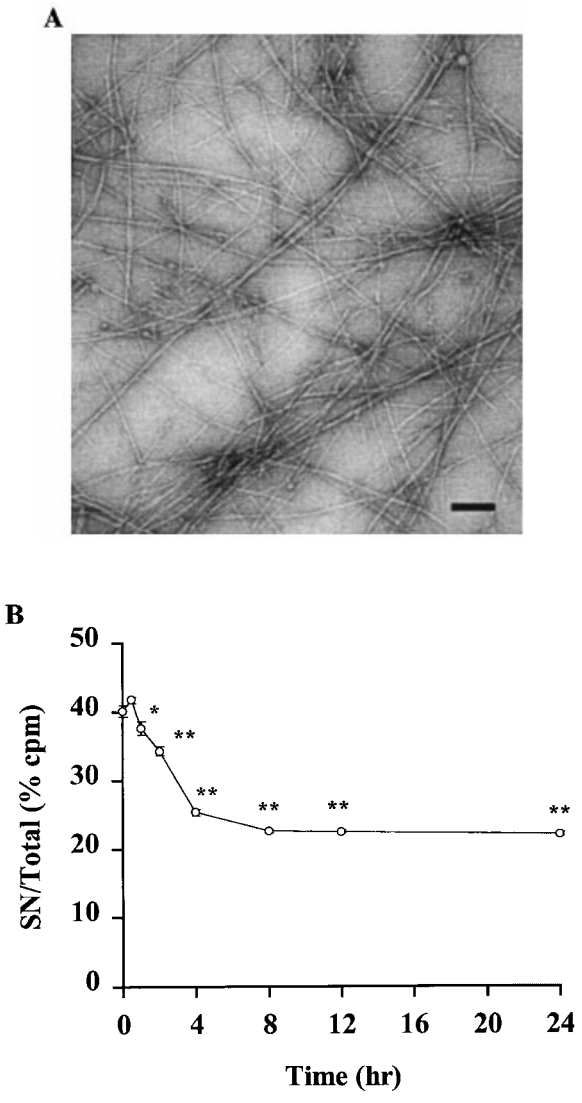

Figure 1 Fibril formation by hA

(**A**) TEM showing the amyloid fibrils spontaneously formed by 25 μM hA in aqueous solution. Scale bar, 100 nm. (**B**) Formation of hA fibrils by 10 μM hA at various time points monitored by the precipitation of tracer quantities of ^{125}I-hA. Results are expressed as percentages of the radioactivity (c.p.m.) in supernatants relative to the totals (SN/total) and are means ± S.E.M., $n = 6$. $^*P < 0.05$, $^{**}P < 0.001$ relative to zero-time controls.

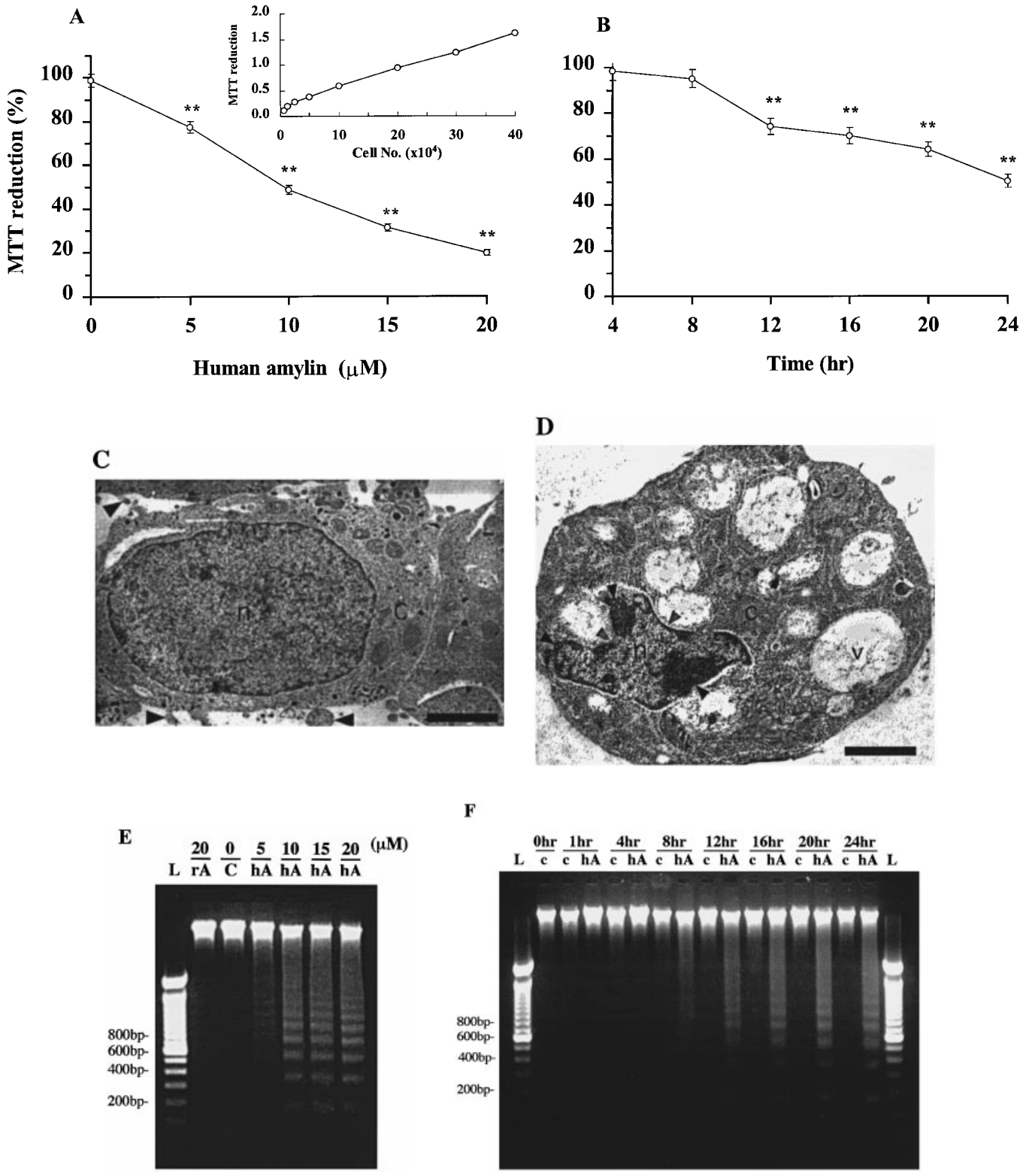

Figure 2 Induction of apoptosis by hA in RINm5F islet β-cells

(**A**, **B**) Cellular reduction of MTT showing concentration-dependent (**A**) and time-dependent (**B**) response of hA-mediated cytotoxicity in cultured islet β-cells [the hA concentration in (**B**) was 10 μM]. Assays were calibrated against cell counts by seeding cells in 96-well plates in a dilution series [inset to (**A**), plotted as A_{562} against cell numbers]. Values are percentages of corresponding controls at equivalent concentrations or time points (means $\pm$ S.E.M., $n = 18$ at each point). $^{**}P < 0.001$ relative to hA treatment controls. (**C**, **D**) Transmission electron micrographs of RINm5F islet β-cells treated for 22 h with vehicle control (water) (**C**) or 10 μM hA (**D**). Arrowheads in (**C**) point to microvilli; arrowheads in (**D**) point to compacted and segregated chromatin lying against the nuclear membrane. hA-treated cells also show loss of nuclear volume, convolution of the nuclear membrane and prominent cytoplasmic vacuole formation (n, nucleus; c, cytoplasm; v, vacuole; scale bars, 1 μm). Cells in (**C**) and (**D**) are representative. (**E**, **F**) Agarose-gel electrophoresis of islet β-cell DNA showing concentration-dependent (**E**) and time-dependent (**F**) internucleosomal DNA cleavage after treatment with hA. Lanes L, DNA size markers of 100 bp; lanes rA, rat amylin; lanes C, corresponding controls at different time points. Studies of concentration dependence were performed with 24 h incubations at stated concentrations. Studies of time dependence were performed at different time points, as indicated.

Table 1 Concentration-dependent effect of ATA on the viability of RINm5F islet β-cells in the presence or absence of 10 μM hA

Cells were pretreated for 1 h with the stated concentrations of ATA, then incubated in the presence or absence of the stated concentrations hA at 37 °C for 24 h, after which their viability was determined by MTT assay. Results are means ± S.E.M., $n = 18$ at each point. $^{**}P < 0.001$ relative to hA treatment controls.

	Viable cells (% MTT reduction)	
[ATA] (μM)	With hA	Without hA
0	51.3 ± 0.9	97.7 ± 1.3
25	67.7 ± 1.1**	110.3 ± 1.9
50	74.1 ± 0.9**	115.6 ± 1.1
100	78.9 ± 1.8**	107.3 ± 1.5
200	85.6 ± 1.6**	104.1 ± 1.5

spontaneous formation of amyloid fibrils in aqueous hA solutions. We also developed a precipitation assay to measure fibril formation by hA in culture medium. Figure 1(B) shows the extent of fibril formation by hA as determined by the measurement of precipitation of ^{125}I-hA. The percentage precipitation of tracer quantities of ^{125}I-hA by 10 μM hA was significant by 1 h incubation at 37 °C in culture medium and reached saturation after 8 h. Moreover, both TEM studies and ^{125}I-hA precipitation assays failed to detect fibril formation by rat amylin (results not shown), which is consistent with the observation that rat amylin does not form amyloid although it differs from hA in only six amino acid residues [3]. These results establish that hA, but not rat amylin, forms amyloid fibrils under the cell culture conditions employed in the current studies.

Table 2 Effect of Ca^{2+} modulators on hA cytotoxicity in RINm5F islet β-cells

RINm5F cells were pretreated for 1 h with A23187, thapsigargin, verapamil, EGTA or BAPTA/AM at various concentrations as indicated, in the absence or presence of 10 μM hA. The viability of cells was then determined by MTT reduction after a further 24 h incubation at 37 °C in culture medium with the methods described in the text. Results are means ± S.E.M., $n = 12$ at each point. $^{*}P < 0.05$, $^{**}P < 0.001$ relative to vehicle controls; †$P < 0.05$, ††$P < 0.001$ relative to hA treatment controls.

		Viable cells (% MTT reduction)	
Ca^{2+} modulator	Modulator concentration (μM)	With hA	Without hA
A23187	0	51.3 ± 2.0	100.1 ± 2.9
	5	47.5 ± 2.3	74.1 ± 1.7**
	10	44.4 ± 1.0†	64.4 ± 1.6**
	20	36.8 ± 0.7††	51.4 ± 2.7**
	40	28.6 ± 0.5††	37.8 ± 2.3**
Thapsigargin	0	52.0 ± 2.5	96.7 ± 1.0
	5	30.9 ± 1.7††	38.0 ± 3.8**
	10	32.6 ± 1.8††	39.5 ± 4.7**
	20	32.6 ± 3.2††	46.9 ± 1.7**
	40	0.5 ± 0.2††	0.0 ± 0.8**
Verapamil	0	51.3 ± 1.5	99.2 ± 1.2
	10	51.9 ± 1.6	94.2 ± 1.3
	50	62.3 ± 1.9†	97.9 ± 3.0
	100	63.1 ± 1.0†	94.3 ± 4.2
	200	4.2 ± 1.0††	4.1 ± 1.0**
EGTA	0	54.2 ± 1.4	100.0 ± 1.2
	500	58.3 ± 1.6	109.0 ± 2.4
	1000	31.4 ± 1.6 ††	27.9 ± 5.8**
	2000	23.6 ± 1.7††	21.6 ± 2.6**
	4000	18.6 ± 1.2††	31.0 ± 3.6**
BAPTA/AM	0	53.3 ± 1.5	97.4 ± 1.5
	1	54.7 ± 6.7	102.2 ± 6.7
	5	54.0 ± 8.1	97.6 ± 1.4
	10	54.6 ± 7.1	99.8 ± 7.4
	20	50.7 ± 5.4	93.1 ± 3.3
	40	43.8 ± 3.7††	61.0 ± 8.6
	50	34.6 ± 3.7††	44.6 ± 1.3*
	100	11.1 ± 3.4††	12.3 ± 2.7**

Induction by hA of apoptosis in RINm5F islet β-cells

The exposure of RINm5F cells to synthetic hA evoked both concentration- and time-dependent cell death, as determined by cellular reduction of MTT (Figures 2A and 2B). After 24 h of treatment, significant cell death was evident at 5 μM hA and reached a maximum level of approx. 80 % at 20 μM (Figure 2A). The measurement of cell death was quantitatively reproducible when calibrated against viability assays (performed with calcein AM/ethidium homodimer-1; results not shown) [12] or MTT reduction (Figure 2A, inset). Consistent with previous observations for primary rat and human islet β-cells [12] was the observation that the calculated EC_{50} value for the concentration dependence of hA cytotoxicity was 10 μM. Studies of time dependence of cell killing by 10 μM hA indicated that cell death was detected at 12 h after the addition of hA and reached half-maximal by 24 h (Figure 2B).

TEM was used to compare the ultrastructure between control and hA-treated RINm5F islet β-cells, and thereby to detect structural features of hA-evoked β-cell killing. Over a period of 1–22 h, vehicle-treated control cells were unchanged (Figure 2C), whereas hA exposure evoked progressive, asynchronous cellular degeneration. hA-treated cells showed features consistent with apoptosis, including a progressive loss of nuclear volume with folding of the nuclear membrane, and the compaction and segretation of chromatin into masses that lay against the nuclear envelope (Figure 2D, arrowheads), as well as cytoplasmic degeneration with prominent vacuole formation, and loss of microvilli (Figure 2D).

Agarose gel analysis of genomic DNA extracted from cells exposed to hA revealed internucleosomal DNA fragmentation (Figures 2E and 2F), a further hallmark of apoptosis [21,28,33], which was both concentration- and time-dependent. DNA fragmentation, with the smallest fragment in the ladder being approx. 180–200 bp, was detected when cells had been exposed to 10 μM hA for 12 h (Figure 2F), after which it increased in magnitude until 24 h. In contrast, treatment with rat amylin, vehicle or medium alone (controls) caused no detectable DNA fragmentation (Figure 2E). In addition, preincubation of RINm5F cells with aurintricarboxylic acid (ATA), an endonuclease inhibitor [34] that can suppress apoptosis in a variety of cell types [12,24,35], prevented hA-evoked apoptosis in a concentration-dependent manner (Table 1), completely abolishing hA-induced DNA fragmentation at 100 μM (results not shown). These results suggest that hA evokes death in RINm5F cells by stimulating apoptosis.

Effect of Ca^{2+} modulators on hA-evoked β-cell apoptosis

To test the possible role of Ca^{2+} in hA-evoked β-cell apoptosis, a number of Ca^{2+} modulators were used. As shown in Table 2, both A23187 and Tg were toxic to cultured β-cells, in either the presence or the absence of hA, confirming that increased $[Ca^{2+}]_i$ can elicit death in islet β-cells [21]. A23187 evoked β-cell death in a concentration-dependent manner, with a calculated EC_{50} of approx. 20 μM (Table 2). Tg alone was effective at 5 μM and induced complete cell death at 40 μM. Attenuation of Ca^{2+} influx

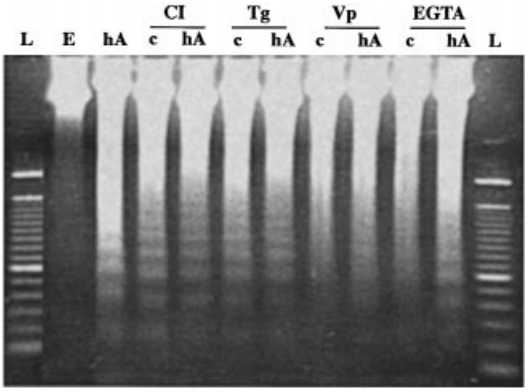

Figure 3 Effects of Ca^{2+} modulators on DNA fragmentation evoked by hA in RINm5F islet β-cells

RINm5F cells were treated for 24 h with A23187 (10 μM), Tg (10 μM), verapamil (Vp, 10 μM) or EGTA (1 mM) in the absence (c) or presence (hA) of 10 μM hA. Total genomic DNA was isolated and analysed on 1.1% (w/v) agarose gels, illustrating DNA fragmentation in islet cells treated with or without hA and Ca^{2+} modulators as shown (Note that two unnecessary lanes have been removed.) Lanes L, DNA size marker of 100 bp; lane E, 1% (v/v) ethanol control.

by the Ca^{2+} channel blocker verapamil or the Ca^{2+} chelator EGTA failed to inhibit apoptosis evoked by hA (Table 2). Morever, incubation with BAPTA/AM, a cytosolic Ca^{2+} buffer [27], also failed to inhibit apoptosis evoked by hA. Verapamil, EGTA and BAPTA/AM were all toxic to β-cells when administered by themselves at concentrations of 200 μM, 1 mM and 50 μM respectively.

DNA fragmentation was evident when either A23187 or Tg was added to β-cells, in either the presence or the absence of hA (Figure 3), suggesting that they both evoked death via an apoptotic pathway. Although the effect of verapamil on β-cell DNA fragmentation was not clearly seen, the Ca^{2+} chelator EGTA did not prevent hA-induced DNA laddering (Figure 3), suggesting that the latter process is likely to be independent of Ca^{2+}-influx. Taken together, these results indicate that hA does not elicit apoptosis in RINm5F islet β-cells via increasing $[Ca^{2+}]_i$, although other stimuli can evoke death through such a mechanism.

Effect of hA on intracellular Ca^{2+} mobilization

We next investigated the direct link between treatment with hA and intracellular Ca^{2+} mobilization in fura-2-loaded β-cells. In the presence of glucose (5.6 mM), the baseline $[Ca^{2+}]_i$ ranged between 153 and 320 nM, averaging 232 ± 8 nM (mean $\pm$ S.E.M., $n = 41$). It was noted that basal $[Ca^{2+}]_i$ could be affected by the duration for which samples were left at room temperature before processing, as well as by the room temperature itself. As shown in Figures 4(A) and 4(B), the exposure of β-cells to 10 μM hA or rat amylin failed to evoke any detectable change in $[Ca^{2+}]_i$. In contrast, ethanolic Tg induced a biphasic increase in $[Ca^{2+}]_i$ with a large early transient rise and a subsequent small but prolonged increase (Figure 4C), which is consistent with previous observations in a number of different cell systems [33,36–39]. The mean Tg-induced $[Ca^{2+}]_i$ increase was approx. 108.9 ± 8.6 nM (mean $\pm$ S.E.M., $n = 20$) within the first minute after administration. When cells were treated with a lethal dose of hA (10 μM) for 2, 5 or 8 h and then loaded with fura-2/AM, we did not observe any significant changes in either basal $[Ca^{2+}]_i$ or the Tg-induced increase in $[Ca^{2+}]_i$ (Table 3). These results indicate that treatment with hA neither stimulates Ca^{2+} influx nor induces the mobilization of Ca^{2+} from intracellular stores during hA-evoked apoptosis in islet β-cells.

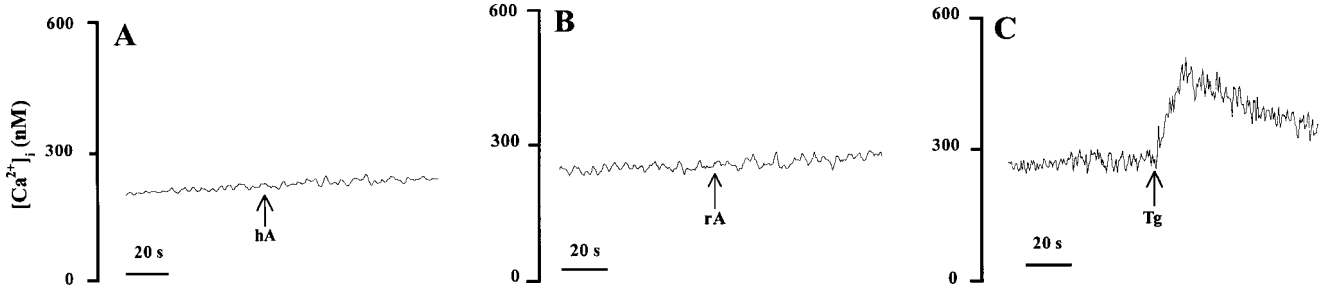

Figure 4 Effect of hA, rat amylin (rA) and Tg on transient $[Ca^{2+}]_i$ in RINm5F islet β-cells

$[Ca^{2+}]_i$ in fura-2/AM-loaded β-cells was monitored as described in the text. At the time indicated by the arrows, 10 μM hA (**A**), 10 μM rA (**B**) or 10 μM Tg (**C**) was added. Each trace is representative of eight experiments from different cell preparations.

Table 3 Effects of hA on basal $[Ca^{2+}]_i$ and Tg-modulated $[Ca^{2+}]_i$ in RINm5F islet β-cells

RINm5F cells were pretreated with or without hA (10 μM) for the durations indicated, then loaded with fura-2/AM. $[Ca^{2+}]_i$ was monitored before and within 1 min of administration of Tg (10 μM). Results are means $\pm$ S.E.M., $n = 12$ at each point. $^{**}P < 0.001$ relative to the corresponding controls without Tg stimulation.

	Basal $[Ca^{2+}]_i$ (nM)		Tg-induced $[Ca^{2+}]_i$ (nM)	
Incubation duration (h)	Without hA	With hA	Without hA	With hA
2	170 ± 5	191 ± 6	$297 \pm 8^{**}$	$308 \pm 6^{**}$
5	179 ± 8	201 ± 4	$278 \pm 16^{**}$	$305 \pm 8^{**}$
8	171 ± 3	199 ± 3	$257 \pm 7^{**}$	$281 \pm 10^{**}$

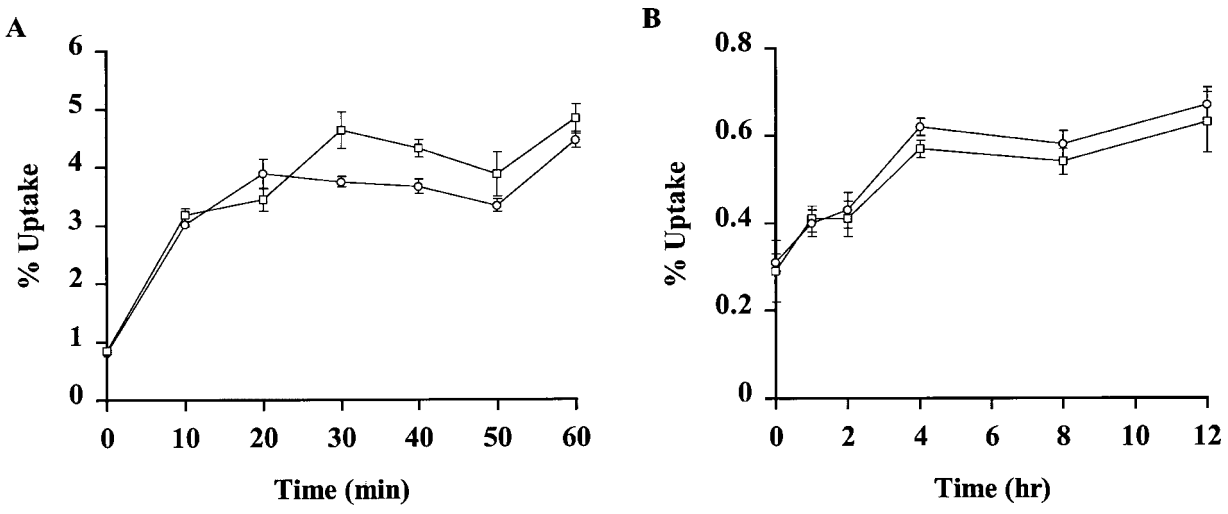

Figure 5 Effects of hA on $^{45}Ca^{2+}$ accumulation in RINm5F islet β-cells

RINm5F cells (8×10^6/ml) were incubated in PBS at room temperature (**A**) or normal tissue culture medium at 37 °C (**B**) with $^{45}Ca^{2+}$ in the presence (○) or absence (□) of 10 μM hA. The amounts of $^{45}Ca^{2+}$ taken up at various time points were determined. Results are means ± S.E.M., $n = 6$. No significance was detected between the two groups with and without hA treatment.

Effect of hA on ^{45}Ca uptake

To examine further the role of external Ca^{2+} and the reported lipid bilayer ion-pore-inducing activity of hA [22] in the mechanism of β-cell apoptosis, we measured the uptake of $^{45}Ca^{2+}$ by β-cells in the presence or absence of 10 μM hA. As shown in Figure 5, progressive increments in the uptake of $^{45}Ca^{2+}$ were observed in both treatments, during either brief incubations in Ca^{2+}-free PBS buffer (Figure 5A) or longer incubations in Ca^{2+}-containing tissue culture medium (Figure 5B). However, the rates of $^{45}Ca^{2+}$ accumulation were comparable in β-cells in the presence and in the absence of hA. In line with our previous observations, these results suggest that increased uptake of Ca^{2+} from external stores does not occur during hA-evoked β-cell apoptosis. They also indicate that hA is unlikely to evoke apoptosis by forming pores in cell membranes *in vivo*.

DISCUSSION

Here we show that either treatment with the fibril-forming peptide hA or changes in $[Ca^{2+}]_i$ can independently trigger apoptosis in RINm5F cells, which are widely used for studies of pancreatic islet β-cell function [28,29]. However, hA-induced β-cell death occurred independently of changes in $[Ca^{2+}]_i$. hA-treated RINm5F cells show many features of apoptosis such as the internucleosomal cleavage of DNA but aspects of their morphology might be characteristic of this particular cell type rather than of a general apoptotic nature.

There is much evidence that cellular Ca^{2+} acts as a regulator of apoptosis in many different types of cell [17], including pancreatic RINm5F cells [21]. One characteristic property of apoptosis is the internucleosomal cleavage of nuclear DNA, leading to a characteristic pattern of DNA fragmentation [24,34]. In the present study, both A23187 and Tg themselves evoked a form of cell death characterized by the presence of such laddering of nuclear DNA. A23187 elevates $[Ca^{2+}]_i$ by increasing the permeability of the cell membrane [25]; in contrast, Tg acts as an endoplasmic-reticulum-specific Ca^{2+}-ATPase inhibitor, which is widely used to deplete Ca^{2+} stores in the endoplasmic reticulum and thus to increase $[Ca^{2+}]_i$ [26,39]. In agreement with our current observations, A23187 has been shown to promote apoptosis in prostatic glandular cells [40], lymphocytes [41], fibroblasts [42], cortical neurons [43] and thymocytes [44]. Similarly, Tg has also been shown to stimulate apoptosis in lymphocytes [41], thymocytes [37], neurosecretory cells [33], prostatic cancer cells [38,45], submandibular acinar cells [39] and lymphoma cells [36]. Interestingly, recent evidence shows that Tg-evoked apoptosis can be abrogated by the overexpression of *bcl-2*, which prevents sustained $[Ca^{2+}]_i$ increases in the cytoplasm or nucleus after treatment with Ca^{2+} [36,45]. Whereas the molecular targets of Ca^{2+} in apoptosis can be diverse [17], the rise in $[Ca^{2+}]_i$ is believed to activate a Ca^{2+}/Mg^{2+}-dependent endonuclease, which cleaves DNA into fragments of oligonucleosomal length [17,35,44].

However, hA-evoked β-cell death was not prevented by the Ca^{2+}-channel blocker verapamil, by the Ca^{2+}-chelator EGTA [12] or by the cytosolic Ca^{2+} buffer BAPTA/AM [27]. The last result is important, because it shows that hA can evoke β-cell death in the presence of constant $[Ca^{2+}]_i$, arguing against the possibility that hA might evoke apoptosis via a transient effect on Ca^{2+}. This is supported by the findings that there were no detectable alterations in transient $[Ca^{2+}]_i$ in fura-2-loaded cells after exposure to hA (as well as its non-fibril-forming homologue rat amylin) and that there were no significant changes in either basal or Tg-released intracellular Ca^{2+} stores during the treatment of β-cells with a concentration of hA that evoked apoptosis. These findings suggest that hA does not mobilize intracellular Ca^{2+} during its induction of apoptosis in islet β-cells. Complementary studies with ^{45}Ca showed that hA did not elicit any detectable changes in Ca^{2+} uptake at cytotoxic concentrations. Taken together, these results indicate that hA-evoked β-cell apoptosis is Ca^{2+}-independent. Moreover, they are inconsistent with the hypothesis [22] that the formation of ion-permeable channels in the cell membrane accounts for its cytotoxic properties.

Accumulating evidence suggests that increased cytosolic $[Ca^{2+}]_i$ is not necessary for the triggering of apoptosis in many types of cell [18,46]. For instance, CEM-C7 lymphocytes did not die in response to raised $[Ca^{2+}]_i$ during glucocorticoid-induction of apoptosis [47]. Treatment of interleukin-3-dependent haemato-

poietic cells with Ca^{2+} ionophores was shown to block endonuclease activation and apoptotic cell death after the withdrawal of interleukin 3 [18,46]. No significant change in $[Ca^{2+}]_i$ was detected by fura-2 imaging during βA-evoked neuronal DNA fragmentation and apoptosis that were inhibited by ATA [24], as is the case in similar studies of hA-evoked death in islet β-cells. ATA acts to suppress apoptosis; this effect could be elicited by the inhibition of Ca^{2+}-activated endonucleases [35,44], although it has many additional effects. Cleavage of chromosomal DNA into nucleosomal units is a hallmark of apoptosis. So far both Ca^{2+}/Mg^{2+}-dependent (DNase I, NUC18, cyclophilins, DNase γ) and Ca^{2+}/Mg^{2+}-independent (DNase II) endonucleases have been proposed as mediators of DNA cleavage during apoptosis [35,46,48,49]. Recently, caspase-activated DNase ('CAD') [50,51] and DNA fragmentation factor ('DFF') [52] have been identified as further important apoptotic nucleases, which act downstream from the caspase cascade to evoke the degradation of DNA during apoptosis.

Although ATA is sometimes used to block Ca^{2+}-dependent apoptosis [35,44], it is a more general inhibitor of nuclease action, being used to prevent the degradation of DNA during the isolation of nucleic acids [34]. Indeed, both Ca^{2+}-dependent (inhibited by ATA [53]) and Ca^{2+}-independent endonucleases [54] have been implicated in DNA fragmentation evoked by Ca^{2+} stress in PC12 cells. The responses to ATA in the present study, when taken together with our other results, are consistent with a role for a Ca^{2+}-independent nuclease (susceptible to inhibition by ATA) in hA-evoked apoptosis in islet β-cells.

Many signals other than increased $[Ca^{2+}]_i$, have been implicated in the mechanisms of apoptosis, including the accumulation of ROS, the production of ceramide and the activation of adenylate cyclase or various cytoplasmic enzymes [18]. Therefore the Ca^{2+}-independence of fibril-forming hA-evoked cytotoxicity suggests that other signal transduction pathways have roles in this process. In particular, there is evidence that ROS contribute to βA-mediated apoptosis and that they can activate endonucleases independently of cellular Ca^{2+} [18]. That study, and our present findings that hA-evoked apoptosis is independent of changes in Ca^{2+} metabolism, lead us to suspect the possible involvement of ROS in the mechanism of hA-elicited β-cell death. In agreement with reported observations in βA-mediated neuronal apoptosis [16], our results indicate that the reduction of MTT, an indicator of cellular redox activity, is an early measurable indicator of hA-evoked β-cell death. Studies currently under way in our laboratory are investigating the possible role of ROS-associated mechanisms in this process. The elucidation of these relationships should provide an enhanced understanding of how hA fibrils evoke β-cell destruction, and provide new targets for therapeutic intervention that might aid in the treatment of NIDDM and related disorders.

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